

**MELIOIDOSIS:
AN INVESTIGATION OF CELLULAR IMMUNE RESPONSES**

Thesis submitted by

Jodie Lee BARNES, BBiomedSc (Hons I) *JCU*

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James Cook University

**This thesis is dedicated to my family for their support, patience and belief that I
would not be a uni student forever.**

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2. Ketheesan N, **Barnes J L**, Ulett G C, VanGessel H J, Norton R E, Hirst R G and LaBrooy J T. 2002. Demonstration of a cell-mediated immune response in melioidosis. *Journal of Infectious Diseases* 186(2): 286-289.
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Other Publications:

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8. Ulett G C, Ketheesan N, Clair T W, McElnea C L, **Barnes J L** and Hirst R G. 2002. Analogous cytokine responses to *Burkholderia pseudomallei* strains contrasting in virulence correlate with partial cross-protection in immunized mice. *Infection and Immunity*. 70(7): 3953-3958.

ABSTRACT

Melioidosis is a potentially fatal disease caused by the soil bacterium *Burkholderia pseudomallei* and is predominantly seen in southeast Asia and northern Australia. Protection from infections with other facultative intracellular bacteria such as *Listeria monocytogenes* and *Legionella pneumophila*, has been shown to be mediated largely by a cell-mediated immune (CMI) response of the host. *B. pseudomallei* is also a facultative intracellular bacterium but despite decades of research being conducted on this pathogen, few studies have focussed on the CMI response in melioidosis. The nature of a protective host immune response, and the conditions under which it is induced, are fundamental for improved clinical management of patients and vaccine development. Therefore, the major focus of the research outlined within this thesis was the characterisation of the CMI responses involved in the development of protective immunity in melioidosis. This was achieved using a previously characterised mouse model of acute and chronic melioidosis. Following intravenous (*iv*) inoculation, BALB/c mice are highly susceptible, and C57BL/6 mice relatively resistant to *B. pseudomallei* infection.

Using the murine model, we compared the pathogenesis of *B. pseudomallei* infection following inoculation of the bacterium by *iv*, intraperitoneal (*ip*), intranasal (*in*), per os (*po*) and subcutaneous (*sc*) routes of infection. These studies emphasized that determination of bacterial virulence is highly dependent on the route of infection. BALB/c mice consistently demonstrated greater susceptibility toward *B. pseudomallei*, independent of the route of infection. Spleen and liver were the primary organs targeted following infections by all methods tested. Interestingly, following *in* and *po* inoculation of C57BL/6 mice, significant levels of bacteria were detected in the brain in the absence of septicemia.

Reverse transcriptase-polymerisation chain reaction (RT-PCR) and histology were used to assess the increased expression of messenger (m) ribonucleic acid (RNA) for interferon- γ -inducible protein 10 (IP-10), monocyte interferon- γ -inducible protein

(Mig), regulated upon activation, normal T-cell expressed and secreted chemokine (RANTES), monocyte chemoattractant protein-1 (MCP-1), cytokine-induced neutrophil chemoattractant (KC), macrophage inflammatory protein-2 (MIP-2), granulocyte colony-stimulating factor (G-CSF), macrophage (M)-CSF, granulocyte-macrophage (GM)-CSF during infection with a highly virulent strain of *B. pseudomallei* (NCTC 13178). Histological changes and bacterial loads were also monitored in the livers and spleens of C57BL/6 and BALB/c mice infected with NCTC 13178. Disparate expression of mRNA was demonstrated for IP-10, Mig, MCP-1, KC, MIP-2, GM-CSF and M-CSF. The magnitude of cellular responses observed in tissue correlated with increased levels of the chemokines and CSF investigated, as well as bacterial load. Compared with C57BL/6 mice, greater infiltration of neutrophils was observed in liver and spleen of BALB/c mice. In contrast, early lesions in C57BL/6 mice predominantly comprised macrophages. These results suggested that the inability of BALB/c mice to contain the infection at sites of inflammation may underlie the susceptible phenotype of this mouse strain toward *B. pseudomallei* infection.

The nitric oxide (NO) secretory activity of C57BL/6 and BALB/c peritoneal macrophages were compared following *in vitro* stimulation with *B. pseudomallei* antigens. Comparable levels of NO were produced by peritoneal macrophages from both mouse strains, suggesting that other antimicrobial mechanisms may underlie differences in the *in vitro* intracellular killing capacities of peritoneal macrophages from C57BL/6 compared to BALB/c mice. However, the low numbers of macrophages observed histologically at sites of *B. pseudomallei* infection in BALB/c mice suggest that failure to recruit sufficient macrophages to these sites may also contribute to the susceptibility of this mouse strain.

The present studies are the first to demonstrate the role of T cell responses in experimental and human melioidosis. Following exposure to a less virulent strain of *B. pseudomallei* (NCTC 13179), both C57BL/6 and BALB/c mice demonstrated delayed-type hypersensitivity (DTH) responses ($P < 0.05$) and lymphocyte proliferation ($P < 0.05$) towards *B. pseudomallei* antigens. Such a response indicated the generation of

B. pseudomallei-specific lymphocytes following initial exposure to the bacterium. The strength of DTH and lymphocyte proliferation responses was dependent on the concentration of the primary inoculating dose. Adoptive transfer experiments were carried out using mononuclear leucocytes (MNL) or purified T cells from spleen of *B. pseudomallei*-immunised C57BL/6 mice. The transfer of *B. pseudomallei*-specific MNL to naïve C57BL/6 mice was demonstrated by a DTH response ($P < 0.05$) to *B. pseudomallei* antigens. However, these mice were not protected from a subsequent lethal challenge with NCTC 13178. Similarly, recipient mice that received purified T cells were not protected from a subsequent lethal challenge. The results of this study suggested that a single exposure to *B. pseudomallei* is insufficient to induce a protective adaptive immune response.

Therefore, we attempted to induce resistance in susceptible BALB/c mice using repetitive low-dose exposure to live *B. pseudomallei* NCTC 13179. Immune responses and resistance following *sc* immunisation with live *B. pseudomallei* was compared to exposure to *B. pseudomallei* antigens. Low-dose immunisation with live bacteria induced protection ($P < 0.01$) to a subsequent lethal challenge with NCTC 13178. In comparison, mice immunised with *B. pseudomallei* antigens were not protected but demonstrated significantly increased levels of IgG_{2a} ($P < 0.01$) and IgG₁ ($P < 0.01$) in serum. These findings suggest that although exposure to *B. pseudomallei* antigens induced a DTH response and lymphocyte proliferation to *B. pseudomallei* antigens *in vitro*, the generation of a protective immune response following *B. pseudomallei* infection requires the presence of live bacteria.

The present studies have also given the first evidence of the development of CMI responses to *B. pseudomallei* in patients who had recovered from melioidosis. Significantly higher lymphocyte proliferation, IFN- γ production and activation of CD4⁺ and CD8⁺ T cell subsets were observed in the patient group compared with control subjects after *in vitro* challenge of peripheral blood mononuclear leucocytes (PBML) cultured with *B. pseudomallei* antigens. It is tempting to reason that the survival of the patients included in this study was due to the development of a protective adaptive

immune response to *B. pseudomallei*. Strong CMI responses were also demonstrated in subclinical melioidosis infection. Individuals who had been exposed to *B. pseudomallei* without any clinical manifestations displayed enhanced lymphocyte proliferation and IFN- γ production in response to stimulation with *B. pseudomallei* antigens *in vitro*, when compared to individuals who had recovered from clinical melioidosis. Such a response may be essential for determining protection following *B. pseudomallei* infection.

In summary, the results of the present studies have provided basic data regarding the involvement of CMI responses during *B. pseudomallei* infection. They provide evidence supporting an essential role for T cells in the development of an effective adaptive immune response to experimental and human *B. pseudomallei* infection and suggest that differences in the development of T cell responses may influence the outcome of infection.

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LIST OF ABBREVIATIONS

AIDS – acquired immunodeficiency syndrome	LV – less virulent
ANOVA – analysis of variance	M – 123 bp DNA size marker
APC – antigen presenting cell	MAC – membrane attack complex
Ara ⁻ - non arabinose assimilating	MCP-1 – monocyte chemoattractant protein-1
Ara ⁺ - arabinose assimilating	M-CSF – macrophage CSF
ARDS – acute respiratory distress syndrome	MHC – major histocompatibility complex
BCG – bacillus Calmette-Guérin	Mig – monocyte interferon- γ -inducible protein
Bp – base pairs	MIP-2 – macrophage inflammatory protein-2
BP-LPS – <i>B. pseudomallei</i> LPS	MLD – minimum lethal dose
BpLy1 – <i>B. pseudomallei</i> lysate (NCTC 13179)	MNGC – multinucleated giant cells
cDNA – complementary DNA	MNL – mononuclear leucocytes
CepLy – <i>B. cepacia</i> lysate	mRNA – messenger RNA
CF – cystic fibrosis	MW – molecular weight
cfu – colony forming units	NK – natural killer
CLT – cytolethal toxin	NO – nitric oxide
CMI – cell-mediated immunity	OD – optical density
ConA – concanavalin A	O-PS – O-antigenic polysaccharide
CP- capsular polysaccharide	PBML – peripheral blood mononuclear leucocytes
cpm – counts per minute	PBP – penicillin-binding protein
CSF – colony stimulating factor	PBS – phosphate buffered saline
DNA – deoxyribonucleic acid	PCR – polymerase chain reaction
dNTP – deoxynucleoside triphosphate	PEC – peritoneal exudate cells
EIA – enzyme immunoassay	PFGE – pulse field gel electrophoresis
ELISA – enzyme linked immunosorbent assay	PHA – phytohaemagglutinin
FCS – foetal calf serum	PMNL – polymorphonuclear leucocyte
G-CSF – granulocyte CSF	<i>po</i> – per os, oral
GM-CSF – granulocyte macrophage CSF	PPD – tuberculin purified protein derivative
H&E – haematoxylin and eosin	PPS – protein polysaccharide
HIFCS – heat-inactivated FCS	RANTES – regulated upon activation, normal T-cell expressed and secreted chemokine
HIV – human immunodeficiency virus	RAPD – randomly amplified polymorphic DNA
HLA – human leucocyte antigen	RNA – ribonucleic acid
HV – highly virulent	RNI – reactive nitrogen intermediates
IFA- indirect fluorescent antibody	ROI – reactive oxygen intermediates
IFN- γ – gamma interferon	rRNA – ribosomal RNA
Ig – immunoglobulin	RT – room temperature
IHA – indirect haemagglutination	RT-PCR – reverse transcription PCR
IL – interleukin	SBA – sheep blood agar
<i>in</i> – intranasal	<i>sc</i> – subcutaneous
<i>ip</i> - intraperitoneal	SEM - standard error of the mean
IP-10 – interferon- γ -inducible protein 10	TNF- α – tumor necrosis factor alpha
<i>iv</i> – intravenous	T _H 1 – T Helper (type) 1
KC - cytokine-induced neutrophil chemoattractant	T _H 2 – T Helper (type) 2
LD ₅₀ – fifty percent lethal dose	Ttox – tetanus toxoid
LIX – LPS-induced C-X-C chemokine	
LPS – lipopolysaccharide	

CHAPTER 1

INTRODUCTION

Melioidosis is a potentially fatal disease caused by the soil bacterium *Burkholderia pseudomallei* and is predominantly seen in southeast Asia and northern Australia. Many cases of melioidosis probably go unrecognised due firstly to a lack of access to modern diagnostic facilities in regions where the bacteria may be present, and secondly due to our lack of awareness as to how extensive *B. pseudomallei* is in the environment. Infection can occur through a breach in the skin, inhalation or ingestion of contaminated soil or water. Person-to-person transmission of melioidosis is extremely rare.

Individuals with a pre-existing condition that suppresses the host immune response, such as diabetes or chronic renal failure have a higher risk of infection with *B. pseudomallei*. However, melioidosis also affects apparently healthy individuals with no identifiable risk factors. Although clinical melioidosis is often classified into four main forms (acute, subacute, chronic and subclinical), all gradations exist between them. Acute melioidosis is characterised by a fulminating septicemia, with mortality rates as high as 95% in endemic regions of Thailand (Chaowagul *et al.* 1989). Cases of subacute melioidosis are more frequent and less severe than the acute form and can be localised or disseminated, involving multiple organ systems. Abscess formation or focal infection is the characteristic feature of chronic melioidosis and this may involve one or more tissue types. The ability of *B. pseudomallei* to persist in the host in a dormant phase for extended periods of time accounts for the subclinical and latent melioidosis. Activation of latent infection is well documented and typically occurs following periods of immunosuppression, emphasising the importance of a cell-mediated immune (CMI) response in controlling this bacterium. The course of disease is likely to be influenced by a combination of factors including the inoculum size, the virulence of the bacterial strain involved and the immune status of the host.

Most features of *B. pseudomallei* infection are poorly understood, particularly the response of the host's immune system to the bacterium and the mechanisms involved in generating protection from the infection. A contrasting mouse model of acute and chronic human melioidosis in BALB/c mice and C57BL/6 mice respectively, has been characterised (Leakey *et al.* 1998). Disease progression in BALB/c mice is rapid with substantial bacterial growth occurring in the liver and spleen within 48 hrs of infection. Bacteraemia results in septicaemic shock and death within 72 hrs of infection, resembling the clinical course of disease in patients with acute melioidosis. C57BL/6 mice are relatively resistant to *B. pseudomallei* when compared to BALB/c mice, and therefore provide a good model for chronic melioidosis. In the past, many studies using murine models have used intravenous (*iv*) or intraperitoneal (*ip*) infection with *B. pseudomallei* to generate systemic melioidosis (Leakey *et al.* 1998; Hoppe *et al.* 1999; Gauthier *et al.* 2001). Transmission of *B. pseudomallei* is believed to occur following subcutaneous inoculation, inhalation or ingestion of contaminated soil or water (Leelarasamee & Bovornkitti 1989). Recently, Liu *et al.* (2002a) and Jeddloh *et al.* (2003) used a method to mimic inhalation of *B. pseudomallei* which may reflect the natural transmission of the bacterium more closely. As part of the current study, the influence of the route of infection on disease outcome using the C57BL/6-BALB/c model was examined.

Infections with facultative intracellular bacteria such as *Listeria monocytogenes*, *Mycobacterium tuberculosis* and *Legionella pneumophila*, have been shown to be controlled largely by a CMI response of the host (Ehlers *et al.* 1992; Mittrucker *et al.* 2000; Andersen & Smedgaard 2000; Tateda *et al.* 2001). Macrophages and lymphocytes are the predominant cells involved in this defence and their recruitment and subsequent activation is regulated through a complex interaction of cytokines, chemokines and adhesion molecules. Electron microscopy has demonstrated that *B. pseudomallei* is also a facultative intracellular bacterium. However, the role of CMI responses in *B. pseudomallei* infection has not been adequately characterised. In the early phases of infection, it is the role of the hosts' innate immune responses to hold the pathogen in check while an adaptive immune response is generated in an attempt to clear infection.

An important feature of the innate host immune response against a pathogen is the infiltration of leucocytes to the site of infection and the subsequent activation of these cells. However, while this process is paramount in the effective clearance of the pathogen, it often contributes to the pathogenesis of disease (De Vries, Ran & Kelvin 1994). The hyper-production of pro-inflammatory cytokines in acutely-infected BALB/c mice parallel cytokine responses described in human melioidosis and is associated with a poor prognosis (Brown *et al.* 1991; Friedland *et al.* 1992; Lauw *et al.* 1999; Simpson *et al.* 1999; Ulett, Ketheesan & Hirst 2000; Ulett *et al.* 2000).

Chemokines play a critical role in the recruitment, migration and activation of cells during infection with intracellular pathogens (Kaufmann 1993; Rhoades, Cooper & Orme 1995; DiTirro *et al.* 1998). It has been previously demonstrated that the neutrophil chemoattractant, LPS-induced CXC chemokine (LIX), is differentially expressed in murine melioidosis (Ulett *et al.* 2000). Other chemokines such as interferon- γ -inducible protein 10 (IP-10), macrophage inflammatory protein-2 (MIP-2) and various colony-stimulating factors (CSF) are also believed to play an important role in regulating immune responses to *B. pseudomallei*. Some of these chemokines have already been implicated in patients with melioidosis (Lauw *et al.* 2000; Stephens, Fisher & Currie 2002). Adequate investigations into the immunoregulatory role of chemokines during infection with *B. pseudomallei* have not yet been carried out.

An adaptive CMI response to a pathogen can be detected several days after development of the innate immune response and is involved in bacterial clearance and the development of long-term immunity. Clearance of intracellular bacteria such as *L. monocytogenes* and *M. tuberculosis* is predominantly mediated by CD4⁺ T lymphocytes (Mittrucker, Kohler & Kaufmann 2000; Andersen & Smedegaard 2000). These lymphocytes recognise antigens presented by antigen-presenting cells (APC) and based on the cytokines they secrete, can be broadly classified as T_H1- or T_H2-type cells. A T_H1-type cytokine response involves the production of interleukin (IL)-2 and interferon-gamma (IFN- γ), and is important for CMI responses to intracellular pathogens. T_H2-type cytokine responses include anti-inflammatory cytokines such as

IL-4 and IL-10 and are involved in B lymphocyte stimulation. The cytokine cascades observed in many infections with intracellular pathogens do not conform to a T_H1/T_H2 polarised response, instead comprising a mixed T_H response (Bohn *et al.* 1994; Pie *et al.* 1996; Allen & Maizels 1997; Kelso 1998). However, for clearance of intracellular infection, it is important that T_H1 -type responses predominate since T_H2 -type responses exacerbate the infection. Recent studies suggest that a mixed population of T_H cells is involved in host responses toward *B. pseudomallei* infection (Ulett, Ketheesan & Hirst 2000; Liu *et al.* 2002). This study will assess the development of *B. pseudomallei*-specific T cells in resistant C57BL/6 mice and susceptible BALB/c mice following infection with *B. pseudomallei*. Adoptive transfer and secondary challenge experiments will be carried out to determine the role of *B. pseudomallei*-specific T cells in providing host protection in the C57BL/6-BALB/c model. The role of *B. pseudomallei*-specific T cells in human melioidosis will also be investigated.

Vaccination strategies for melioidosis have had limited success (Warawa & Woods 2002). Recently Ulett *et al.* (2002) demonstrated that immunisation of mice with a less virulent (LV) *B. pseudomallei* strain generated only partial protection from subsequent challenge. In that study mice received only a single immunising dose, delivered *iv* (Ulett *et al.* 2002). A series of studies have demonstrated in both experimental leishmaniasis and mycobacterial infection that the dose of antigen administered is critical in the development of a protective immune response (Bretscher *et al.* 1992; Menon & Bretscher 1996; Power, Wei & Bretscher 1998). They demonstrated that infection with low doses of *L. major* or *M. bovis* resulted in the induction of a stable, cell-mediated T_H1 -like response that is exclusive of antibody production in susceptible BALB/c mice. A similar approach will be used in an attempt to induce protective immunity against *B. pseudomallei* infection.

Exposure to, or infection with *B. pseudomallei* does not necessarily result in the development of clinical disease. This then raises the question: What is the difference between the host immune response of those who succumb to *B. pseudomallei* infection, and those with subclinical infection? Two distinct factors are believed to influence the

development of different presentations of melioidosis. These are (i) the type of chemokines and inflammatory infiltrate involved in the early stages following infection and (ii) the type of specific T cell responses that develop following infection. Therefore, the broad aims for the work outlined in the subsequent chapters of this thesis were:

1. To compare the pathogenesis of *B. pseudomallei* infection generated by different routes of infection
2. To investigate the expression of chemokine and CSF during *B. pseudomallei* infection
3. To assess the development of *B. pseudomallei*-specific T cells in a murine model of melioidosis
4. To investigate the role of *B. pseudomallei*-specific T cells in providing protection in a murine model of melioidosis
5. To generate resistance to *B. pseudomallei* infection in susceptible BALB/c mice
6. To investigate the role of T cells in survival of *B. pseudomallei* infection in patients
7. To investigate the role of T cells in preventing the progression of *B. pseudomallei* infection in patients

Despite decades of melioidosis research, the nature and potentially protective role of a CMI response in melioidosis is poorly understood. Without characterisation of the immune regulatory mechanisms involved in melioidosis, improvement to strategies for the treatment and prevention of melioidosis will not be possible.

CHAPTER 2

LITERATURE REVIEW

2.1 Historical Overview

In 1911, following autopsies on 38 cadavers in the Rangoon General Hospital, Burma a British pathologist, Captain Alfred Whitmore, and his assistant, C. S. Krishnaswami, documented the existence of a “glander’s-like illness” (Whitmore & Krishnaswami 1912). Glanders is a chronic and debilitating disease of horses caused by the bacterium *Bacillus* (now *Burkholderia*) *mallei*, which is an infrequent pathogen of humans. Morphine addicts accounted for many of the fatal cases and Whitmore made an association between the “glander’s-like illness” and the debilitation and poor health of the morphine addicts (Whitmore & Krishnaswami 1912). All of the cadavers studied exhibited widespread caseous abscessation, particularly in the lungs, liver, spleen and kidneys. Whitmore and Krishnaswami isolated a novel bacterium from these lesions and, due to the remarkable biochemical, morphological and clinical similarity to *Bacillus mallei*, Whitmore proposed the name *Bacillus* (now *Burkholderia*) *pseudomallei* (Cowan & Heap 1993; Whitmore & Krishnaswami 1912). However, it was not until 1925 that the disease became known as melioidosis, based on the Greek words ‘*melis*’ (a distemper of asses) and ‘*eidosis*’ (resemblance) (Cox 1996).

In the early 1930’s, William Fletcher and Ambrose Thomas Stanton described numerous cases of melioidosis in humans and animals, both wild and domestic at the Institute of Medical Research in Malaya (Cox 1996). They proposed the disease was carried by rodents and was transmitted to humans and other animals following contamination of food with rat excreta. While this theory was later disproved (Laws & Hall 1964), Stanton and Fletcher are recognised for their pioneering work in the serodiagnosis of melioidosis and the use of experimental animals for the confirmation of virulence of the bacterium (Cox 1996).

As the awareness of the disease grew, documented cases of melioidosis became more widespread but were still mainly confined to areas within southeast Asia. By 1932, it was recognised in Vietnam, Sri Lanka and Indonesia (Cox 1996; Dance 1991). The clinical presentation of melioidosis varied greatly, often closely resembling other diseases. For this reason, melioidosis has been described as the “great imitator of disease” (Cox 1996).

During the Vietnam War, over 400 cases of melioidosis were reported among French and American troops, of which approximately 10% were fatal (Cox 1996). Another 225,000 Vietnam veterans are believed to be seropositive for *B. pseudomallei* (Short 2002) and the impact of this high incidence rate initiated a fresh surge of research into melioidosis. One important finding to come from this was the ability of *B. pseudomallei* to remain latent within a host, causing no clinical illness until many years later when the individual becomes immunocompromised. This phenomenon was observed in Vietnam War veterans and was dubbed the “Vietnamese time bomb”, with the longest period of latency recorded as 26 years (Mays & Ricketts 1975).

Melioidosis is endemic in northern Australia and was first reported by Cottew (1950) who described an outbreak of melioidosis in sheep in north Queensland. Rimington first described cases of human melioidosis in Australia in 1962. Six cases were documented in north Queensland, five of which were fatal (Rimington 1962). Since then cases in both animals and humans have occurred annually within the Northern Territory and north Queensland, predominantly during the wet season (Laws & Hall 1964; Ashdown, Duffy & Douglas 1980; Ashdown & Guard 1984; Guard *et al.* 1984; Currie *et al.* 2000c; Cheng *et al.* 2003). Cheng *et al.* (2003) reported an average annual incidence of 5.8 cases per 100,000 population in northern Australia.

2.2 *Burkholderia pseudomallei*: The Aetiological Agent

2.2.1 Taxonomy

The causative agent of melioidosis, *Burkholderia pseudomallei*, has undergone a series of taxonomic changes since its discovery in 1911. In the past, reports have described the

organism by a variety of names such as *Bacillus pseudomallei*, *Bacillus whitmori*, *Malleomyces pseudomallei*, *Loefflerella whitmori*, *Pfeifferella whitmori*, and *Pseudomonas pseudomallei* (Leelarasamee & Bovornkitti 1989). *B. pseudomallei* was formerly included in the rRNA homology group II of the genus *Pseudomonas* until re-classification into the genus *Burkholderia* in 1992, together with *B. mallei* and *B. cepacia*. This grouping was based on the cellular lipid and fatty acid composition of the bacteria, its 16S rRNA sequences, DNA-DNA homology values and phenotypic characteristics (Yabuuchi *et al.* 1992). Many members of this genus exist in the environment, however *B. pseudomallei* and *B. cepacia* are also opportunistic human pathogens. In 1996, an avirulent *B. pseudomallei*-like organism was first described (Wuthiekanun *et al.* 1996). The phenotype and biochemical profiles of the two biotypes were identical although, unlike the virulent, classical *B. pseudomallei*, the avirulent organism was able to assimilate L-arabinose (Ara⁺). However, based on cloning and sequencing of 16S rRNA and differences in the physiology and pathogenicity of the two biotypes, Ara⁺ isolates were later classified as *B. thailandensis* (Smith *et al.* 1997; Brett, DeShazer & Woods 1998; Dharakul 1999).

2.2.2 Geographic distribution and ecology

Since the growth of this organism in nature requires relatively high temperatures, humidity and rainfall, melioidosis is largely a disease of the tropics and is therefore predominantly found in countries between the latitudes 20° north and 20° south (Figure 2.1; Dance 1991). Southeast Asia and northern Australia are considered endemic for melioidosis (Leelarasamee & Bovornkitti 1989). Clinical melioidosis cases occur at a rate of 2,000 to 3,000 per year in Thailand with a population of 60 million (Leelarasamee 2000). In northeast Thailand, septicaemic melioidosis is a major cause of morbidity and mortality and is responsible for the majority of severe community-acquired sepsis in this region (Chaowagul *et al.* 1989). The risk of acquiring melioidosis in Thailand varies according to the region, with cases most common in the northeast. The high clinical incidence in this region correlates with high burden of *B. pseudomallei* in the environment, Wuthiekanun *et al.* (1995) having demonstrated the presence of this bacterium in more than two-thirds of rice fields. In comparison, an environmental study

that compared regions of Thailand cultured *B. pseudomallei* from only 13.8%, 24.5% and 18.4% of sites sampled in northern, central and southern provinces, respectively (Vuddhakul *et al.* 1999). Later, Trakulsomboon *et al.* (1999) demonstrated that compared to northern, central and southern regions of Thailand, soil in the northeast had a much higher ratio of virulent, Ara⁻ *B. pseudomallei* compared to the avirulent, Ara⁺ *B. thailandensis*. The reasons for this variation in distribution are unknown. The hyper-endemicity of this disease in northeast Thailand was reinforced by a study demonstrating that by four years of age, 80% of children in this region have been exposed to *B. pseudomallei* (Kanaphun *et al.* 1993). However, despite exposure, the majority of seropositive children are asymptomatic. In those children who do develop clinical melioidosis, the disease is typically less severe than forms of melioidosis seen in adults (Dance *et al.* 1989). *B. pseudomallei* infection is believed to be under-diagnosed and under-recognised in many southeast Asian countries. Melioidosis is considered endemic in Malaysia and Singapore. Strauss *et al.* (1969b) demonstrated *B. pseudomallei* as a normal inhabitant of soil and water in Malaysia, particularly in plantations of rice and oil palms and screening of healthy blood donors in Malaysia demonstrated 26.5% have antibodies to *B. pseudomallei* (Norazah *et al.* 1996). Melioidosis is also considered an emerging disease in Philippines, Cambodia, Laos, Vietnam, Indonesia, Brunei and Myanmar (Van Phung *et al.* 1993; Dance 2000; Leelarasamee 2000).

In Australia, human melioidosis was first described in the Townsville region (Rimington 1962), an area that is now regarded as endemic. The disease is currently responsible for the majority of fatal community-acquired bacteraemic pneumonias in northern Australia (Currie *et al.* 2000a). Every year cases are recorded predominantly between November to April, which is considered the wet season (Ashdown & Guard 1984). The endemic region of northern Australia extends north of Rockhampton, along the east coast to Darwin and west to Tennant Creek, and includes the Torres Strait Islands (Figure 2.2; Johnson 1967; Ashdown & Guard 1984). A serological survey of north Queensland reported a 5.7% prevalence of antibodies to *B. pseudomallei*, although this rate was significantly higher in both Aboriginal populations, and in individuals suffering from conditions such as diabetes mellitus or chronic alcoholism (Ashdown & Guard 1984).

Seropositivity in the Northern Territory of Australia ranges from 5% to 13% (Currie *et al.* 2000a). Of the 252 cases of melioidosis identified at the Royal Darwin Hospital in the Northern Territory between 1989 and 1999, 19% were fatal (Currie *et al.* 2000a). Almost half of these patients were indigenous, despite Aboriginals representing only 24% of the Northern Territory population. The majority of cases occurred in males, presumably due to a higher occupational or recreational risk of environmental exposure (Currie *et al.* 2000a). Interestingly, *B. thailandensis* has not been recovered from environmental samples in Australia.

B. pseudomallei does exist outside this traditional tropical habitat, although most cases of melioidosis in temperate countries are believed to be imported from southeast Asia or northern Australia. Dance *et al.* (1999) documented nine cases of culture-positive melioidosis in England between 1988 and 1997, all of which were imported from India, Pakistan, Bangladesh, Indonesia, Malaysia or Thailand. The importation of infected animals is also believed to be responsible for the spread of melioidosis to previously non-endemic areas. For example, an imported ram may have been responsible for an outbreak of melioidosis in Aruba (Sutmöller, Kraneveld & van der Schaaf 1957) and infected cattle from Townsville may have introduced *B. pseudomallei* to the Brisbane River Valley in southern Queensland (Ketterer, Donald & Rogers 1975). A study by Currie *et al.* (1994) supports the clonal introduction, persistence and dispersion of *B. pseudomallei* into a temperate region of Australia. The geographic spread of this disease over the last 30 years may be due to a myriad of factors including improved detection methods for *B. pseudomallei*, an increased awareness of the disease among physicians, a surge in international travelers, movement of refugees from endemic regions of southeast Asia and a rise in trade of livestock between countries (Ashdown, Duffy & Douglas 1980). Consequently, melioidosis has been documented in the USA, Central and South America, China, Korea, Vietnam, the French West Indies, India, East Pakistan, the Philippines, Fiji, Iran, Turkey, Madagascar, Africa, and France (Maegraith & Leithead 1964; Strickland 1984; Van Phung *et al.* 1993; Yabuuchi & Arakawa 1993; Pérez *et al.* 1997; Dance 2000; Yang *et al.* 2000). The existence of *B. pseudomallei* in

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Figure 2.1 The worldwide distribution of melioidosis.

Melioidosis is largely a disease of the tropics and is predominantly found in countries between the latitudes 20° north and 20° south. Southeast Asia and northern Australia are considered endemic for this disease. However, cases of melioidosis are documented outside this traditional tropical region and are believed to be attributed to increased awareness of the disease, travel to endemic regions and the importation of infected animals (Leelarsamee & Bovornkitti 1989).

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Figure 2.2 Distribution of regions endemic for melioidosis within Australia.

The first cases of human melioidosis were described from the Townsville area in 1962. The endemic region of northern Australia extends north of Rockhampton, along the east coast to Darwin and west to Tennant Creek. The Torres Strait Islands are also included in this region (Ashdown & Guard 1984).

regions beyond its traditional habitat illustrates the nutritional and ecological versatility of this bacterium.

B. pseudomallei is an environmental saprophyte that has been isolated from soil, stagnant streams and ponds, rice paddies and market produce in endemic areas (Strickland 1984). In southeast Asia, *B. pseudomallei* yields are greatest from cleared, cultivated and irrigated agricultural sites (Dance 2000). *B. pseudomallei* is regarded as an opportunistic pathogen and infection is more common in individuals with a close association with contaminated soil, as indicated by the high prevalence of melioidosis in rice farmers and their families in northeast Thailand (Rode and Webling 1981; Chaowagul *et al.* 1989). Melioidosis is also documented in individuals following hunting trips in wet terrain, automobile accidents and even gardening (Piggot 1976). The distribution of *B. pseudomallei* in the environment has been studied using a variety of different techniques, ranging in sensitivity (Ellison, Baker & Marippan 1969; Ashdown 1979; Thomas & Forbes-Faulkner 1981; Wuthiekanun *et al.* 1995; Brook, Currie & Desmarchelier 1997). The potential persistence of *B. pseudomallei* in a viable, non-cultivable state in the environment necessitates the development of a technique that is capable of isolating this form of the bacterium (Dance 2000).

Little is known with regard to the interaction of *B. pseudomallei* with its environment and the conditions under which infection is acquired. The bacterium is capable of invading and replicating within free-living amoebae (Inglis *et al.* 2000b), a characteristic that may have important implications for the persistence of *B. pseudomallei* in the environment. Soil and surface water that receives filtered light is more likely to contain *B. pseudomallei* than that in direct sunlight and the numbers of organisms present correlates with temperature, rainfall, humidity and soil composition (Thomas, Forbes-Faulkner & Parker 1979). In studies performed in Townsville, Thomas *et al.* (1979) demonstrated the ability of *B. pseudomallei* to survive the wet and dry seasons within the clay layer of the soil, found more than 30 cm below the surface. They postulated that the increase in incidence of melioidosis observed after periods of high rainfall occurred when rising water tables carried the bacteria to the soil surface. However, since samples

for this study were obtained from a single sheep enclosure where the animals may have served as an additional reservoir for *B. pseudomallei*, the survey site is not regarded as being representative of an undisturbed environment (Brook, Currie & Desmarchelier 1997). Using RAPD analysis of soil isolates from a goat paddock associated with melioidosis, Haase *et al.* (1995) demonstrated that *B. pseudomallei* is distributed evenly throughout soil layers, and that there is no seasonal variation in this distribution. These observations are supported by an environmental study performed in the Northern Territory using selective culture and PCR that demonstrated the persistent distribution of *B. pseudomallei* throughout soil layers during both the wet and dry seasons (Brook, Currie & Desmarchelier 1997). Furthermore, no change in the isolation rate of *B. pseudomallei* was observed during the wet season (Brook, Currie & Desmarchelier 1997). The authors attribute the low incidence of melioidosis during the dry season to both the possible existence of an inactive form of *B. pseudomallei*, and to behavioral factors that increase host exposure to contaminated environments during the wet season (Haase *et al.* 1995; Brook, Currie & Desmarchelier 1997). Increased numbers of biting insects during the wet season and the increased frequency of skin lesions associated with them may contribute to increased prevalence of melioidosis. Further postulated explanations for the seasonal variation observed with melioidosis includes acute seasonal immunosuppression and seasonal dietary changes (Suputtamongkol *et al.* 1994). Although infections with *B. pseudomallei* peak during the wet season, the bacterium is capable of surviving for extended periods in areas which experience long dry seasons (Rode and Webling 1981; Guard *et al.* 1984). *B. pseudomallei* requires few nutrients and can survive in normal saline for more than 726 days (Tong *et al.* 1996). Laboratory investigations have also shown that *B. pseudomallei* can survive in dry soil or faeces for up to four weeks, and in shaded, moist soil for up to 30 months (Laws & Hall 1964; Thomas & Forbes-Faulkner 1981). The reliance on heavy rainfall for the dispersal of *B. pseudomallei* was also questioned following investigation of an outbreak in Western Australia during the dry season of 1997 (Inglis *et al.* 1999). The authors recovered *B. pseudomallei* from a water storage tank and an aerator unit (Inglis *et al.* 2000a). These isolates were shown to be identical to isolates recovered from human melioidosis cases and the authors postulated that this communal water supply was the

contamination source for the outbreak (Inglis *et al.* 1998b; Inglis *et al.* 2000a). Further research is still necessary to increase our understanding of the ecological factors that promote the survival of *B. pseudomallei* in the environment, and the conditions under which transmission of the bacterium is likely to occur.

2.2.3 Isolation and identification

B. pseudomallei is a gram negative, pleomorphic, non-spore-forming, short rod which often exhibits bipolar staining (Manson-Bahr & Bell 1987). The bacterial cells have an approximate length and width of 1.5 µm and 0.8 µm, respectively (Miller *et al.* 1948a). Compared to *B. mallei*, this bacterium is more actively motile, possessing one or more polar flagella. *B. pseudomallei* is a facultative anaerobe, capable of growth in the presence of nitrates. Such a characteristic favours the survival of *B. pseudomallei* in lesions of infected host tissue, as well as in soil where it can utilise nitrate produced by the actions of nitrifying soil bacteria on fertilisers (Wongwanich *et al.* 1996). The growth of *B. pseudomallei* occurs within temperatures of 18°C to 42°C and a pH of 5 to 8 (Manson-Bahr & Bell 1987; Tong *et al.* 1996). An important feature used in the identification of *B. pseudomallei* in the laboratory is its' resistance toward the antibiotics colistin and gentamicin (Ashdown 1979). Due to the significant level of DNA homology that exists between *B. cepacia* and *B. pseudomallei*, identification in the laboratory is often difficult. *B. pseudomallei* and *B. cepacia* have similar microscopic features, colonial morphology and both are relatively resistant to aminoglycosides.

Most routine media used in the laboratory supports the growth of *B. pseudomallei* as it requires no specific growth factors. The colonial morphology of *B. pseudomallei* is highly variable and often medium-dependent thus making it difficult to identify the organism based on this feature alone. Molecular analysis of colonies with different morphologic appearances has failed to show any differences in the genomic macrorestriction profiles (Koonpaew *et al.* 2000). Nigg *et al.* (1955) described colonies that varied in colour, ranging from yellow, to orange and brown. Size and texture of the colonies were also highly varied. Two different colonial types were described: highly corrugated or rough (R), dense, granular colonies; and smooth (S), convex, translucent

colonies. On agar, *B. pseudomallei* colonies reach maximum size by 48 to 72 hours and these produce a distinct earthy, musty odour (Miller *et al.* 1948a).

The development of a selective media by Ashdown (1979) greatly improved the isolation and identification of *B. pseudomallei* from environmental and clinical samples. Ashdown agar consists of a trypticase soy base with various selective agents added. Glycerol (4%) enhances the characteristic wrinkled colonial morphology, while the addition of gentamicin (4 mg/L) restricts the growth of many pseudomonads and soil bacteria commonly found in environmental samples. Crystal violet (5 mg/L) and neutral red (50 mg/L) are also added to inhibit the growth of many contaminating organisms. The dyes are taken up by *B. pseudomallei*, staining the colonies mauve or lilac (Ashdown 1979). On blood agar, hemolysis may be seen surrounding areas of heavy growth. In standing nutrient broth, *B. pseudomallei* produces a thick wrinkled pellicle by 24 hours (Magee *et al.* 1967).

B. pseudomallei is nutritionally versatile, being able to utilise almost 88 different organic compounds as sole carbon sources including fucose, trehalose, maltose, starch and cellobiose (Smith *et al.* 1987). This organism is positive for both catalase and oxidase, attacks glucose oxidatively, and reduces nitrates to nitrites (Zierdt 1972). Tests for indole and methyl red-Voges-Proskauer (MR-VP) are negative and esculin is unable to be hydrolysed (Brook, Currie & Desmarchelier 1997). One discerning feature of this bacterium is the presence of arginine dihydrolase and absence of lysine decarboxylase (Brook, Currie & Desmarchelier 1997). The API 20NE and Microbact 24E are two commercially available tests used to identify *B. pseudomallei* on the basis of biochemical tests. The API 20NE identified 97.5% of *B. pseudomallei* strains in an area endemic for melioidosis (Dance *et al.* 1989b). However, it has less than 100% accuracy and can potentially misidentify *B. pseudomallei* as *B. cepacia* or *Chromobacterium violaceum* (Dance *et al.* 1989c; Inglis *et al.* 1998a). The Microbact 24E is an alternative method, although more evaluation as to its accuracy in the diagnostic field is necessary (Inglis *et al.* 1998a).

Genetic typing methods are being employed with increasing frequency for the identification and characterisation of *B. pseudomallei*. Lew & Desmarchelier (1993) compared patterns of restriction fragment length polymorphisms in rRNA genes (ribotyping) to develop a typing scheme for *B. pseudomallei*. Later using this method Pitt *et al.* (2000) succeeded in identifying 44 different patterns. Of these, three ribotypes were shown to occur with the greatest frequency, one of them predominantly found only in Asia (Pitt, Trakulsomboon & Dance 2000). However, the level of discrimination achieved with ribotyping is not sufficient to allow strain identity to be determined. Random amplified polymorphic DNA (RAPD) analysis, a technique exhibiting increased sensitivity, has also been employed to investigate the epidemiology of melioidosis (Haase *et al.* 1995). Using RAPD analysis it is possible to subdivide the individual ribotype patterns into unrelated strains. This proved a useful tool for the confirmation of clonality in outbreaks of melioidosis (Haase *et al.* 1995). The same study also suggested that identification of a given strain does not enable clinical outcome of infection to be predicted (Haase *et al.* 1995). However, particular RAPD patterns have been shown to be more prevalent in clinical versus environmental isolates, and vice versa (Leelayuwat *et al.* 2000). A study by Norton *et al.* (1998) also reported an association between disease presentation and particular isolates. Isolates from individuals presenting with lung or brain involvement differed from those recovered from individuals presenting with visceral abscesses or bone and joint involvement (Norton *et al.* 1998). However further studies are necessary to investigate the potential relationship between particular *B. pseudomallei* strains and clinical manifestations of infection.

Studies by Desmarchelier *et al.* (1993) using ribotyping suggested that for the majority of cases, the original isolate is responsible for recurrent infections. This finding was supported by Vadivelu *et al.* (1997; 1998) and then by Koonpaew *et al.* (2000) who demonstrated identical DNA macrorestriction profiles in 88.6% of primary and secondary infective strains from patients with recurrent melioidosis. However, the authors acknowledge the possibility of reinfection with an isolate of the same genotype as the original infective strain (Koonpaew *et al.* 2000). Concurrent infection with more

than one strain has also been demonstrated using DNA macrorestriction profiles (Vadivelu *et al.* 1998).

The search for a highly sensitive system that allows detection of *B. pseudomallei* in both environmental and clinical samples is ongoing. Environmental investigations are often hampered by the heterogeneous population of bacteria that may be present. Similarly, problems arise when clinical specimens containing *B. pseudomallei* are overgrown with normal microbial flora. Initially, these obstacles were overcome by selective culture enrichment of environmental and clinical samples. In 1992, it was proposed by Ashdown & Clarke that maximum recovery of *B. pseudomallei* from simulated positive soil samples is achieved following enrichment in trypticase soy broth with crystal violet (5 mg/L) and colistin (20 mg/L) then subculture onto Ashdown agar. A comparison of the Ashdown method with another that utilised a threonine-based salt solution (Wuthiekanun *et al.* 1995), demonstrated that recovery rates for dry soil were higher using the Ashdown method (Brook, Currie & Desmarchelier 1997). Numerous groups, with varied success, subsequently used PCR and hybridisation as identification techniques (Lew & Desmarchelier 1994; Brook, Currie & Desmarchelier 1997; Bauernfeind *et al.* 1998). Since the nucleotide sequences of the 16S rDNA of *B. pseudomallei* and *B. mallei* are identical (Yabuuchi *et al.* 1992), it is not possible to differentiate between them at this level. Lew & Desmarchelier (1994) constructed an oligonucleotide probe against the 23S rDNA of *B. pseudomallei* to evaluate its potential for use with environmental and clinical samples. Using these methods, the authors were able to detect 10^4 cfu per ml of artificially inoculated blood or 10^3 cfu per ml from seeded sputum samples. Concentration of bacteria by centrifugation or culture enrichment of samples prior to PCR increased the sensitivity to 10^2 cfu per ml of blood. However, the probe designed by Lew & Desmarchelier (1994) hybridised with *B. mallei* DNA. Later, Bauernfeind *et al.* (1998) demonstrated heterogeneity between the 23S rDNA sequences among different *B. pseudomallei* strains and successfully developed a set of PCR primers that could discriminate between *B. pseudomallei* and *B. mallei*.

B. pseudomallei and *B. thailandensis* share similar phenotypes and biochemical profiles, but differ in their ability to assimilate L-arabinose (Wuthiekanun *et al.* 1996). The presence of both *B. pseudomallei* and *B. thailandensis* in soil of endemic regions of northeast Thailand necessitated the development of techniques to readily distinguish between the two bacteria. PCR techniques based on differences in the nucleotide sequence of the 16S rRNA-encoding gene (Dharakul *et al.* 1999), the flagellin-encoding gene (Sonthayanon *et al.* 2002) and repetitive elements in the DNA (Liu *et al.* 2002) have successfully discriminated between *B. pseudomallei* and *B. thailandensis*. However, improvements to the sensitivity of these techniques for the identification of *B. pseudomallei*, particularly from environmental samples, are still required.

2.2.4 Pathogenicity determinants

B. pseudomallei is largely an opportunist, causing infection in situations where the hosts' immune system is impaired, the infecting strain is highly virulent, the inoculating dose is sufficiently high, or any combination of these factors (Ashdown 1991). Close contact between certain bacteria and eukaryotic host cells triggers the assembly of type III secretion systems (TTS), allowing the insertion of bacterial pathogenic proteins into the host cells. Genes encoding two TTS have been identified in *B. pseudomallei*. One cluster is thought to allow bacterium-plant interactions (TTS-1), while the other may target the infection of animal cells (TTS-2) (Winstanley, Hales & Hart. 1999; Attree & Attree 2001; Rainbow, Hart & Winstanley 2002). Similar to *B. pseudomallei*, *B. mallei* contains both TTS-1 and TTS-2, however TTS-1 is absent in *B. thailandensis* (Rainbow, Hart & Winstanley 2002). A study by Stevens *et al.* (2002) demonstrated the importance of TTS in equipping *B. pseudomallei* with the ability to exist within host cells. The extent of the virulence factors of *B. pseudomallei* is not fully known, however a range of different components are likely to contribute to the pathogenesis of melioidosis, rather than a single determinant.

2.2.4.1 Capsule, pili and flagella

The presence of a protective polysaccharide coat allows *B. pseudomallei* and other bacterial pathogens such as *Neisseria* and *Haemophilus* spp. to overcome the bactericidal activity of phagocytes in the initial stages of an immune response (Lee 1987; Griffis *et al.* 1997; Masoud *et al.* 1997). Exopolysaccharides include structures from discrete capsules to slime wall formation outside the outer membrane of a bacterial cell. Steinmetz, Rhode & Brenneke (1995) identified a constitutively expressed exopolysaccharide located in a capsular structure in *B. pseudomallei* strains. Sera from patients with melioidosis reacted strongly to this exopolysaccharide indicating an immunogenic role for this structure (Steinmetz, Rhode & Brenneke 1995). Electron microscopy studies demonstrated that microcolony and biofilm formation was associated with the presence of a glycocalyx in some *B. pseudomallei* strains (Vorachit *et al.* 1995). Other strains, which possessed pili and a reduced glycocalyx, did not form microcolonies (Vorachit *et al.* 1995). Puthuchery *et al.* (1996) also demonstrated morphogenic variants of *B. pseudomallei* that differed in the amounts of glycocalyx present. It is possible that biofilm formation may assist *B. pseudomallei* in the evasion of host immune responses, and protect the bacteria from the actions of antibiotics.

Kawahara *et al.* (1998) have found that *B. pseudomallei* produces three kinds of capsular polysaccharide (CP), depending on the presence of glycerol in the growth medium. CP-1a and CP-1b are present when *B. pseudomallei* is grown in the absence of glycerol. In contrast, addition of glycerol to growth media results in predominantly CP-2 production (Kawahara, Dejsirilert & Ezaki 1998). The capsular polysaccharide layer appears to facilitate attachment of *B. pseudomallei* to the respiratory tract, although the attachment ability of the bacterium was demonstrated to be weak (Ahmed *et al.* 1999). Further studies are required to understand the role of capsular polysaccharide in the pathogenesis of *B. pseudomallei* infection.

B. pseudomallei also possesses pili, allowing them to attach to host cells to initiate infection (Smith *et al.* 1987). However, the presence of pili can be detrimental to the survival of the bacterial cells *in vivo* since they serve as ligands to which phagocytic

cells can attach (Kelly *et al.* 1989). Adherence of *B. pseudomallei* to host cells is regulated by the temperature at which the bacterial cells are grown, the composition of the media and the growth phase of the bacterium (Brown *et al.* 2002). Bacterial cells grown at 27°C demonstrated the greatest attachment ability since bacterial adhesion molecules are downregulated when temperatures approach 37°C. Studies into the bacterial molecules enabling attachment to host cells are currently being investigated (Brown *et al.* 2002). The authors speculate that temperature regulation of adherence may play a role in initial transmission of *B. pseudomallei* from the environment to a host.

A polar tuft of one to four flagella provides motility of *B. pseudomallei*. Transposon mutagenesis has been used by two groups to generate aflagellate, non-motile *B. pseudomallei* mutants (DeShazer *et al.* 1997; Chua, Chan & Gan 2003). The use of these mutants to investigate the role of flagella as virulence determinants in animal models produced conflicting results. Originally Brett, Mah & Woods (1994) reported that polyclonal antisera raised against *B. pseudomallei* flagellin proteins gave partial protection against a lethal dose of *B. pseudomallei* in diabetic rats, suggesting that flagella may be important in pathogenesis. Several years later, the same group produced an aflagellate mutant that did not demonstrate any significant difference in virulence when compared to a wild-type strain following intraperitoneal infection of Syrian hamsters and infant diabetic rats (DeShazer *et al.* 1997). The authors suggested that while flagella may not be essential virulence determinants, flagellin proteins may serve as protective immunogens in melioidosis (DeShazer *et al.* 1997). The findings of DeShazer *et al.* (1997) are in conflict with a recent study demonstrating that their aflagellate mutant was avirulent following both intranasal and intraperitoneal infection of mice (Chua, Chan & Gan 2003). The discrepancy between the results of the two studies may be due to the use of different animal models to assess virulence attenuation. Chau *et al.* (2003) also found that while flagella are important for establishing infection *in vivo*, they are not necessary for invasion of cultured human lung cells. To explain these findings, the authors proposed that host cell receptors for *B. pseudomallei* flagella may exist *in vivo*, and that these surface properties may be different in cultured cells

(Chua, Chan & Gan 2003). Clearly, further investigation of the true role of flagella and motility in establishing *B. pseudomallei* infection is still required.

2.2.4.2 Toxins

Lipopolysaccharide (LPS) or endotoxin is one of the main virulence factors of gram negative bacteria, as well as a major surface antigen. An early study suggested that endotoxin plays only a small role in the pathogenesis of melioidosis due to its low activity (Heckly & Nigg 1958). The LPS of *B. pseudomallei* (BP-LPS) is virtually species-specific, cross-reacting only with *B. mallei* and possesses unique acid-stable structures in the inner core region attached to the lipid A moiety (Kawahara *et al.* 1992; Pitt, Aucken & Dance 1992). Until recently, two types of BP-LPS were recognised, based on different O-antigenic polysaccharide components (Perry *et al.* 1995). O-PS I and O-PS II were reported as being structurally distinct and existing in roughly equal amounts (Perry *et al.* 1995). O-PS II is more immunogenic and is responsible for the resistance of *B. pseudomallei* to normal human serum (Masoud *et al.* 1997; DeShazer, Brett & Woods 1998). Monoclonal antibodies to O-PS II provide partial protection from a lethal dose of *B. pseudomallei* in diabetic rats (Bryan *et al.* 1994). Recent work by Reckseidler *et al.* (2001) suggests that the structure originally identified as O-PS I may in fact be a major component of the *B. pseudomallei* capsule.

Compared to the LPS of *Salmonella abortus equi* (SAE-LPS), BP-LPS demonstrated significantly weaker pyrogenic and lethal toxicity in rabbits and mice, respectively (Matsuura *et al.* 1996). Macrophage activation, as assessed by interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- α) and nitric oxide (NO) production was also less in macrophage cultures stimulated with BP-LPS compared to SAE-LPS (Matsuura *et al.* 1996). However, BP-LPS induced strong proliferation of splenocytes, including those from LPS-resistant mice (Matsuura *et al.* 1996). Shedding of BP-LPS also occurs when the bacterium is grown *in vitro* (Anuntagool *et al.* 1999) and this may have important implications on the immunopathogenesis of melioidosis.

The rapid onset and fulminant nature of septicaemic melioidosis is believed to be the result of exotoxin production by *B. pseudomallei*. Although several groups have conducted research into the presence of a *B. pseudomallei* exotoxin, variations in laboratory protocols make it difficult to draw parallels between different studies. Culture filtrates of *B. pseudomallei* appear to contain at least two exotoxins. One toxin is lethal to mice, whereas the other is nonlethal but necrotizing, causing haemorrhage at the site of injection (Heckly & Nigg 1958). The production of exotoxin *in vivo* was demonstrated in two Malaysian studies. Using ELISA, 49.3% of sheep that had environmental exposure to *B. pseudomallei* were seropositive for antibodies against an exotoxin of this bacterium (Ismail *et al.* 1991). In a separate study, anti-*B. pseudomallei* exotoxin antibodies were detected in the serum of 54.4% of Malaysian military personnel (Embi *et al.* 1992).

Häußler *et al.* (1998) described a heat-stable glycolipid possessing both haemolytic and cytolytic properties, although the role of this exolipid as a virulence determinant is unknown. In a study by Haase *et al.* (1997), a cytolethal toxin (CLT) was isolated from various *B. pseudomallei* strains and shown to exhibit differential cytolethality *in vitro*. Cytolethal activity was lowest in isolates of the soil, and highest in isolates taken from patients with severe *B. pseudomallei* encephalitis. The correlation between cytotoxicity of a strain and disease manifestation is not direct, since underlying disorders may also influence the outcome of infection with *B. pseudomallei*. The mechanisms regulating toxin expression are not known. Low concentrations of *B. pseudomallei* exotoxin inhibits cellular protein synthesis, possibly via ADP-ribosylation of elongation factor-2 (EF-2; Mohamed *et al.* 1989). Mohamed *et al.* (1989) also demonstrated a strong inhibitory effect of the exotoxin on DNA synthesis at 48 hrs in cultures of mouse peritoneal macrophages. Such an effect may have important implications on the bactericidal efficiency of macrophages *in vivo* and the subsequent clearance of *B. pseudomallei* by the host immune system. Attempts to produce monoclonal antibodies against *B. pseudomallei* exotoxin are currently being undertaken (Nathan *et al.* 2002; Su, Lim & Nathan 2003). The development of such antibodies will enhance our understanding of the role of exotoxin in the pathogenesis of *B. pseudomallei* infection.

2.2.4.3 Extracellular enzymes

Ashdown & Koehler (1990) demonstrated the production of at least two haemolysins by *B. pseudomallei*. The first was heat-stable and weakly cytolytic and its actions can be observed on sheep blood agar around areas of heavy growth. A second, less common haemolysin was characterised as heat-labile and cytolytic, producing α -hemolysis around individual colonies of *B. pseudomallei* on sheep blood agar. Out of one hundred clinical isolates of *B. pseudomallei* studied by Ashdown & Koehler (1990), all were capable of producing at least one extracellular enzyme. Some biologically active substances released by *B. pseudomallei* that are believed to contribute to the virulence of this bacterium include lecithinase, lipase and protease (Ashdown & Koehler 1990; Sexton *et al.* 1994; Lee & Lui 2000). *B. pseudomallei* also expresses phospholipase C (PLC), an enzyme that is also known to be important for the intracellular survival and cell-to-cell spreading of *L. monocytogenes* (Smith *et al.* 1995). The role of PLC as a virulence factor in melioidosis is unknown.

A protease characterised by Sexton *et al.* (1994) is a 36 kDa metalloenzyme that requires iron for maximum activity and has the ability to digest human complement component C3, all major classes of immunoglobulins, transferrin, haemoglobin, muscle proteins and collagen. Strains deficient in this protease are less virulent in animal models (Sexton *et al.* 1994). Percheron *et al.* (1995) also identified a Zn^{2+} -dependent protease. However this was later shown to have little influence on *B. pseudomallei* virulence in a murine model (Gauthier *et al.* 2000).

2.2.4.4 Siderophores

In order to initiate and maintain infection, bacteria must possess the ability to acquire iron from their host since this compound is essential for bacterial growth and multiplication (Payne & Finklestein 1978). Most iron within mammals is intracellular, with a small amount bound to transferrin in serum, or lactoferrin in mucosal secretions and granules of neutrophils. Bacteria often produce siderophores, or specific iron chelators that compete for the hosts iron. Siderophore production contributes to the virulence of organisms such as *Escherichia coli*, *P. aeruginosa* and *B. cepacia* (Cox

1982; Sokol & Woods 1988). Yang, Chaowagul & Sokol (1991) identified a siderophore produced by *B. pseudomallei*, which they named malleobactin. Malleobactin production was increased in the absence of iron *in vitro*, and consequently led to enhanced growth of *B. pseudomallei*. This iron chelator removes iron more efficiently from transferrin and lactoferrin, than from the hosts intracellular iron stores (Yang, Kooi & Sokol 1993). Currently, no comparative studies have been performed to determine if a correlation exists between malleobactin production and virulence of *B. pseudomallei* strains.

2.2.4.5 Insulin receptors

A significant number of patients who develop septicaemic melioidosis also have pre-existing diabetes mellitus, or type 1 diabetes (Chaowagul *et al.* 1989; Puthuchery, Parasakthi & Lee 1992). This form of diabetes is an autoimmune disease and involves the destruction of pancreatic beta cells and subsequent insulin insufficiency, causing blood glucose levels to rise (Barnett 1991). Diabetics are more prone to fungal infections, necrotizing infections and osteomyelitis and this is believed to be associated with poor circulation and a lack of local sensation observed in these patients (Woods, Jones & Hill 1993). In 1993, Woods *et al.* identified receptors on the surface of *B. pseudomallei* that specifically bound insulin molecules, resulting in a depressed growth rate of the bacterium. This effect was not seen in the growth rates of *P. aeruginosa*, *B. cepacia* and *E. coli* and was deemed a specific response to *B. pseudomallei*. These observations suggested that insulin plays an important role in the pathogenesis of *B. pseudomallei* infection. However, a more recent study suggests that the presence of m-cresol in the recombinant insulin used by Woods *et al.* (1993) may be responsible for the reported bacterial growth inhibition (Simpson & Wuthiekanun 2000). Therefore, the interaction of insulin with *B. pseudomallei* requires further clarification, particularly to reveal if this plays a significant role in the increased incidence of melioidosis in individuals with diabetes mellitus.

2.2.4.6 Intracellular survival

The ability of *B. pseudomallei* to remain latent for long periods within a host, the difficulty in antimicrobial treatment of melioidosis despite the apparent efficacy of the agents *in vitro*, and the significant proportion of healthy individuals within endemic

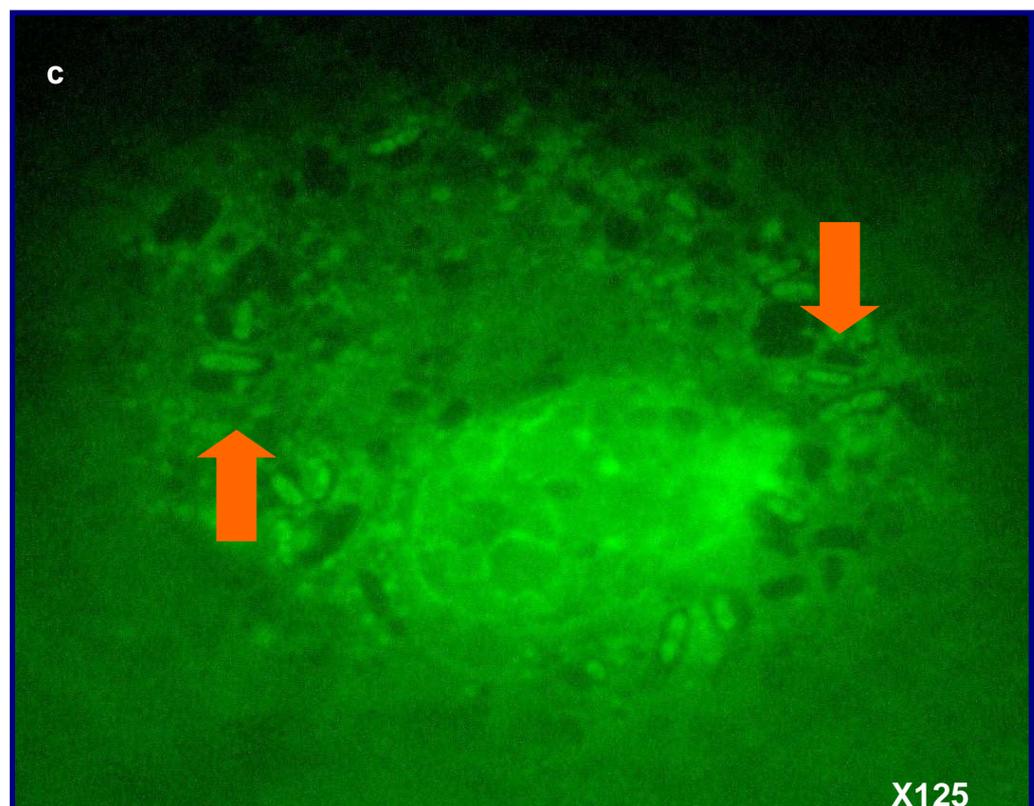
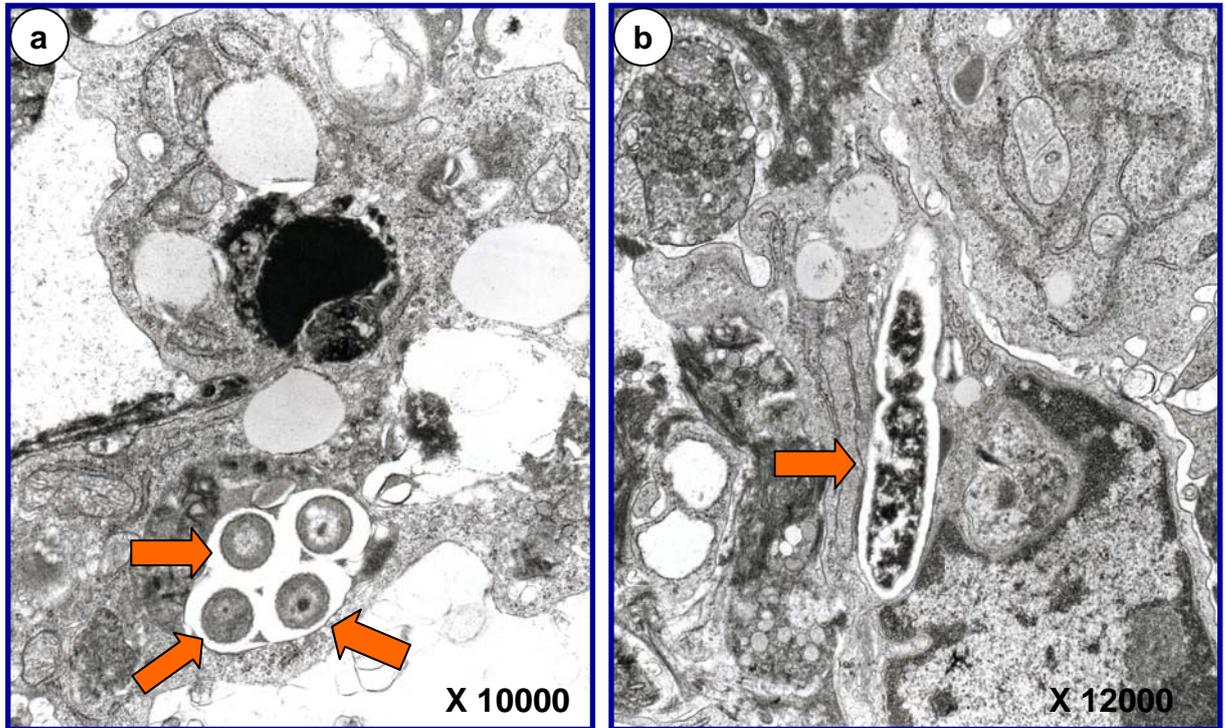
regions that are seropositive for the organism, suggested that *B. pseudomallei* may be able to survive within host cells (Thompson & Ashdown 1989; Pruksachartvuthi, Aswapokee & Thankerngpol 1990). *In vitro* studies by Pruksachartvuthi *et al.* (1990) and Jones *et al.* (1996) demonstrated the ability of *B. pseudomallei* to invade and replicate within epithelioid and fibroblast cell lines, as well as professional phagocytic cells, including human alveolar macrophages. Electron microscopy studies have illustrated the presence of *B. pseudomallei* within membrane-bound vacuoles as well as the cytoplasm of host cells (Figure 2.3; Jones *et al.* 1996; Wongwanich *et al.* 1996; Harley *et al.* 1998). Escape of *B. pseudomallei* into the cytoplasm of the host cell may occur prior to the fusion of the phagosome and lysosome (Harley *et al.* 1998).

B. pseudomallei, like other facultative intracellular bacteria, possesses various mechanisms which enable it to evade or resist the host defense mechanisms, allowing multiplication to occur even within host macrophages. Mechanisms such as evasion of phagolysosomal fusion, resistance to bactericidal lysosomal enzymes and translocation from the phagosome to the cytoplasm have been described for intracellular bacteria such as *M. tuberculosis*, *L. pneumophila* and *L. monocytogenes* (Miyagi, Kawakami & Saito 1997). Similarly, a study by Jones *et al.* (1996) found that *B. pseudomallei* is also able to resist the bactericidal actions of various compounds present in activated macrophages or neutrophils, including the cationic peptide protamine and purified human defensin (HNP-1), respectively. The resistance shown towards neutrophil defensins may enable *B. pseudomallei* to evade the bactericidal activity of these cells, allowing the subsequent interaction with macrophages arriving at the site of infection. This may contribute to the ability of *B. pseudomallei* to survive within host cells (Jones *et al.* 1996).

Multinucleated giant cell (MNGC) formation has been reported in human melioidosis and investigated in cultured cell lines (Wong *et al.* 1995; Harley *et al.* 1998; Kespichayawattana *et al.* 2000). Kespichayawattana *et al.* (2000) demonstrated that MNGC is a result of host cell fusion and actin-associated membrane protrusion induced by *B. pseudomallei*. Such a mechanism would enable cell-to-cell spreading of the bacterium within the host (Harley *et al.* 1998). However, further *in vivo* studies are

Figure 2.3 *B. pseudomallei* is a facultative intracellular bacterium.

In vitro studies and electron microscopy have demonstrated that *B. pseudomallei* is capable of survival within membrane-bound vacuoles and the cytoplasm of host cells. (a) and (b) Electron microscopy obtained from mice *iv* infected with *B. pseudomallei*. Liver was removed at 72 hrs post-infection. Under electron microscopy, *B. pseudomallei* can be observed within vacuoles of host cells. (c) Murine peritoneal macrophages were incubated *in vitro* with live *B. pseudomallei* for 24 hrs. A single macrophage containing multiple intact *B. pseudomallei* is shown (arrows indicate bacterial cells within the macrophage).



required to understand the role of MNGC formation in the pathogenesis of *B. pseudomallei* infection.

2.3 Melioidosis in Animals

B. pseudomallei is not confined to infection within humans. The first recorded cases of melioidosis in animals came from Kuala Lumpur in 1913, among laboratory guinea pigs, rabbits and rats (Stanton & Fletcher 1932, cited by Cottew, Sutherland & Meehan 1952). Prior to death, all animals exhibited weakness, fever and discharge from the eyes and nose, and upon autopsy lesions were found in the lungs and spleen (Stanton & Fletcher 1932, cited by Cottew, Sutherland & Meehan 1952). Since then melioidosis has been documented in endemic regions in domestic animals, particularly sheep, goats, cattle, horses and pigs, as well as in native mammals and birds (Cottew, Sutherland & Meehan 1952; Lewis & Olds 1952; Thomas, Norton & Pott 1980; Thomas 1981; Smith *et al.* 1987; Choy *et al.* 2000).

Similar to human melioidosis, disease manifestation in animals varies widely within a species and includes acute, chronic and subclinical forms of infection. Abscessation is common and can occur in any tissue but typically involves the lungs, liver, spleen and lymph nodes (Choy *et al.* 2000). Pulmonary involvement is frequently observed in sheep with melioidosis, whereas *B. pseudomallei* infection in goats often involves lesions of the mediastinal lymph node (Lewis & Olds 1952; Olds & Lewis 1955). CNS involvement is also documented in animals, including cows, goats and sheep (Laws & Hall 1964; Choy *et al.* 2000). Most cases of melioidosis in livestock are chronic and asymptomatic, usually only becoming apparent when lesions are discovered during routine meat inspections (Laws & Hall 1964). Horses, cattle and birds tend to be relatively resistant to infection with *B. pseudomallei* and the few cases that have been documented are believed to be attributed to exceptionally high rainfall and stress, or other underlying diseases of the animal (Cottew, Sutherland & Meehan 1952; Laws & Hall 1964). Pigs also have a high natural resistance to *B. pseudomallei*, with death occurring only under conditions of environmental stress (Ketterer *et al.* 1986). Cold-blooded animals are resistant to infection with *B. pseudomallei* (Laws & Hall 1964).

The prevalence of melioidosis in livestock has a significant economic impact, since all carcasses containing melioidosis lesions are condemned at the abattoir. An outbreak in an intensive piggery in southern Queensland resulted in infection of 159 pig carcasses (Ketterer *et al.* 1986). Handling of infected animals at the abattoir also increases the risk of transmission of *B. pseudomallei* to meatworkers and meat inspectors. The use of gloves and thorough cleaning and disinfection of knives as preventative measures have minimised risk. There is no evidence to support direct transmission of melioidosis from animals to humans although originally *B. pseudomallei* was believed to have been transmitted to humans via food contaminated with rat excreta (Cottew, Sutherland & Meehan 1952). However, blood cultures from several thousand rodents in an endemic area identified only a single strain of the bacterium (Stanton & Fletcher 1932, cited by Cottew, Sutherland & Meehan 1952). In contrast, a serological survey by Glazebrook, Campbell & Hutchinson (1977) identified 8.8% of rodents in the Townsville area as seropositive for *B. pseudomallei* using a complement fixation assay (CFA). The bacterium was also isolated from the faeces of two asymptomatic rodent carriers that had no detectable melioid lesions, supporting a role for these animals in the contamination of the environment in endemic regions (Glazebrook, Campbell & Hutchinson 1977). Biting insects have been shown to transmit *B. pseudomallei* under experimental conditions (Blanc & Baltazard 1942, cited by Cottew, Sutherland & Meehan 1952) and recently the bacterium was isolated from flies trapped in Malaysia (Sulaiman, Othman & Aziz 2000). Together these findings have important implications for our understanding of the transmission of this disease and warrant further examination into the potential role of biting insects in the transmission of *B. pseudomallei*.

Melioidosis infections in animals have played an important role in the spread of this disease throughout the world. It is believed that the endemic regions in France were created by the import of infected horses from Iran, and an outbreak in Britain was traced to the importation of infected feral monkeys from the Philippines (Leelarasamee & Bovornkitti 1989; Trakulsomboon, Pitt & Dance 1994). Similarly, sporadic cases of melioidosis have occurred in southern Queensland and southwest Western Australia in goat, cattle, sheep and pigs (Ketterer & Bamford 1967; Thomas *et al.* 1981; Ketterer *et al.* 1986; Currie *et al.* 1994). These areas lie outside the

recognised endemic regions for melioidosis in Australia and are thought to have originated from the importation of infected livestock.

Experimental infection with melioidosis can be induced in monkeys, sheep, goats, guinea pigs, rabbits, hamsters, rats and mice via parenteral inoculation, contamination of mucosal surfaces, skin scarification, ingestion or intranasal instillation (Cottew, Sutherland & Meehan 1952; Lewis & Olds 1952; Dannenberg & Scott 1958a,b; Leakey *et al.* 1998; Hoppe *et al.* 1999; Gauthier *et al.* 2001; Liu *et al.* 2002). Various groups have demonstrated that inbred mouse strains differ in their susceptibility to *B. pseudomallei* and have therefore been used as experimental models (Leakey *et al.* 1998; Hoppe *et al.* 1999; Gauthier *et al.* 2001; Liu *et al.* 2002). Each of these studies has illustrated the organotropism of *B. pseudomallei* for spleen and liver. The C57BL/6-BALB/c mouse model was characterised in this laboratory for studies into the immunopathogenesis of melioidosis (Leakey *et al.* 1998). C57BL/6 mice are relatively resistant to infection with *B. pseudomallei*. In contrast, infection of BALB/c mice with as few as 37 cfu of *B. pseudomallei* is sufficient to cause acute septicaemia and subsequent death within 72 hrs (Leakey *et al.* 1998). Hoppe *et al.* (1999) and Liu *et al.* (2002) have confirmed these observations. Results of a mendelian analysis suggested that innate resistance of C57BL/6 mice is predominantly controlled by a single locus (Leakey *et al.* 1998). However, no further investigations have been carried out to identify the genes that are important for determining resistance or susceptibility to *B. pseudomallei*. Numerous studies have demonstrated disparate levels of innate susceptibility of various inbred mouse strains toward other intracellular pathogens such as Mycobacterium, Salmonella and Leishmania (McLeod *et al.* 1995; Vidal *et al.* 1996). Natural resistance to infection with *Mycobacterium bovis*, *Salmonella typhimurium* and *Leishmania donovani* is regulated by the mouse natural resistance-associated macrophage protein 1 (*Nramp1*) gene, also known as *Bcg/Ity/Lsh* (Lissner, Swanson & O'Brien 1983; McLeod *et al.* 1995; Vidal *et al.* 1996). In the initial stages of infection, *Nramp1* regulates the functional capacity of phagocytes, subsequently affecting the disease susceptibility of the host (Vidal *et al.* 1996). Using inbred mouse strains, other genes that are involved in resistance to intracellular pathogens have also been identified, including genes that modulate the production of T_H1- and T_H2-type cytokines (McLeod *et al.* 1995). Studies on other intracellular pathogens, such as Mycobacterium and

Salmonella, will provide insight into possible mechanisms underlying the resistance or susceptibility to *B. pseudomallei* infection. Indeed, there is much scope for immunogenetic analysis of resistance to *B. pseudomallei* infection using the C57BL/6-BALB/c mouse model of human melioidosis.

2.4 Melioidosis in Humans

2.4.1 Transmission

It was originally thought that infected rodents were responsible for the transmission of *B. pseudomallei* by contamination of food with their excreta. However, this theory was questioned following extensive surveys that isolated only one strain of the bacterium from the blood of several thousand rats within an endemic area (Stanton & Fletcher 1932, cited by Cottew, Sutherland & Meehan 1952). Evidence suggests that direct contamination of penetrating wounds or superficial skin lesions is the predominant cause of *B. pseudomallei* infection (Rode & Webling 1981; Thin *et al.* 1970, DeBuse *et al.* 1975; Merianos *et al.* 1993). During the Vietnam War, where several hundred cases of melioidosis were documented in French, Vietnamese and American soldiers, the causative agent primarily gained entry to the host via injuries and burns brought in contact with ground water (Greenawald, Nash & Foley 1969; Piggot 1976).

B. pseudomallei is also able to gain entry to a host via inhalation of the organism with dust particles (Ashdown *et al.* 1980; De Buse *et al.* 1975), aspiration of contaminated water (Lee *et al.* 1985; Vimuktalaba *et al.* 1985; Pruekprasert & Jitsurong 1991) and ingestion (Thomas *et al.* 1979; Ketterer *et al.* 1986). The influence of the route of transmission of *B. pseudomallei* on the pathogenesis of infection is not known. Investigations carried out in an experimental model to compare disease outcome following *B. pseudomallei* infection by various routes are discussed in Chapter 4. Ingestion of *B. pseudomallei* was highly suspected in a human case of melioidosis presenting at the Royal Darwin Hospital, Northern Territory where the patient presented with peritonitis and intra-abdominal abscesses (Currie *et al.* 2000a). Nosocomial infections have arisen due to the contamination of

antiseptics, intravenous fluid, bronchoscopes and catheters (Ashdown 1979). Mother-to-child transmission of *B. pseudomallei* has also occurred as a result of placental infection (Abbink, Orendi & de Beaufort 2001). Neonates are able to acquire *B. pseudomallei* from the birth canal, infected amniotic fluid or contaminated equipment (Leelarasamee & Bovornkitti 1989). There have been reported cases of laboratory-acquired melioidosis following exposure to aerosols generated by sonication or centrifugation (Green & Tuffnell 1968; Schlech *et al.* 1981) and unconfirmed reports of deaths from melioidosis associated with bioweapon development (Alibek 1999). More recently, studies have suggested an association of *B. pseudomallei* infections with unchlorinated, or insufficiently chlorinated, potable water supplies (Inglis *et al.* 2000a; Currie *et al.* 2001). Investigations into this possible source of infection are continuing.

Transmission from animal to man has not yet been documented. Person-to-person transmission is rare but cases have been documented following transmission by the venereal route (McCormick *et al.* 1975). A large proportion of melioidosis cases in Australian Aborigines affect the lower genito-urinary tract, supporting a role for sexual transmission of *B. pseudomallei* (Webling 1980). Human transmission of *B. pseudomallei* between siblings with cystic fibrosis was also speculated in a recent case report (Holland *et al.* 2002). While there is evidence of human-to-human transmission, secondary cases of melioidosis are rare and with regards to communicability, the disease is not considered a major threat to public health (McCormick *et al.* 1975; Rode & Webling 1981). As a general precaution, individuals residing in areas endemic for melioidosis, particularly those with associated risk factors, are advised to wear protective clothing to prevent potential exposure to *B. pseudomallei* in soil or surface water. It is also recommended that skin abrasions or lesions are covered and thoroughly cleaned after contact with soil or ground water in endemic areas.

2.4.2 Associated Risk Factors

In 20 to 36% of patients with melioidosis there are no identifiable risk factors, the disease seemingly affecting apparently healthy individuals (Currie 2003). However, melioidosis is more commonly associated with immunocompromised patients. Predisposing conditions include diabetes, chronic alcoholism, drug addiction, cancer,

pregnancy, and malnutrition (Ashdown, Duffy & Douglas 1980; Rode & Webling 1981; Guard *et al.* 1984; Merianos *et al.* 1993). Melioidosis has also been associated with lung cancer (Mays & Ricketts 1975) and steroid treatment (So *et al.* 1983). Clinical reviews in Australia revealed diabetes mellitus and alcoholism to be the most frequently associated conditions, while diabetes mellitus and renal failure were the two primary underlying disorders associated with melioidosis in northeast Thailand (Guard *et al.* 1984; Chaowagul *et al.* 1989). A serological survey by Ashdown & Guard (1984) in north Queensland also demonstrated increased seroprevalence in individuals with alcohol addiction (15%), liver disease (13%) and diabetes (9%) compared to the seroprevalence of the general population (5.7%). An association between melioidosis and dengue hemorrhagic fever has also been demonstrated (Pongrithsukda, Simakachorn & Pimda 1988). In the Northern Territory of Australia, excessive consumption of kava, an extract from a plant root, is a recognised risk factor for melioidosis among the indigenous population (Currie *et al.* 2000c). Melioidosis is also more prevalent in males, presumably due to a higher risk of occupational exposure for this gender (Suputtamongkol *et al.* 1994; Currie *et al.* 2000c).

There have been several case reports of *B. pseudomallei* isolation from sputum samples of patients with cystic fibrosis (CF) following travel to regions that are endemic for melioidosis (Dance *et al.* 1999; Schulin & Steinmetz 2001; Visca *et al.* 2001; Holland *et al.* 2002; O'Connell *et al.* 2003). CF is a fatal genetic disorder in the caucasian population, with an incidence of 1:2500 births (Visca *et al.* 2001). Bacterial infection, most commonly *B. cepacia*, of the lung results in rapid deterioration of respiratory function and ultimately death. Individuals with CF are considered to have a higher risk of infection with *B. pseudomallei*, compared to healthy individuals (Holland *et al.* 2002; O'Connell *et al.* 2003).

Human immunodeficiency virus (HIV) the cause of acquired immunodeficiency syndrome (AIDS) is another emerging infectious disease that affects CD4⁺ T lymphocytes and dendritic cells, consequently impairing other host immune mechanisms that are regulated by these cells (Abbas, Lichtman & Pober 2000). Similar to other immunocompromising conditions such as diabetes mellitus, it is reasonable to expect to see an association between *B. pseudomallei* and HIV

infection in regions that are endemic for melioidosis. However, few studies have investigated the association between these two diseases and the likelihood of HIV as a risk factor for melioidosis. In Thailand, one fatal case of recurrent melioidosis was documented in an AIDS patient (Kanai *et al.* 1992). However, a serological survey for HIV in patients with melioidosis in Thailand did not find evidence of combined infection with HIV and *B. pseudomallei* (Kanai & Kondo 1994). Due to the limited research that has been carried out to date, it is not possible to postulate the extent of the association between HIV infection and melioidosis but it is certainly an avenue for future investigation.

2.4.3 Clinical features

Melioidosis incorporates a wide spectrum of clinical disease caused by infection with *B. pseudomallei*. Cases of human melioidosis show a broad variation with respect to severity, duration and clinical features. Clinical manifestations vary greatly, ranging from an asymptomatic carrier state to an overwhelming septicaemia which, in its severest form, may result in death within 48 hours (Strickland 1984). Melioidosis is considered a “medical timebomb” since the time period between exposure to *B. pseudomallei* and the onset of clinical symptoms ranges from several days to many years. Recrudescence or reactivation of *B. pseudomallei* infection to clinical melioidosis is associated with a decrease in host immunocompetence caused by conditions such as diabetes mellitus, chronic lung disease or alcoholism. A study by Chaowagul *et al.* (1993) also demonstrated that 23% of individuals, who appear to respond successfully to treatment of initial infection with *B. pseudomallei*, subsequently develop a second infection, months or years later. Almost a quarter of individuals that do relapse, develop fatal septicaemia (Chaowagul *et al.* 1993). Mechanisms responsible for the association between disease relapse and a failure of the host immune system to contain *B. pseudomallei* infection have not been investigated.

B. pseudomallei affects virtually any organ system and can result in either localised or disseminated infection. Table 2.1 lists the organ systems that are commonly affected in melioidosis and the predominant infections which are observed.

Table 2.1. Common clinical manifestations of melioidosis.

Organ System	Common clinical manifestations
Respiratory	Pneumonia, lung abscess, pleural effusion, empyema
Skin & soft tissue	Cellulitis, skin ulcers, subcutaneous abscess, chronic granuloma, parotitis
Skeletal & joints	Septic arthritis, osteomyelitis
Genito-urinary	Pyelonephritis, prostatic abscess, prostatitis, kidney abscess
Hepatobiliary	Liver abscess, spleen abscess
CNS	Brain abscess, spinal abscess, brainstem encephalitis, acute paraplegia
Cardiovascular	mycotic aneurysm
Generalised infection	Pyrexia of unknown origin (PUO), septicaemia

Histologically, infections can range from acute suppurative to chronic granulomatous inflammation, with tissue necrosis often evident. For this reason melioidosis is known as the “great imitator” of diseases, having been confused with other diseases such as tuberculosis, malaria, typhoid and histoplasmosis (Cowan & Heap 1993; Cox 1996). While it is recognised that the severity of the disease falls along an almost continuous spectrum, for convenience clinical melioidosis is often categorised into acute, subacute and chronic forms. In humans, lung involvement is common and pulmonary melioidosis can occur as a primary focus of infection, or following dissemination of *B. pseudomallei* to the lungs in patients with septicaemia (Ip *et al.* 1995).

2.4.3.1 Acute infections

Acute *B. pseudomallei* infection usually presents as a severe, fulminating illness, often involving severe vomiting, diarrhoea and collapse resulting in death within 2 to

3 days of the onset of symptoms (Dannenberg & Scott 1958a). Chills, fever and prostration are usually accompanied by azotemia, hypoglycaemia, leucopaenia and jaundice (Chaowagul *et al.* 1989). Pneumonia and multiorgan dissemination frequently occurs. Septicaemia may be primary or terminal, the latter resulting from reactivation of chronic *B. pseudomallei* infection (Saltos *et al.* 1976). Septicaemic melioidosis is the most commonly presented form of the disease in northeast Thailand where it accounts for one-fifth of all documented cases of community-acquired septicaemias (Chaowagul *et al.* 1989). Metastatic abscesses up to 3 cm in diameter, are often found in the lungs, liver and spleen following septicaemia, and histologically show evidence of acute suppurative inflammation and the presence of *B. pseudomallei* (Kingston 1971; Piggot 1976; Manson-Bahr & Bell 1987).

In the Northern Territory of Australia and parts of Thailand, melioidosis is a leading cause of fatal, bacteraemic community-acquired pneumonia (Boonsawat *et al.* 1990; Cheng *et al.* 2003; Currie 2003). In the majority of acute melioidosis cases, lung involvement is believed to be secondary to hematogenous spread of *B. pseudomallei*. Radiographically, the most common appearance of acute melioidosis is the presence of multiple, small irregular densities that resemble disseminated tuberculosis. Segmental or lobar consolidation may also occur (Tshibwabwa, Richenberg & Aziz 2002). Acute Respiratory Distress Syndrome (ARDS) is a common sequela and develops more rapidly in patients with acute melioidosis, than patients with sepsis caused by bacteria other than *B. pseudomallei* (Puthuchery *et al.* 2001). The authors suggest that the massive lung damage that can occur in fatal septicaemic melioidosis may result from the rupture of *B. pseudomallei*-infected PMNL in the lungs causing the release of bacteria, toxic oxygen radicals and chemokines (Puthuchery *et al.* 2001). Shock and respiratory failure contribute to the high mortality rates associated with acute, septicaemic melioidosis.

Not all patients with acute melioidosis present with septicaemia. Infections may involve almost any tissue or organ system and there are documented cases of acute prostatitis, vaginitis and urethritis (Kan & Kay 1978; Webling 1980). In northeast Thailand, one quarter of childhood melioidosis cases present as acute suppurative parotitis (Dance *et al.* 1989a). However, this form of the disease does not appear to be common in northern Australia (Currie *et al.* 2000a). The mortality rate of most

forms of melioidosis is usually below 10%, however it reaches 80 to 90% in cases of acute, septicaemic melioidosis if the appropriate treatment is not implemented quickly. With expeditious antibiotic therapy mortality is generally about 50% (Suputtamongkol *et al.* 1994).

2.4.3.2 Subacute infections

Cases of subacute melioidosis are more frequent and less severe than the acute form and account for most cases of melioidosis documented in non-endemic regions. Subacute melioidosis can result from primary infection or from reactivation of a subclinical infection. Similar to acute melioidosis, subacute infection with *B. pseudomallei* can be localised or disseminated, involving multiple organ systems.

The pulmonary form of subacute melioidosis is the most commonly encountered type in Australia. There are frequently foci of caseation or coagulation necrosis evident, often resembling lobar or bronchopneumonia and tuberculosis (Kingston 1971; Ip *et al.* 1995). Progressive weight loss and persistent low grade fever are also common signs. However, subacute infections may also involve the skin, muscle, bone and viscera and give rise to septicaemia if left untreated (Chaowagul *et al.* 1989). In the Northern Territory of Australia, infection of the urogenital tract is another common form of melioidosis observed (Webling 1980). Compared to Thailand, the occurrence of prostatic abscesses is much higher in northern Australia (Currie *et al.* 2000a)

2.4.3.3 Chronic infections

Persistent or recurrent abscesses are prominent in chronic melioidosis, although some patients may present with a fever of unknown origin without any obvious lesions (Ip *et al.* 1995). Patients with chronic melioidosis may live for decades with minimal symptoms. The most common finding of chronic melioidosis is the presence of large abscesses in the lung and/or spleen. Abscesses generally exhibit caseous necrosis as well as a granulomatous inflammatory reaction that is distinct from abscesses of acute infection (Piggot 1976; Chaowagul *et al.* 1989). Subcutaneous abscesses, sinuses down to the bone and chronic osteomyelitis are also common outcomes of chronic infection with *B. pseudomallei* (Kingston 1971; Tran & Tan 2002). Several reports of melioidosis with abscesses in unusual sites including parotid glands

(Dance *et al.* 1989a), adrenal gland (Lee *et al.* 1999), prostate gland (Tan *et al.* 2002) and mesentery (Kiertiburanabul *et al.* 2002) have been published.

In chronic melioidosis, severe muscle wasting and weight loss is often observed. Paton *et al.* (2001) demonstrated that wasting associated with chronic melioidosis is a result of decreased energy intake rather than defects in protein or energy metabolism (Paton *et al.* 2001).

2.4.3.4 Neurological melioidosis

Neurological abnormalities are documented in melioidosis, and a predilection for the brainstem and spinal cord has been suggested (Maguire *et al.* 1998; Currie *et al.* 2000b). Approximately 6% of patients with melioidosis presenting to the Royal Darwin Hospital in the Northern Territory demonstrate neurological involvement (Maguire *et al.* 1998), which can manifest as focal or diffuse encephalitis, purulent meningitis or acute flaccid paraparesis (Currie *et al.* 2000b). In many cases of neurological melioidosis, there has been no evidence of direct brain infection suggesting that damage may be a result of immune or toxin-mediated mechanisms (Maguire *et al.* 1998; Bartley *et al.* 1999). Several reports describe brain abscesses caused by *B. pseudomallei* with fatal outcomes, despite treatment (Thin *et al.* 1970; Lee & Chua 1986; Pit *et al.* 1988; Woods *et al.* 1992; Padiglione *et al.* 1997; Lath *et al.* 1998; Peetermans *et al.* 1999). Evidence suggests that neurological melioidosis does not result from infection by a specific *B. pseudomallei* strain (Currie *et al.* 2001), although further study is required to gain a true understanding of the pathogenesis of this form of the disease.

2.4.3.5 Neonatal melioidosis

The majority of documented cases of melioidosis have occurred in adults. Neonatal melioidosis is extremely rare, but frequently fatal. Sepsis and pneumonia are the predominant features of *B. pseudomallei* infection in neonates (Osteraas, Bass & Wilson 1971; Lumbiganon, Pengsaa & Puapermpoonsiri 1988; Halder *et al.* 1993), although there have also been several reports of meningitis (Lumbiganon, Pengsaa & Puapermpoonsiri 1988; Halder *et al.* 1998). *B. pseudomallei* infection in neonates can occur as a result of perinatal transmission, infection of the placenta or amniotic fluids

or from contaminated equipment or environments (Leelarasamee & Bovornkitti 1989).

2.4.3.6 Subclinical infections

Serologic studies within populations living within endemic areas in Thailand, Malaysia and northern Queensland, have demonstrated that the prevalence of melioidosis is far more widespread than the incidence of clinical cases alone would indicate (Nigg 1963; Strauss *et al.* 1969a; Ashdown & Guard 1984). Subclinical infections, manifested only by the presence of circulating antibodies, appear to be the most common form of melioidosis in these regions. This chronic carrier state is thought to exist while the host's immune system is capable of controlling or suppressing the infection with *B. pseudomallei* (Ip *et al.* 1995). Recrudescence to acute or subacute melioidosis occurs when conditions become favourable for the growth and multiplication of *B. pseudomallei* within the host, such as immunosuppression, many years after the initial infection (Piggot 1976; Ashdown 1992). A World War II veteran developed melioidosis 26 years after exposure in the Phillipines (Mays & Ricketts 1975). In this instance, recrudescence occurred due to physical deterioration associated with bronchogenic carcinoma (May & Ricketts 1975). In North Vietnam, which is also considered endemic for melioidosis, seropositivity ranges from 6.4% to 31.8% (Van Phung *et al.* 1993).

2.4.4 Diagnosis

2.4.4.1 Isolation and identification

Melioidosis should be suspected in individuals who have lived or visited endemic areas and who present with septicaemia, multiple abscesses, a pyrexia of unknown origin or doubtful etiology (Manson-Bahr & Bell 1987). Definitive diagnosis is obtained by culture of clinical specimens such as blood and pus. However, since a large proportion of patients present with deep visceral abscesses or multifocal disease, this is not always possible. Diagnosis is further compounded by the broad spectrum of clinical presentations of melioidosis and the ability of the disease to remain latent for long periods (Ashdown 1979).

Despite decades of research and advancements in technology, culture of *B. pseudomallei* from clinical specimens remains the standard method used in the diagnosis of melioidosis. Any oxidase positive, non-pyocyanogenic, gram negative bacillus with bipolar or irregular staining is suspicious in the first 24 hrs. Characteristic wrinkled colonies that produce a sweet smell of putrefaction on Ashdown agar after 48 to 72 hours are indicative of *B. pseudomallei* and subsequent biochemical tests are performed to confirm identification (Guard *et al.* 1984; Walsh *et al.* 1994). Some clinical specimens may be overgrown with contaminating flora, but if enrichment broths for *B. pseudomallei* are used prior to culturing, a further 48 to 96 hours will elapse before a diagnosis can be made. Identification of *B. pseudomallei* is also hindered due to similarities in its biochemical and physical properties with other bacteria, particularly *Burkholderia* and *Pseudomonas* spp. In 1989 the API 20NE, a biochemical-based system, identified 97.5% of *B. pseudomallei* strains in an area endemic for melioidosis (Dance *et al.* 1989c). Following the screening of *B. pseudomallei* isolates from northern Australia, Lowe *et al.* (2002) reported a similar finding. These biochemical results, combined with the observations of resistance to colistin and gentamicin, the colonial morphology on Ashdown agar and agglutination with *B. pseudomallei* antiserum, enable *B. pseudomallei* to be distinguished from other gram negative bacteria (Ashdown 1979; Dance *et al.* 1989b). However, the API20NE is less than 100% accurate, a study in Singapore demonstrating its potential to misidentify 12% of *B. pseudomallei* isolates (Inglis *et al.* 1998a). The Microbact 24E and Minitek disc system are alternative methods, although more evaluation as to their accuracy in the diagnostic field is necessary (Ashdown 1992; Inglis *et al.* 1998a). The obvious setback to using culture techniques for the diagnosis of melioidosis is the length of time involved. Such delays contribute to the high mortality associated with the septicaemic forms of melioidosis, many patients dying within 72 hours of hospital admission (Chaowagul *et al.* 1989).

2.4.4.2 Serology & immunologic techniques

While the definitive diagnosis of melioidosis is made primarily on the isolation and identification of *B. pseudomallei*, presumptive diagnosis of melioidosis can be made by serology. The indirect hemagglutination (IHA) test is the most widely used serologic assay for the diagnosis of melioidosis. *B. pseudomallei* antigens, derived

from either a crude autoclaved culture filtrate or LPS, are adsorbed directly to ovine erythrocytes which are then incubated with the patient's serum. In the presence of specific antibody, haemagglutination occurs (Alexander *et al.* 1970). An IHA titer of $\geq 1:40$ is generally regarded as evidence of exposure to *B. pseudomallei*. While sensitive (95%), Appassakij *et al.* (1990) reported that this test was not capable of recognising patients with the acute fulminating form of melioidosis. The IHA test is also non-specific for active melioidosis infection as shown by Nigg (1963), who detected antibodies to *B. pseudomallei* in a significant proportion of healthy individuals living within an endemic area. This finding is also supported by other studies in Thailand and Australia (Ashdown 1987; Leelarasamee 1985; Khupulsup & Petchclai 1986; Chaowagul *et al.* 1989). Therefore it does not give any indication as to how recently an infection was acquired. Antibody to *B. pseudomallei* can persist in individuals who have previously had clinical melioidosis, in patients with prolonged chronic infections as well as in those who have a subclinical infection (Ashdown 1987). Thus, in endemic areas the diagnostic IHA titer varies according to the background titers in the normal, healthy population and is most useful as a screening technique and not as a direct measure of disease activity.

Due to the presence of subclinical melioidosis in endemic areas, serological differentiation of active from inactive infection is usually achieved by the indirect fluorescent antibody (IFA) test for specific IgM antibody (Nigg 1963; Ashdown 1981). The indirect fluorescent antibody test for IgM and IgG specific antibody to *B. pseudomallei* was developed by Ashdown (1981) who reported this method to be more sensitive and specific for patients with acute melioidosis than the IHA test. Khupulsup & Petchclai (1986) also found a high correlation between patients with culture-confirmed melioidosis and IFA-IgM seropositivity. However, similar to the IHA test, some healthy controls also tested positive for IFA-IgM indicating that serology alone cannot be relied upon (Khupulsup & Petchclai 1986). The screening of large numbers of clinical samples by this method is also cumbersome and strains of *B. pseudomallei* have been shown to lose their IFA antigenicity when stored or repeatedly subcultured (Ashdown *et al.* 1989). The use of immunofluorescence techniques is obviously restricted to laboratories that have a fluorescent microscope, and is therefore not a useful diagnostic method for developing countries. A direct

immunofluorescent antibody test is also available and can be used to detect *B. pseudomallei* cells in sputum, pus or urine although the sensitivity is low (73%) (Walsh *et al.* 1994).

Various enzyme-linked immunosorbent assays (ELISA's) have been developed as diagnostic techniques for the detection of *B. pseudomallei*-specific IgM or IgG antibodies (Ashdown *et al.* 1989; Van Phung *et al.* 1995; Dharakul *et al.* 1997; Chenthamarakshan, Vadivelu & Puthuchery 2001). The sensitivity and specificity of these assays showed slight improvements compared to the IHA test, although it was observed by Kunakorn *et al.* (1990) that the use of indirect ELISA and IHA tests in combination significantly increases these parameters. An ELISA evaluated by Dharakul *et al.* (1997) found that detection of specific IgG antibodies to an immunoaffinity-purified *B. pseudomallei* antigen provided a more sensitive and specific diagnosis in the early stages of septicaemic melioidosis compared to IgM detection. Chenthamarakshan *et al.* (2001) also found that patients with melioidosis, with either a septicaemic or localised clinical presentation, were positive for IgG antibodies. However, IgG antibody levels can remain elevated for more than five years following diagnosis, despite full recovery of the patient (Dharakul *et al.* 1997). Therefore, this test is unable to differentiate between patients who have a current *B. pseudomallei* infection, and those who have had a previous history of melioidosis but are presenting with an infection caused by another pathogen. The majority of current methods for serological diagnosis of melioidosis rely on crude antigen preparations, which often cross-react with sera from individuals infected with other bacteria. Anuntagool *et al.* (1993) attempted to improve the sensitivity and specificity of these methods by producing a purified, *B. pseudomallei*-specific antigen for use in an ELISA system. Of the five antigens screened, a 19.5kDa protein was the most promising. However, it showed no improvement in the sensitivity and specificity (82% and 96%, respectively) that was obtained by the ELISA of Ashdown *et al.* (1989) using crude antigens (90% and 99%, respectively).

While serology is widely used for the diagnosis of melioidosis it still has some disadvantages, such as when seroconversion is delayed or when antibody levels are raised in the absence of isolation of the organism (Guard *et al.* 1984). Negative serology or low titres have also been demonstrated in patients with culture-positive

melioidosis, particularly during localised infection (Ashdown 1979; Alexander *et al.* 1970; Guard *et al.* 1984). Several assays have been developed to identify *B. pseudomallei* antigens in clinical specimens since this indicates active infection (Desakorn *et al.* 1994; Smith *et al.* 1995; Anuntagool *et al.* 1996; Pongsunk *et al.* 1999). Latex agglutination of *B. pseudomallei* antigens present in patient's urine has proven useful for patients with life threatening, disseminated melioidosis (Smith *et al.* 1995). This technique has also been applied to blood samples for detection of *B. pseudomallei* (Pongsunk *et al.* 1999; Samosornsuk *et al.* 1999; Anuntagool *et al.* 2000). While sensitivity and specificity approached 100% using the latex agglutination test, blood cultures still required subculture and an overnight incubation prior to testing. However, this method allowed identification of *B. pseudomallei* up to 2 days earlier compared to conventional culture and biochemical techniques. The development of sandwich ELISAs that use monoclonal antibodies to capture *B. pseudomallei* antigens in clinical samples have shown specificity but lack sensitivity (Desakorn *et al.* 1994; Anuntagool *et al.* 1996).

2.4.4.3 Molecular diagnosis

In the past decade improvements to the diagnosis of melioidosis have included the use of molecular techniques, such as polymerase-chain reaction (PCR) to detect *B. pseudomallei* DNA from clinical specimens. The high sensitivity and specificity provided by PCR is based on its ability to produce multiple copies of target DNA sequences, and the fact that bacteria of the same species contain conserved regions of DNA or RNA (Dharakul *et al.* 1996). A nested PCR developed by Dharakul *et al.* (1996) was able to detect down to two *B. pseudomallei* cells from a buffy coat specimen by using primers for a section of the 16S rRNA gene. PCR techniques have high sensitivity that is often offset by a lack of specificity, with cross-reactions occurring with closely-related bacteria such as *B. mallei* (Lew & Desmarchelier 1994; Dharakul *et al.* 1996; Wongratanacheewin, Komutrin & Sermswan 2000; Hagen *et al.* 2002). Recently, a semi-nested PCR was developed for detection of *B. pseudomallei* DNA in formalin-fixed, paraffin wax-embedded tissue (Hagen *et al.* 2002). However, once again this technique alone does not distinguish between *B. pseudomallei* and *B. mallei*. Various types of PCR protocols have been tested, each of which require refinement to improve the sensitivity, specificity of primers, the ease and cost required to perform the test and application of the method to a

broader range of clinical specimens (Lew & Desmarchelier 1994; Dharakul *et al.* 1996; Kunakorn & Markham 1995; Brook, Currie & Desmarchelier 1997; Wongratanacheewin, Komutrin & Sermswan 2000).

Even today, there is still a need for a simple, rapid laboratory test that will allow diagnosis of active infection in patients with non-specific symptoms or low titres of specific antibodies toward *B. pseudomallei*. Currently, a combination of culture techniques and either immunologic or molecular assays are recommended for the diagnosis of *B. pseudomallei* infection. For example, an overnight haemoculture to increase *B. pseudomallei* numbers, followed by identification with PCR (Sirisinha *et al.* 2000). The use of real-time PCR holds promise for improving time taken for diagnosis of melioidosis in the future.

2.4.5 Chemotherapeutic regimens and vaccines

Accurate and rapid laboratory diagnosis is essential for successful treatment of melioidosis. *B. pseudomallei* is resistant to most of the commonly used antibiotics for treatment of undiagnosed septicaemic illnesses and bacterial pneumonia, such as β -lactams, polymyxins and aminoglycosides (Eickhoff *et al.* 1970; Franklin 1971; Ashdown & Frettingham 1984; Guard *et al.* 1984). The resistance of *B. pseudomallei* to ampicillin and broad-spectrum cephalosporins such as cefotaxime is due to the production of a β -lactamase (Livermore *et al.* 1987; Godfrey *et al.* 1991). Quinolones, which are used successfully to treat *P. aeruginosa* infections, also show poor activity against *B. pseudomallei* (Ashdown & Currie 1992). Therefore, in areas considered endemic for melioidosis physicians have to be aware of melioidosis and consider it as a possibility in patients presenting with pneumonia or septicaemia.

The high mortality associated with septicaemic melioidosis is often due to delay in therapy, or the implementation of ineffective drugs (Chaowagul *et al.* 1989). The most effective regimens involve the use of combinations of drugs, most commonly ceftazidime in various combinations with tetracycline, chloramphenicol, cotrimoxazole or co-amoxiclav. Ceftazidime is a third-generation cephalosporin that is

resistant to β -lactamase and shows bactericidal activity against *B. pseudomallei* as well as other common pathogens that cause community-acquired septicaemia (Ashdown 1988; White *et al.* 1989; Suputtamongkol *et al.* 1994). Ceftazidime alone, or in combination with co-trimoxazole is the standard treatment for septicaemic melioidosis (White *et al.* 1989). Alternatively, co-amoxiclav or imipenem is used in combination with ceftazidime (Suputtamongkol *et al.* 1994; Simpson *et al.* 1999). Ulett *et al.* (2003) compared the efficacy of treatment with ceftazidime alone or in combination with co-trimoxazole in a murine model. Combination therapy proved most successful, resulting in bacterial clearance from mice with acute *B. pseudomallei* infection (Ulett *et al.* 2003). Although resistance to ceftazidime can develop during treatment, primary resistance to the antibiotic is rare (White *et al.* 1989; Dance *et al.* 1989b). The efficacy of fourth-generation cephalosporins, cefpirome and cefepime showed no improvement over ceftazidime in the treatment of *B. pseudomallei* infection in a murine model (Ulett, Norton & Hirst 1999; Ulett *et al.* 2003). Ceftazidime and imipenem differ in the effects they have on Gram negative bacteria as a result of each binding to different penicillin-binding proteins (PBP). The PBP-2-specific antibiotic, imipenem, causes spheroblast formation and rapid killing of *B. pseudomallei*, with low amounts of free endotoxin released. In contrast, *B. pseudomallei* killing by ceftazidime, which is specific for PBP-3, is much slower and involves the release of large amounts of endotoxin (Simpson *et al.* 2000). A comparison of these agents for treatment of patients with severe melioidosis found endotoxin release as a result of ceftazidime treatment had no impact on overall mortality. The authors proposed this was due to the limited role of BP-LPS in the pathogenesis of *B. pseudomallei* infection (Simpson *et al.* 2000).

Current antibiotic regimens are not without complications. High doses of chloramphenicol, tetracycline and sulphonamides for extended periods are associated with high toxicity in patients. Also, bacterial resistance is quick to develop to these antimicrobials and long-term therapy promotes positive selection for antibiotic-resistant *B. pseudomallei* strains (Dance *et al.* 1988; Tribuddharat *et al.* 2003). As yet, no alternative regimen has been identified that would prove beneficial to patients who are unable to tolerate, or who do not respond to prolonged therapy (Ashdown 1988). Numerous documented cases indicate that relapse in melioidosis is common,

despite lengthy courses of antibiotic therapy (Kingston 1971; Viner Smith, Grimmond & Monk 1975; Chaowagul *et al.* 1993; Silbermann *et al.* 1997; Inglis *et al.* 2001). Due to the high risk of relapse in melioidosis infections, prolonged antibiotic treatment is advised to ensure complete eradication of *B. pseudomallei*. Treatment can continue for up to 9 months or longer, although the optimum duration of treatment is still not known. Current treatment of acute melioidosis involves intravenous administration of ceftazidime, imipenem or co-amoxiclav followed by oral maintenance therapy for at least six months with bacteriostatic drugs such as chloramphenicol, doxycycline or cotrimoxazole. The duration of maintenance therapy varies depending on the site of infection (Chaowagul *et al.* 1989; Ashdown & Currie 1992; Tsang & Lai 2001). Antibiotics used for maintenance therapy could also be toxic and cannot be used in pregnant women and children (Silbermann *et al.* 1997). Co-amoxiclav is considered a safe alternative in this instance. In some instances, where abscesses are deep-seated and antibiotic therapy proves ineffective, surgical drainage and debridement may be necessary (Chaowagul *et al.* 1989; Tsang & Lai 2001). It is important that patients with underlying disorders presenting with melioidosis also receive treatment for the concomitant disease.

Granulocyte colony-stimulating factor (G-CSF) is a glycoprotein that regulates neutrophil activation and development. Stephens *et al.* (2002) proposed the use of G-CSF for immunotherapy in conjunction with antibiotic treatment for patients with septicaemic melioidosis. However, a subsequent study investigating the benefits of G-CSF using a murine model of melioidosis found no advantage of G-CSF inclusion, over ceftazidime treatment alone (Powell *et al.* 2003).

Management of melioidosis is expensive since it requires prolonged combination therapy with costly antimicrobials. It is also important to review the susceptibility of *B. pseudomallei* to the antibiotics in use, since the emergence of resistant strains occur during treatment (So *et al.* 1983; Dance *et al.* 1991; Tsang & Lai 2001).

Currently, no safe, effective vaccine exists for melioidosis. Attenuated, conjugate, DNA and heterologous vaccines for melioidosis have been investigated with limited success. Early attempts to immunise laboratory animals against melioidosis using preparations of killed *B. pseudomallei* and live, avirulent strains, afforded minimal

protection (Dannenberg & Scott 1958b). Also, the potential for reversion of avirulent to virulent strains once inoculated was considered high (Dannenberg & Scott 1958b; Dannenberg & Scott 1960). More recently, immunisation with avirulent *B. pseudomallei* strains in a murine model of melioidosis produced partial protection from a subsequent lethal challenge (Ulett *et al.* 2002). Similar findings were shown for an auxotrophic *B. pseudomallei* mutant (Atkins *et al.* 2002). Such studies demonstrate potential for an attenuated vaccine for melioidosis.

Masoud *et al.* (1997) found that patients with melioidosis contained high titers of specific anticapsular antibodies, demonstrating that the capsular polysaccharide (CP) of *B. pseudomallei* is an important immunogen. Acapsular mutants did not afford protection against subsequent challenge in mice (Reckseidler *et al.* 2001; Atkins *et al.* 2002). Efficacies of conjugate vaccines involving *B. pseudomallei* flagellin proteins, CP and O-PS have been investigated in animal models with limited success (Bryan *et al.* 1994; DeShazer *et al.* 1997; Chua, Chan & Gan 2003). A protein-polysaccharide (PPS) conjugate vaccine for glanders is currently being studied in a horse model (Warawa & Woods 2002). The *B. mallei* polysaccharide molecules used in the vaccine share close homology to those of *B. pseudomallei*. Therefore, the authors propose that the glanders vaccine may provide protection against melioidosis, although this has not yet been investigated (Warawa & Woods 2002). The flagellin structural gene, *fliC*, which is immunogenic in patients with melioidosis has been used to produce a prospective DNA vaccine. However, immunisation in hamsters with the *fliC*-vaccine did not provide protection and modifications are being attempted to improve the efficacy (Warawa & Woods 2002). Further studies are also warranted for the use of the non-pathogenic *B. pseudomallei* relative, *B. thailandensis* that afforded up to 50% protection in a guinea pig model (Warawa & Woods 2002).

The potential use of *B. pseudomallei* as a bioweapon has added to the surge in research into the development of a vaccine for melioidosis. Host clearance of intracellular pathogens requires a CMI response, mediated through the production of T_H1-type cytokines such as IFN- γ and TNF- α . Therefore, effective vaccination against intracellular pathogens requires the generation of a protective CMI response. A detailed understanding of how the immune system responds to *B. pseudomallei*

infection will open avenues for combating melioidosis and allow for the development of effective vaccine strategies.

2.5 Immunopathogenesis of *B. pseudomallei* Infections

Our understanding of the immunopathogenesis of *B. pseudomallei* infection is limited. Similar to other intracellular pathogens, *B. pseudomallei* possesses various mechanisms that enable it to evade or resist the host defense mechanisms, allowing survival and growth even within host immune cells.

The host immune response to invading pathogens includes both a non-specific (innate) component, in which recognition of foreign antigen occurs, followed by a specific (adaptive) component during which antibodies and effector T cells contribute to elimination of the pathogen. Both the innate and adaptive immune responses are comprised of humoral and cellular components. An innate immune response to infection does not depend on prior exposure to a particular antigen and acts directly to contain and eliminate a pathogen (Abbas, Lichtman & Pober 2000). The hosts' first line of defence includes mechanical barriers, such as skin and mucosal surfaces, secretions and normal flora. The predominant cells involved in the innate response are macrophages, neutrophils and natural killer (NK) cells. Most microbes encountered are detected by the innate immune response before disease is perceptible. Those that are not, elicit an adaptive immune response, with the generation of antigen-specific effector cells and molecules that specifically target the pathogen. Adaptive immunity is a continuation of innate immunity although only the former is able to generate long-lasting, protective immunity. Adaptive immunity is triggered by a threshold dose of antigen and signals delivered by the innate immune response. Antigen-specific T and B lymphocytes are the key players of adaptive immunity and due to the time required for these cells to proliferate and differentiate into effector cells, an adaptive immune response is detected several days after infection (Abbas, Lichtman & Pober 2000).

Most microbes are phagocytosed and processed by professional APC by two routes called class I and class II pathways, and presented on the cell surface in lymphoid

tissues. Presentation of antigen by class I and class II pathways activate CD8⁺ and CD4⁺ T lymphocytes, respectively. Activation of CD4⁺ T lymphocytes, which are also known as T-helper (T_H) cells, results in the production of various cytokines. The combination of cytokines produced can vary along a spectrum that ranges from T_H1 to T_H2 patterns. T_H1 and T_H2 cytokines trigger other immune cells to produce either a predominantly cell-mediated (T_H1-type) or humoral (T_H2-type) immune response. In a humoral response, antibodies specific for the invading pathogen are produced by B lymphocytes and are important for toxin neutralisation, opsonisation and activation of complement, and the elimination of the pathogen. Once pathogens gain entry into host cells they are inaccessible to circulating antibody. Therefore, intracellular organisms and virus-infected cells are destroyed through the activation of T_H1-type cells that subsequently drive a CMI response. Thus the type of T_H subpopulation that develops during an infection profoundly affects the course of the disease (Abbas, Lichtman & Pober 2000). While most responses to an invading pathogen involve both arms of the immune response working in concert, some infections elicit responses that are mutually exclusive. Parish and Liew (1972) demonstrated this phenomena using *Salmonella* flagellin protein. With low and high concentrations of antigen a T_H1-type or CMI response predominated. However, at intermediate doses of antigen, CMI was accompanied by antibody production (Parish & Liew 1972; Romagnani 1997).

Research is beginning to unveil some of the interactions between *B. pseudomallei* and the host immune system, although most pathways for protective immunity of the host toward *B. pseudomallei* have not yet been identified.

2.5.1 Regulation of Immune Responses by Cytokines

Cytokines play an important role in imparting resistance against infection, although the mechanisms by which they achieve this are often complicated and not uniform for different pathogens. Resistance to infection with *B. pseudomallei* is governed by the production of IFN- γ within the first 24hrs (Santanirand *et al.* 1999). During the innate immune response to *B. pseudomallei* IFN- γ , a potent activator of macrophages, is produced predominantly by NK cells (Lertmemongkolchai *et al.*

2001). Failure to produce IFN- γ results in overwhelming septicaemia and death of the host (Santanirand *et al.* 1999).

However, there are instances where cytokine release may be directly harmful to the host. The lipid A moiety in LPS of gram negative bacteria stimulates the production and release of various cytokines in large quantities and it is this response which is thought to result in the pathologic features associated with septicaemia including multiple organ failure, coagulation defects and shock (Suputtamongkol *et al.* 1992). TNF, produced by macrophages and T cells, plays an important role in the pathogenesis of endotoxic shock, since endotoxin is a potent stimulus for its release (Beutler, Milsark & Cerami 1985; Tracey, Lowry & Cerami 1988). While the local effects of TNF- α are beneficial, higher concentrations of this cytokine may be detrimental to the host since a more generalised inflammatory reaction is induced which can lead to shock, diffuse intravascular coagulation and eventual death (Van Deuren, Dofferhoff & van der Meer 1992; Schaible *et al.* 1999). Using animal models, the clinical and pathologic features of endotoxemia can be generated with intravenous administration of exogenous TNF- α and, with the introduction of a monoclonal antibody to this cytokine, death following a lethal dose of TNF- α can be prevented (Tracey, Fong & Hesse 1987). Studies in humans have shown that a correlation exists between TNF- α levels in the blood, and the severity and mortality associated with septic shock, meningococcal septicaemia and falciparum malaria (Waage *et al.* 1989; Cannon, Tompkins & Gelfand 1990; Kwiatkowski, Hill & Sambou 1990). Organ dysfunction commonly occurs as a complication of shock and sepsis. The pathophysiology of organ dysfunction has been linked to systemic inflammation as a result of infection or massive tissue injury (systemic inflammatory response syndrome, SIRS) (Cobb *et al.* 2000). Subsequent complications of organ dysfunction, including death, may also occur as a result of immunosuppression associated with counter-regulatory anti-inflammatory response syndrome (CARS) (Cobb *et al.* 2000).

Recent studies have demonstrated the importance of several cytokines and chemokines on the outcome of both human and experimental melioidosis (Friedland *et al.* 1992; Lauw *et al.* 1999; Simpson *et al.* 2000; Ulett, Ketheesan & Hirst

2000a,b; Ulett *et al.* 2000c; Barnes *et al.* 2001). Research carried out at James Cook University has shown that contrasting pro-inflammatory cytokine profiles are produced in mice of varying susceptibility to virulent *B. pseudomallei* (Ulett, Ketheesan & Hirst 2000b). In susceptible BALB/c mice, hyperproduction of IFN- γ , TNF- α and IL-1 β preceded death (Ulett *et al.* 2000a,b). In human melioidosis, elevated plasma levels of IFN- γ , TNF- α and IL-2, and of the chemokines IP-10 and Mig, correlated with disease severity (Brown *et al.* 1991; Suputtamongkol *et al.* 1992; Lauw *et al.* 1999; Lauw *et al.* 2000; Simpson *et al.* 2000). IP-10 and Mig, whose production is induced by IFN- γ , are chemoattractive for activated T cells and NK cells. Increased plasma concentrations of IL-6, an activator of B, T and NK cells, and IL-8, a potent neutrophil chemoattractant and activator also reflect a poor prognosis in melioidosis (Friedland *et al.* 1992). Such a correlation suggests that these compounds, in particular IFN- γ , may be contributing to the pathophysiology of septicaemic melioidosis, and that their levels in the serum may be useful in the prediction of disease outcome. Disparate expression of mRNA for several chemokines and CSF in the C57BL/6-BALB/c model have been shown to be associated with differences in bacterial growth and the composition of cellular infiltrate (Barnes *et al.* 2001). Compared with C57BL/6 mice, there is greater infiltration of neutrophils into lesions in liver and spleen of BALB/c mice following *B. pseudomallei* infection (Barnes *et al.* 2001). This study is discussed further in Chapter 5.

2.5.2 Host Genetic Factors

Protection against infection with a pathogen can also be afforded by some non-immunological factors. Resistance can occur as a result of species insusceptibility, for example the growth of *Neisseria gonorrhoeae* within humans and *Brucella abortus* in mammals, but not chickens (Abbas, Lichtman & Pober 2000). T cell recognition of antigens presented on the surface of APC relies on the presence of two types of specialised proteins encoded by genes in a locus called the major histocompatibility complex (MHC) (Abbas, Lichtman & Pober 2000). MHC class I and MHC class II molecules present peptides to CD8⁺ T cells and CD4⁺ T_H cells, respectively. Therefore, the type of MHC molecule that displays an antigen influences the type of immune response that is initiated. Antigens presented by MHC

class I molecules, found on most nucleated host cells, are typically endogenous. Association of the MHC class I molecule with antigen occurs in the endoplasmic reticulum (ER) and the complex is then transported to the cell surface (Abbas, Lichtman & Pober 2000). In contrast, MHC class II molecules are expressed in high concentration on APC where they display exogenous antigens, including bacterial products that have been phagocytosed and processed.

The genes in the MHC locus are highly polymorphic and allelic variations are capable of modifying a disease course in infections with a broad clinical spectrum, thus dividing individuals into 'resistant' and 'susceptible' groups. MHC genes control the activation of antigen-specific T cells and therefore polymorphisms in these alleles have important effects on cellular immunity to pathogens. In tuberculosis and leprosy, protective immunity and clinical manifestations of infection are governed by MHC alleles (Mehra 1990). Similarly, Dharakul *et al.* (1998) demonstrated an association between the clinical manifestations of *B. pseudomallei* infection and certain MHC class II alleles. However, larger studies are necessary to investigate the significance of this finding.

Other genes also play a significant role in controlling immune responses to invading pathogens. For example, the natural resistance associated macrophage protein 1 (*Nramp1*) gene is responsible for controlling resistance towards the intracellular pathogens, *L. major*, *M. bovis* and *S. typhimurium* (Schiabale *et al.* 1999). In mice the *Nramp1* gene is expressed exclusively by macrophages and influences the process of activation of these phagocytic cells. Therefore, the *Nramp1* gene affects the ability of macrophages to control the growth of intracellular bacteria in the early phases of infection (Schiabale *et al.* 1999).

2.5.3 Humoral immunity in melioidosis

The complement system and serum antimicrobial peptides are important effector mechanisms of the humoral immune response. Adaptive humoral immunity is mediated by secreted antibodies and is an important response against infection with extracellular pathogens and microbial toxins. Antibodies block the binding of microbes and toxins to host cell receptors and also promote phagocytosis by

opsonisation of pathogens. The majority of research to date into the immune response to *B. pseudomallei* infection has predominantly focussed on the humoral component, rather than CMI responses.

2.5.3.1 Complement

Through opsonisation and lysis, the complement system provides one of the first lines of defence against invading bacteria. Opsonisation of bacteria promotes phagocytosis by macrophages and neutrophils and the subsequent killing of the pathogen. Complement-mediated lysis of gram negative bacteria involves activation of complement, deposition of complement fragments onto the bacterial cell wall and the assembly of a membrane attack complex (MAC). While some intracellular pathogens, such as *Toxoplasma gondii* enter host cells actively, others are able to induce engulfment by exploiting the host cell surface receptors. This is achieved either by direct binding of the bacteria to host receptors or via host ligands that are deposited on the bacterial surface.

Ismail *et al.* (1988) reported resistance of *B. pseudomallei* to the bactericidal actions of normal human serum, although the bacterium was later shown to activate complement by the alternative pathway, resulting in phagocytosis by neutrophils (Egan & Gordon 1996). However, the bacterium is not killed once phagocytosed and is also resistant to complement-mediated lysis, possibly by a failure of correct MAC assembly (Pruksachartvuthi *et al.* 1990; Egan & Gordon 1996). Studies of *L. monocytogenes* infection have shown that opsonisation by complement fragments generated by the alternative pathway induces phagocytosis but not killing, since the internalisation of complement receptors does not trigger a respiratory burst in the phagocyte (Schiabale *et al.* 1999).

2.5.3.2 Antimicrobial peptides

The bactericidal effect of normal serum is an important component of the innate immune response since it is one of the first lines of defense against an invading pathogen. Host cells, particularly neutrophils, produce a number of proteins that serve as antimicrobials. These include defensins, which permeate the bacterial cell membrane, bacterial permeability-inducing protein (BPI), phospholipase A2 and cathelicidins (Schiabale *et al.* 1999). *B. pseudomallei* is resistant to the bactericidal

action of various compounds present in activated macrophages and neutrophils, including the cationic peptide protamine and defensin (Jones *et al.* 1996).

2.5.3.3 Antibody production

The majority of microbial antigens are processed by APC and presented with MHC class II molecules to CD4⁺ T_H cells that subsequently develop into T_H2 cells. These T_H2 cells interact with B lymphocytes to produce antibodies that generally have three functions. Antibodies are involved in opsonisation of pathogens and the neutralisation of microbial toxins. They also play a role in the activation of complement, which leads to bacterial cell lysis and phagocytosis by APC. Antibody isotypes differ in both function and location within the body. IgM antibodies, which are expressed on the surface of naïve B lymphocytes, are located predominantly in the bloodstream where they bind antigen and cause the activation of complement. IgG antibodies found in the bloodstream and extracellular fluid are important for neutralisation and opsonisation of toxins, viruses and bacteria. Mucosal immunity is provided by IgA, while IgE is predominantly found to be bound to mast cells and is important for triggering type I hypersensitivity reactions.

High levels of IgG have been demonstrated in serum from patients with various clinical manifestations of melioidosis (Chenthamarakshan *et al.* 2001).

Characterisation of IgG isotypes in human and experimental melioidosis indicate that a predominantly T_H1-type antibody response occurs, demonstrated by a high IgG_{2a}/IgG₁ ratio (Hoppe *et al.* 1999; Vasu *et al.* 2003). Though this strong antibody response is believed to be directed against secreted bacterial antigens and may not be important for protection against *B. pseudomallei* infection, Vasu *et al.* (2003) suggest that monitoring of IgG₁ may be a useful indicator of maintenance therapy failure and a potential indicator of relapse.

The production of antibody towards protein antigen requires both B and T lymphocytes and such antigens are therefore regarded as T-dependent antigens (Abbas, Lichtman & Pober 2000). T_H cells stimulate isotype switching, affinity maturation and long-lived memory. Antibody responses to polysaccharide and lipid antigens, or T-independent antigens, do not require the help of T lymphocytes. T-independent antigens stimulate the rapid production of antibody, mainly of the IgM

isotype, within 48 hrs of infection. Despite their inability to specifically activate T_H cells, many polysaccharide vaccines, such as the pneumococcal vaccine can induce long-lived protective immunity. This is presumed to be due to the persistence of polysaccharide in lymphoid tissues causing the continued stimulation of maturing B cells (Abbas, Lichtman & Pober 2000). Antibodies against the polysaccharide antigens of *B. pseudomallei*, likely to be among the first produced in the initial clearance of the bacteria, have phagocytic-promoting properties (Ho *et al.* 1997). The increased efficiency of macrophages from immunised mice to kill phagocytosed *B. pseudomallei*, compared to macrophages harvested from mice with no prior exposure to *B. pseudomallei*, may be in part due to the presence of antibodies on the surface of macrophages that target T-independent antigens of *B. pseudomallei* (Kishimoto & Eveland 1976a).

Originally, O-PS I and O-PS II were considered two structurally distinct O-antigenic polysaccharides comprising the LPS of *B. pseudomallei*, where they exist in roughly equal amounts. A study by Ho *et al.* (1997) found that antibodies against both O-PS antigens were produced in patients with acute melioidosis. While IgG antibodies were detected in all acutely infected patients, an IgM response was observed only in those patients with localised infections. Patients with localised and septicaemic infections produced both IgG₁ and IgG₂ antibodies, however an IgG₃ response was only evident in individuals who had survived the septicaemic form. In contrast, individuals within an endemic area who were identified as seropositive with the IHA test did not produce antibodies to either O-PS I or O-PS II (Ho *et al.* 1997). The fact that these anti-LPS antibodies were produced in all forms of the disease demonstrates their importance in a humoral immune response.

Similar to LPS, flagellin and CPS of *B. pseudomallei* are also potent immunogens (Brett, Mah & Woods 1994; Bryan *et al.* 1994; DeShazer *et al.* 1997; Charuchaimontri *et al.* 1999). However, antibodies against each of these structures do not provide complete protection (Jones *et al.* 2002). The failure of a humoral immune response to provide protection against *B. pseudomallei* infection is not surprising considering that *B. pseudomallei* is able to survive and replicate within host cells, thereby avoiding the actions of circulating antibodies. It is only during the

initial stages of an infection, prior to *B. pseudomallei* entering its intracellular niche, that antibodies targeting the bacterial antigens will be beneficial.

2.5.4 Cell-mediated immunity in melioidosis

Protection from infections with intracellular bacteria, such as *L. monocytogenes*, *L. pneumophila* and *M. tuberculosis*, has been shown to be mediated largely by a CMI response of the host (Ehlers *et al.* 1992; Mittrucker *et al.* 2000; Andersen & Smedgaard 2000; Tateda *et al.* 2001). Since *B. pseudomallei* is a facultative intracellular bacterium it is likely that the cellular arm of the immune response would also be pivotal in providing protection from melioidosis (Jones *et al.* 1996). The importance of such a response in *B. pseudomallei* infection is supported by the ability of the disease to relapse once the host becomes immunocompromised, the association of acute melioidosis with diseases such as diabetes mellitus and chronic renal failure, and the histopathological differences observed between localised and septicaemic melioidosis (Sanford & Moore 1971; Piggott 1976; Chaowagul *et al.* 1989). Until recently, few studies have attempted to determine the role of the cells involved in *B. pseudomallei* infection.

Measurement of urinary neopterin levels is one method that has been used to indirectly assess the cellular immune function of patients with various clinical manifestations of melioidosis (Brown *et al.* 1990). Neopterin is a metabolite released by macrophages that have been activated by IFN- γ produced by T cells. Thus, its level in urine is thought to reflect the activation of both macrophages and T cells or the cellular component of the immune response (Brown *et al.* 1990). While urinary neopterin levels are elevated in all patients with melioidosis, significant rises are evident in patients with the septicaemic form. Brown *et al.* (1990) proposed that while a specific CMI response occurs in all patients with melioidosis, in the septicaemic form this response fails to inhibit bacterial multiplication thereby allowing the dissemination of *B. pseudomallei* and the initiation of a secondary, T cell-macrophage activation. This secondary response is reflected in the elevated urinary neopterin levels in patients with septicaemic melioidosis.

2.5.4.1 The essential role of macrophages

Macrophages are often the first host immune cells to infiltrate sites of infection, playing a dual role in bacterial clearance by interacting directly with the bacterial cell and releasing potent immunomodulators (Abbas, Lichtman & Pober 2000). The majority of protective functions of macrophages in the early phase of infection are achieved through the production of various cytokines and chemokines in response to bacterial infection. Some cytokines, primarily IL-1, are responsible for the initiation of fever in the host, which enhances the hosts' immune response and provides protection for host cells against the deleterious effects of the pro-inflammatory cytokine, TNF- α (Abbas, Lichtman & Pober 2000). Another role of these cytokines is the generation of an inflammatory response involving changes in the permeability and surface properties of blood vessels to allow recruitment of immune cells and molecules to the site of infection.

One of the mechanisms utilised by activated macrophages in the elimination of intracellular bacteria is the production of toxic free radicals. The generation of reactive oxygen intermediates (ROI) or reactive nitrogen intermediates (RNI) are effective against the survival of a number of intracellular bacteria, including *L. pneumophila* (Yamamoto, Klein & Friedman 1996). It has been proposed that the generation of nitric oxide (NO) may regulate the production of asymptomatic, chronic infections produced by a number of intracellular bacteria, such as *M. tuberculosis*. MacMicking *et al.* (1997) observed that intracellular growth of *M. tuberculosis* in mice is reactivated once NO generation is inhibited. Macrophages activated by IFN- γ are capable of inhibiting the intracellular growth of *B. pseudomallei* (Utainsincharoen *et al.* 2001). Their bactericidal activity against the bacterium was mediated via both the RNI and ROI pathways, although the contribution by RNI was more extensive (Miyagi, Kawakami & Saito 1997). Bactericidal activity of macrophages *in vitro* toward *B. pseudomallei* is stimulated only in the presence of lymphocytes (Ulett, Ketheesan & Hirst 1998). Intracellular growth of *B. pseudomallei* is suppressed following IFN- γ activation of macrophages and increased expression of inducible nitric oxide synthase (iNOS) (Utainsincharoen *et al.* 2001). However, *B. pseudomallei* has been shown to invade and replicate within both unstimulated and stimulated macrophages, without activating iNOS (Miyagi, Kawakami & Saito 1997; Utainsincharoen *et al.* 2001). When infected, or

exposed to microbial products of gram negative bacteria such as LPS, macrophages produce IFN- β . IFN- β modulates the ability of macrophages to synthesize iNOS via the upregulation of interferon regulatory factor-1 (IRF-1), a transcriptional activator of the iNOS gene. Protection from *L. monocytogenes* or *M. avium* infection has been generated in mice using recombinant IFN- β therapy (Fujiki & Tanaka 1988; Denis 1991). Since *B. pseudomallei* has been shown to stimulate minimal production of IFN- β , iNOS expression is therefore decreased and the intracellular growth of the bacterium is not controlled (Utaisincharoen *et al.* 2003).

A macrophage's efficiency in the removal of bacteria from the body is greatly influenced by the virulence of the bacterial strain present. This phenomenon has been demonstrated in *in vitro* studies with *L. monocytogenes*, *Brucella abortus* and *B. pseudomallei* (Braun, Pomales-Lebron & Stinebring 1958; Mackness 1962; Kishimoto & Eveland 1976b). Kishimoto & Eveland (1976b) found virulent strains of *B. pseudomallei* were more resistant to phagocytosis and subsequent killing compared to their less virulent counterparts. Although no differences between their surface structures could be detected, the authors concluded that variations within the chemical composition of the lipopolysaccharide may hold the key to understanding this difference in macrophage activity (Kishimoto & Eveland 1976b). However, this line of research has not been pursued in the last few decades.

Neutrophils are also an important part of the innate immune response that possess phagocytic and antimicrobial properties. *B. pseudomallei* is capable of intracellular survival within neutrophils (Jones *et al.* 1996). *In vitro* studies have demonstrated that neutrophils from diabetic rats and patients with diabetes have defective bactericidal activity (Mohsenin & Latifpour 1990; Wykretowicz *et al.* 1993). This is believed to be due to competition for NADPH between NADPH oxidase and adolase reductase, which is upregulated in neutrophils of diabetics, subsequently leading to an impaired production of reactive oxygen intermediates (Wykretowicz *et al.* 1993). The increased risk of *B. pseudomallei* infection in diabetics may therefore, at least in part, be a reflection of the decreased efficiency of neutrophils and consequently an ineffective innate immune response in these individuals.

2.5.4.2 The essential role of lymphocytes

The development of an adaptive immune response is detected several days following infection and is involved in bacterial clearance and the development of long-lasting immunity. Clearance of facultative intracellular bacteria, such as *L. monocytogenes* and *M. tuberculosis*, from the host is predominantly mediated by CD4⁺ T lymphocytes. An adaptive immune response is typically accompanied by a change in the relative distribution of lymphocyte subpopulations. The subset that develops is dependent on the types of cytokines present during the initiation of a T cell response. The combination of cytokines produced can vary along a spectrum that ranges from T_H1-type to T_H2-type responses. T_H1-type cells produce IL-2, IFN- γ and TNF- β and initiate a CMI response. In contrast, T_H2-type cells are potent helpers of antibody production and secrete IL-4, IL-5, IL-6 and IL-13 (Kelso 1998; Abbas, Lichtman & Pober 2000). Since the primary functions of T_H1 cytokines is to promote macrophage activation and to recruit and activate inflammatory leucocytes, a T_H1-type response is critical for phagocyte-mediated defense against infections. The majority of intracellular bacteria induce the production of IL-12 and IFN- γ from APC and IFN- γ from NK and CD8⁺ T cells, subsequently driving the development of a T_H1-type response (Romagnani 1997). Soluble factors released by intracellular pathogens also activate T_H1-type cells, and these cells subsequently activate macrophages through the secretion of IL-2 and IFN- γ . While T_H1-type responses are necessary for host resistance to many intracellular pathogens, excessive or uncontrolled responses result in host tissue injury (Abbas, Lichtman & Pober 2000).

Responses dominated by T_H2-type cells are typical of infection by extracellular organisms. If T_H2-type cells are the predominant subset during infection by intracellular pathogens, cell-mediated immunity fails to develop, macrophages are not activated and chronic progressive disease results (Abbas, Lichtman & Pober 2000). This is best exemplified in leprosy, the clinical forms of which tend to range between two spectral poles. Patients with tuberculoid leprosy tend to be asymptomatic or have only a mild form of the disease since they have a strong CMI response to *M. leprae*. A protective host immune response against mycobacterial infection is predominantly achieved through the actions of CD4⁺ T cells, however CD8⁺ T cells are known to contribute as well and are capable of compensating for

losses to CD4⁺ T cell functions (Xing *et al.* 1998). Cytotoxic CD8⁺ T lymphocytes recognise antigen presented by MHC class I molecules and respond by either killing the target cell with cytolyticins inserted through perforin molecules in the cell membrane, or by inducing lysis of infected cells that are incapable of controlling the infection. This latter function allows more proficient immune cells access to destroy the intracellular pathogens that are released from lysed host cells (Schiabbe *et al.* 1999). In contrast to tuberculoid leprosy, those patients with lepromatous leprosy generate high levels of antibodies against *M. leprae* but are unable to mount a detectable CMI response and show little resistance to the disease (Romagnani 1997; Schiabbe *et al.* 1999). In lepromatous leprosy there is dense infiltration of *M. leprae* into tissues and a poor prognosis for patients.

The nature and potentially protective role of the CMI response in melioidosis is poorly understood. The first evidence of CMI in patients with melioidosis comes from work carried out at James Cook University which will be discussed further in Chapter 9 (Ketheesan *et al.* 2002). Unlike healthy controls, lymphocytes from patients who had recovered from melioidosis showed significant proliferation and production of IFN- γ when stimulated with *B. pseudomallei* antigens *in vitro*, reflecting the presence of *B. pseudomallei*-specific memory T cells in these individuals (Ketheesan *et al.* 2002). Current work involves further characterisation of the role of T lymphocytes in providing protection from *B. pseudomallei* infection.

2.6 Conclusion

Melioidosis and its causative agent, *B. pseudomallei*, have been studied for over eight decades. Despite this, a tremendous amount of knowledge is still lacking, particularly in our understanding of the immunopathogenesis of this bacterium. The increase in prevalence of melioidosis and the recognition that *B. pseudomallei* is a potential bioweapon, is cause enough for upgraded research efforts in this area. Once we have a basic understanding of the host's immune response to *B. pseudomallei* and the mechanisms utilised by the bacterium in order to evade these host responses, effective methods for the diagnosis and control of melioidosis can be achieved.

CHAPTER 3

GENERAL MATERIALS & METHODS

3.1 Bacterial Isolates

3.1.1 Origin of *B. pseudomallei* strains

Two *Burkholderia pseudomallei* strains from the Biomedical and Tropical Veterinary Sciences collection were used in these studies. The first, NCTC 13178, is a highly virulent strain of *B. pseudomallei* that was originally isolated from a fatal case of melioidosis at the Townsville General Hospital in 1990. The LD₅₀ of this isolate was previously shown to be <10 cfu and 5 x 10³ cfu in BALB/c and C57BL/6 mice respectively (Leakey *et al.* 1998). NCTC 13179 is a less virulent strain of *B. pseudomallei* that was recovered from a patient with melioidosis with an abscess of the knee at the Townsville General Hospital. The LD₅₀ values for NCTC 13179 in BALB/c and C57BL/6 mice were shown to be 0.9 x 10⁴ cfu and 6 x 10⁶ cfu, respectively (Ulett 2000).

3.1.2 Confirmation of strain identification

Presumptive identification of *B. pseudomallei* isolates was made by their characteristic colonial morphology on Ashdown agar (bioMérieux, NSW, Australia), together with antibiotic susceptibility patterns. Confirmation of identity was obtained using the API 20NE commercial identification system (bioMérieux, NSW, Australia) following protocols outlined by Dance *et al.* (1989c). Both NCTC 13178 and NCTC 13179 were clinical Ara⁻ strains.

3.1.3 Preparation of bacterial antigens

A *B. pseudomallei* lysate was prepared using the less virulent *B. pseudomallei* strain, NCTC 13179 (BpLy1). For specificity studies, a *B. cepacia* lysate (CepLy) was also prepared using an isolate recovered from environmental samples in Townsville. Confirmation of identity was obtained using the API 20NE commercial identification system (bioMérieux, NSW, Australia). For bacterial lysate preparation, a

0.9 McFarland standard suspension of *B. pseudomallei* or *B. cepacia* in 30 ml of sterile phosphate-buffered saline (PBS; Appendix 1) was obtained from colonies grown on Ashdown agar for 48 hrs at 37°C. The weight of bacteria was estimated and an equal weight of 0.1 mm diameter glass beads (Daintree Industries Pty Ltd, Australia) was added. The bacteria and beads were resuspended in breaking buffer (Appendix 1), and sonicated on ice using 4 x 10 min bursts of 60 W (Biosonik III; Bronwill Scientific, New York, USA), leaving 10 min between each burst. Sonication was performed using a 100 ml sterile glass beaker. The beads and cell debris were pelleted (9000 *g* for 12 min) and the supernatant was filtered using 0.22 µm filters (Millipore, NSW, Australia). After dialysis into PBS, the suspension was again filtered through 0.22 µm filters and protein concentration was estimated using a BCA protein assay kit (Progen, NSW, Australia) as per manufacturers' instructions. Sterility was confirmed by incubating 200 µl aliquots of BpLy1 or CepLy on Ashdown agar and sheep blood agar (SBA; bioMeri  ux, NSW, Australia) at 37°C for 48 hrs. BpLy1 (1 µg/µl) and CepLy (1 µg/µl) was aliquoted into microfuge tubes and stored at -70°C. Prior to use, lysates were thawed at room temperature (RT) and diluted in RPMI 1640 medium (Life Technologies, NSW, Australia) or PBS as required.

3.2 Experimental Animals

3.2.1 Ethics approval, routes of administration and safety measures

Experiments requiring the use of laboratory animals were carried out under the Ethics Approval numbers A059, A536 and A709, which were provided by the James Cook University Experimental Ethics Review Committee. All animal experiments carried out during this study were performed according to the National Health and Medical Research Council (NH&MRC) guidelines using BALB/c or C57BL/6 mice that had been bred with single pair sibling mating by the Division of Microbiology and Immunology, James Cook University. Approximately equal numbers of males and female mice aged between six and sixteen weeks were used. The animals were housed in plastic, wire-topped boxes with wooden shavings as bedding, and were supplied with protein-enriched pellets and water, *ad libitum*. Animals were maintained in an air-conditioned, positive pressure isolation unit and were monitored daily.

Where applicable, all experimental work was performed in a Class II Biosafety Cabinet (Gelman Sciences, Australia) within the Biosafety Level 3 facility of the Department of Microbiology and Immunology, James Cook University.

3.2.2 Preparation and delivery of standard inoculum

Bacterial isolates were passaged once through mice. A few well-isolated colonies taken from an Ashdown agar plate after incubation for 48 hrs at 37°C were suspended in 50 ml of Brain-Heart Infusion (BHI) broth. The suspension was incubated at 37°C for 18 hrs then 1 ml aliquots were dispensed into sterile eppendorfs. Stock bacterial cultures were stored at -70°C until required. Each time a cryotube of stock culture was withdrawn for use, it was thawed for 10 min at 37°C.

Standard curves of optical density (OD) against colony-forming units (cfu)/ml were previously constructed for NCTC 13178 and NCTC 13179 using a 96-well plate reader (Multiskan EX; Labsystems; Ulett 2000). Estimations of the concentration of freshly prepared bacterial suspensions were extrapolated from these curves.

Challenge suspensions were prepared by inoculating several colonies of a 24 hr *B. pseudomallei* culture on SBA into sterile PBS. Suspensions were adjusted to an optical density at 650nm of approximately 0.17, which is equivalent to 0.5 McFarland standard or 1.5×10^8 cfu/ml (Ulett 2000). Required numbers of bacteria were obtained using appropriate dilutions of the stock suspension in PBS. Actual concentrations of bacterial cells (cfu/ml) were determined by plating 20 µl of serial ten-fold dilutions in PBS onto Ashdown agar. Colonies were counted after incubating for 48 hrs at 37°C.

Unless otherwise stated, mice were challenged *iv* with challenge doses of 200 µl using a 1 ml syringe (Terumo, USA) and a 25 gauge needle (Terumo, USA). Prior to inoculation, mice were warmed in a 37°C incubator for 15 min to dilate tail blood vessels. A volume equal to that of the challenge dose, 200 µl, was dispensed from each syringe and spread over the surface of an Ashdown agar plate. The plate was subsequently incubated for 48 hrs at 37°C to determine the actual number of bacterial cells inoculated.

3.2.3 Recovery of viable bacteria from whole organs

For recovery of viable *B. pseudomallei* cells from tissues of euthanased mice, organs were removed aseptically, weighed and placed into a stomacher bag (Disposable Products, South Australia), to which 5 ml of sterile PBS was added. The contents were homogenised for 1 min using a Lab-Blender 80 Stomacher (Townson & Mercer, Australia). If histological sections were to be prepared from the tissue, the organ was bisected sagittally prior to weighing and processing. One half was used to determine bacterial loads, while the other half was fixed in 10% neutral-buffered formalin (Appendix 1) for a minimum of 24 hrs, after which it was processed according to standard histological techniques. This included embedding in paraffin wax, sectioning at 5 µm, and staining with haematoxylin and eosin (H & E; Appendix 1).

To determine bacterial loads, 20 µl aliquots of serial 10-fold dilutions of the homogenate were plated in triplicate onto Ashdown agar. In addition, 100 µl of the undiluted homogenate was dispensed onto a separate Ashdown agar plate, in order to allow enumeration of low bacterial numbers. After 24 to 48 hrs incubation at 37°C, colonies were counted and the concentration of the original homogenate was calculated by the following formula:

$$\text{Concentration (cfu/ml)} = \text{No. of colonies in 20 } \mu\text{l sample} \times 50 \times \text{Dilution factor}$$

Alternatively, for counts from 100 µl of undiluted homogenate:

$$\text{Concentration (cfu/ml)} = \text{No. of colonies in 100 } \mu\text{l sample} \times 10$$

When possible, the diluent sample chosen for calculation was that which contained 30-150 cfu. All counts were performed in triplicate and the mean value obtained.

3.2.4 Isolation of murine splenic mononuclear leucocytes

Splenocyte suspensions from mice were prepared by passing spleens through a sterile stainless steel sieve into transfer medium (Appendix 1). Spleens were ground using sterile blunt-ended glass rod and the splenocyte suspension was collected in a sterile

30 mm petri dish. For each spleen, 5 ml of transfer medium was used and the splenocyte suspensions were pooled and aliquoted into 25 ml centrifuge tubes. The tissue debris was allowed to settle for 25 min at RT then the cell suspension was carefully layered onto endotoxin-free Ficoll-Paque Plus (Amersham Pharmacia Biotech) at a ratio of 7:3 in 10 ml plastic tubes. Following centrifugation (500 *g*; 20 min) the layer of cells at the interface, containing splenic mononuclear leucocytes (MNL), was collected using a sterile glass Pasteur pipette and washed twice (500 *g*; 10 min) in transfer medium with 10% heat-inactivated foetal bovine serum (HIFBS; Appendix 1). MNL were resuspended to an appropriate concentration in culture medium (Appendix 1). All procedures were performed at RT under sterile conditions.

CHAPTER 4

COMPARISON OF *B. pseudomallei* INFECTION GENERATED BY DIFFERENT ROUTES IN A MURINE MODEL

4.1 Introduction

The reservoir for *B. pseudomallei* and the exact mode of transmission remain unknown. Subcutaneous inoculation of contaminated soil and surface water is believed to account for the increased prevalence of melioidosis among individuals working in rice paddies without protective clothing in Thailand (Leelarasamee & Bovornkitti 1989). There is also evidence that melioidosis develops after inhalation or ingestion of contaminated particles or aerosols. The most well-known anecdotal evidence of *B. pseudomallei* infection by inhalation comes from the Vietnam War. A high incidence of melioidosis occurred in helicopter crew compared to other soldiers presumably due to the disturbance of contaminated dust by helicopter rotors (Howe *et al.* 1971; Sanford 1995). There have also been reports of melioidosis occurring following near-drowning accidents (Lee *et al.* 1985; Pruekprasert & Jitsurong 1991) and one case of transmission of *B. pseudomallei* in drinking water (Inglis *et al.* 1998b).

Clinical manifestations of melioidosis vary greatly and are often classified as acute, chronic or asymptomatic infections (Howe *et al.* 1971). Acute melioidosis is characterised by a fulminating septicaemia or pneumonia and is associated with high mortality (Chaowagul *et al.* 1989; Currie *et al.* 2000a). Without the correct antibiotic treatment, death can occur within 48 hours of onset of symptoms (Leelarasamee & Bovornkitti 1989). Abscess formation or focal infection is a characteristic feature of chronic melioidosis. Chronic infection may involve one or more tissue types, although predominant sites include the lungs, liver, spleen, skin and joints (Piggott & Hochholzer 1970; Leelarasamee & Bovornkitti 1989). Latent or subclinical forms of melioidosis exist due to the ability of *B. pseudomallei* to persist in the host for extensive periods

before reactivation of the infection many months or years later (Kingston 1971; Thompson & Ashdown 1989; Chaowagul *et al.* 1993). Individuals with latent melioidosis have no apparent clinical signs or symptoms, and are identified only by positive serology.

Characterisation of microbial pathogenesis relies heavily on the use of animal models. Animal infection models play an important role for investigations of the pathogenesis of melioidosis. In the past, several groups have used intravenous (*iv*) and intraperitoneal (*ip*) routes of infection to mimic systemic melioidosis in animal models (Ellison *et al.* 1969; Leakey *et al.* 1998; Hoppe *et al.* 1999; Gauthier *et al.* 2001). The C57BL/6-BALB/c murine model mimics the acute and chronic forms of human melioidosis. Compared to BALB/c mice, C57BL/6 mice are less susceptible to *B. pseudomallei* infection, but not completely resistant. The 50% lethal doses (LD₅₀) of the two *B. pseudomallei* strains used throughout this project, NCTC 13178 and NCTC 13179, have been determined previously in the C57BL/6-BALB/c model of following *iv* infection (Leakey *et al.* 1998; Ulett 2000). In C57BL/6 mice, the LD₅₀ values are 5×10^3 cfu and 6×10^6 cfu, respectively. For BALB/c mice these values are <10 cfu and 0.9×10^4 cfu, respectively.

While inoculation by the *iv* and *ip* routes are useful and relatively simple techniques for generating acute melioidosis in animal models, they do not reflect the suspected modes of transmission of natural *B. pseudomallei* infection. In the past, several studies have investigated the generation of melioidosis in rodents using either subcutaneous (*sc*), inhalation (*in*) and per os (*po*) infection with *B. pseudomallei* (Miller *et al.* 1948b; Dannenberg & Scott 1958b; Liu *et al.* 2002). Recently, Liu *et al.* (2002) attempted to simulate respiratory transmission of melioidosis by inoculating C57BL/6 and BALB/c mice with *B. pseudomallei* via the *in* route. The study compared the virulence of a single *B. pseudomallei* strain in C57BL/6 versus BALB/c mice by contrasting LD₅₀ values, bacterial loads in spleen, liver and lungs, and proinflammatory cytokine production. Similar to studies using *iv* inoculation (Leakey *et al.* 1998), Liu *et al.* (2002)

demonstrated that C57BL/6 mice were 100-fold more resistant to *in* infection than BALB/c mice.

The route of *B. pseudomallei* infection is considered to be at least one of the factors that influences disease outcome, thus contributing to the broad spectrum of clinical presentations associated with melioidosis. However, to date there has been no comprehensive investigation that compares the pathogenesis of melioidosis established by different routes of infection. Therefore, the purpose of this study was to examine the outcome of different routes of *B. pseudomallei* infection in C57BL/6 and BALB/c mice and to confirm the validity of this murine model of differential susceptibility.

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

- 1 To compare the virulence of NCTC 13178 and NCTC 13179 in C57BL/6 and BALB/c mice following infection by the *iv*, *in* and *sc* routes
- 2 To compare the pathogenesis of *B. pseudomallei* infection following inoculation by the *iv*, *ip*, *in* and *po* routes

4.2 Materials and Methods

4.2.1 Delivery of inoculum and care of animals

The level of virulence of NCTC 13178 and NCTC 13179 following *in* or *sc* challenge was determined by LD₅₀ assay in C57BL/6 and BALB/c mice under the ethics approval number, A536. Mice were inoculated *in* by delivering 10 x 2 µl droplets (total of 20 µl) onto the nostrils using a yellow (2-200 µl; Sarstedt, Australia) pipette tip. Volumes of 40 µl were used for *sc* inoculation of *B. pseudomallei* into the hind footpad of mice. Mice were monitored twice daily for a period of ten days. Moribund mice were euthanased according to NH&MRC guidelines.

The pathogenesis of *B. pseudomallei* infection in C57BL/6 and BALB/c mice was compared following *iv*, *ip*, *in* and *po* challenge under the ethics approval number, A709. For *iv* or *ip* challenge, 200 µl of the required dose was delivered into the tail vein or the

peritoneum of mice, respectively. Intranasal inoculation was performed following the procedure described in the previous paragraph. For *po* inoculation, 20 µl of challenge dose was introduced to the back of the throat using a yellow pipette tip.

4.2.2 Comparison of virulence following infection by different routes

The ten-day LD₅₀ values of NCTC 13178 and NCTC 13179 in BALB/c and C57BL/6 mice were determined following *sc* or *in* inoculation using a modified version of the Reed & Meunch (1938) method. Moribund mice were euthanased according to NH&MRC guidelines and this data represented “mortality data” used to calculate ten-day LD₅₀ values. For each route of infection, four groups of five mice were used. An additional control group of five mice were challenged with 200 µl of sterile PBS. Mice were observed twice daily for a period of ten days. Infection with *B. pseudomallei* was confirmed by performing postmortem examinations on all mice to observe abscessation. The challenge doses for the two *B. pseudomallei* strains tested that were given to each of the four groups are shown below in Table 4.1 and 4.2, respectively.

Table 4.1 Challenge doses of NCTC 13178 used for determination of LD₅₀ values in BALB/c and C57BL/6 mice following *sc* or *in* route of infection.

Route of Infection	Mouse Group No.	Inoculating Dose (cfu)	
		BALB/c	C57BL/6
subcutaneous (sc)	1	6.3 x 10 ³	3.6 x 10 ⁷
	2	630	3.6 x 10 ⁶
	3	63	3.6 x 10 ⁵
	4	6.3	3.6 x 10 ³
Intranasal (in)	1	3 x 10 ³	3 x 10 ⁴
	2	300	3 x 10 ³
	3	30	300
	4	3	30

Table 4.2 Challenge doses of NCTC 13179 used for determination of LD₅₀ values in BALB/c and C57BL/6 mice following *sc* or *in* route of infection.

Route of Infection	Mouse Group No.	Inoculating Dose (cfu)	
		BALB/c	C57BL/6
subcutaneous (sc)	1	3 x 10 ⁶	3 x 10 ⁸
	2	3 x 10 ⁵	3 x 10 ⁷
	3	3 x 10 ⁴	3 x 10 ⁶
	4	3 x 10 ³	3 x 10 ⁵
intranasal (in)	1	3 x 10 ⁵	3 x 10 ⁷
	2	3 x 10 ⁴	3 x 10 ⁶
	3	3 x 10 ³	3 x 10 ⁵
	4	3 x 10 ²	3 x 10 ⁴

The level of virulence of NCTC 13178 and 13179 following *in* or *sc* challenge was compared to LD₅₀ values obtained from previous data using the *iv* route of infection in C57BL/6 and BALB/c mice (Leakey *et al.* 1998; Ulett *et al.* 2000).

4.2.3 Comparison of bacterial loads following infection by different routes

Growth kinetics in various organs were compared following inoculation of mice with the highly virulent *B. pseudomallei* strain, NCTC 13178 via *iv*, *ip*, *in* or *po* routes of infection. BALB/c and C57BL/6 mice were administered 570 cfu (equivalent to 60 x LD₅₀ delivered *iv*) or 3 x 10⁵ cfu (equivalent to 60 x LD₅₀ delivered *iv*) in PBS, respectively. Control mice received PBS alone. At 1, 2 and 3 days post-infection, three mice per group were euthanased with CO₂ and blood was collected into sterile tubes containing lithium heparin (Appendix 1) by cardiac puncture. Bacterial load in blood was determined by plating serial dilutions of whole blood onto Ashdown agar and counting colonies after 24 to 48 hrs incubation at 37°C. Immediately following collection of blood, the liver, spleen, lungs, lymph nodes (right and left axillary and inguinal) and brain were aseptically excised. Tissue bacterial load was determined by homogenising tissue in 2.5 ml of PBS using a Lab-Blender 80 Stomacher (Townson & Mercer, Australia) and plating serial dilutions onto Ashdown agar for colony counts (Chapter 3.2). The detection limit of bacteria in tissues was between 2 and 2 x 10⁹ cfu/ml. Data are expressed as the mean log₁₀ cfu ± SEM (n=3).

4.2.4 Statistical analysis

Bacterial loads in organs following different routes of *B. pseudomallei* infection were tested for significance using one-way ANOVA based on normally distributed sets of data within the SPSS version 9.01 software package.

4.3 Results

4.3.1 Virulence determination

Mortality data for the determination of LD₅₀ values for NCTC 13178 and NCTC 13179 is given in Appendix 2. Virulence of NCTC 13178 was compared in C57BL/6 and BALB/c mice following *iv*, *sc* or *in* inoculation (Figure 4.1). Ten-day LD₅₀ values following *sc* infection with NCTC 13178 were 9.1×10^5 cfu and 1×10^3 cfu for C57BL/6 and BALB/c mice, respectively. For *in* infection, the respective values for C57BL/6 and BALB/c mice were 1.8×10^3 cfu and 1.4×10^2 cfu. Compared to BALB/c mice, C57BL/6 mice demonstrated greater resistance to *B. pseudomallei* infection as indicated by their 10-, 100- and 1000-fold higher LD₅₀ values for the *iv*, *sc* and *in* routes of infection, respectively (Figure 4.1).

Figure 4.2 illustrates the log₁₀ LD₅₀ values for the less virulent *B. pseudomallei* strain, NCTC 13179, in C57BL/6 and BALB/c mice following *iv*, *sc* or *in* challenge. Ten-day LD₅₀ values following *sc* infection with NCTC 13179 were $>10^8$ cfu and 1×10^3 cfu for C57BL/6 and BALB/c mice, respectively. For *in* infection, the respective values for C57BL/6 and BALB/c mice were $>10^8$ cfu and 1.9×10^6 cfu. Accurate LD₅₀ values for C57BL/6 mice after *sc* and *in* challenge with NCTC 13179 were not able to be determined. The inoculation of bacteria at numbers greater than 10^8 cfu results in 100% mortality in mice within 24 hrs. Similar to the results of NCTC 13178 infection (Figure 4.1), C57BL/6 mice demonstrated greater resistance to infection with NCTC 13179 when compared to BALB/c mice as indicated by their higher LD₅₀ values for the three routes of infection (Figure 4.2).

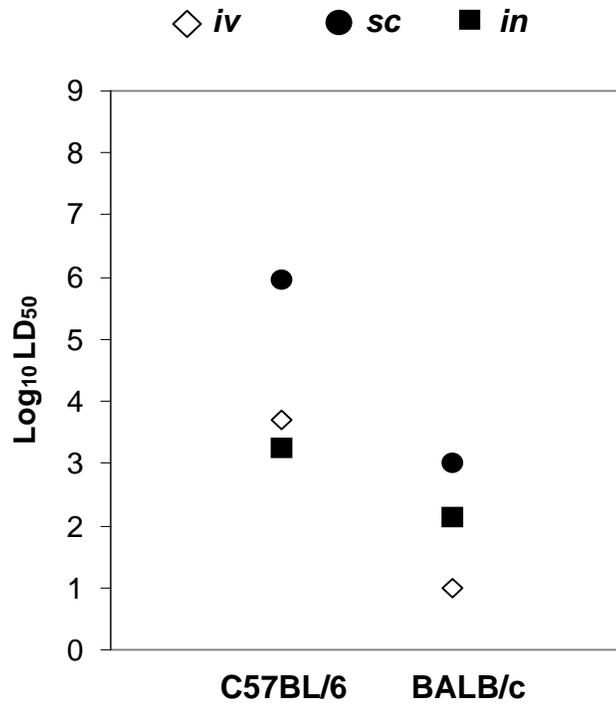


Figure 4.1 Ten-day LD₅₀ values following *iv*, *sc* or *in* inoculation with NCTC 13178. The log₁₀ LD₅₀ values for the highly virulent *B. pseudomallei* strain, NCTC 13178 in C57BL/6 and BALB/c mice were compared following infection via intravenous (*iv*), subcutaneous (*sc*) or intranasal (*in*) routes. (*iv* data derived from Leakey *et al.* 1998).

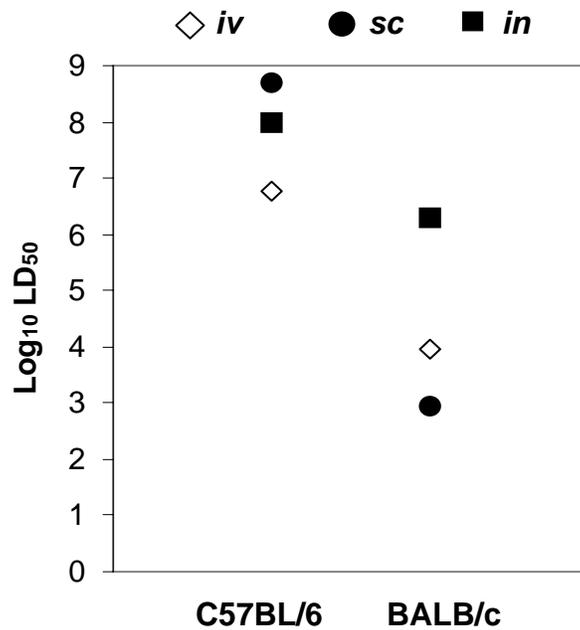


Figure 4.2 Ten-day LD₅₀ values following *iv*, *sc* or *in* inoculation with NCTC 13179. The log₁₀ LD₅₀ values for the less virulent *B. pseudomallei* strain, NCTC 13179 in C57BL/6 and BALB/c mice were compared following infection via intravenous (*iv*), subcutaneous (*sc*) or intranasal (*in*) routes. (*iv* data derived from Ulett *et al.* 2000).

4.3.2 Bacterial loads in tissues

To compare the pathogenesis of *B. pseudomallei* infection following different routes of infection, C57BL/6 and BALB/c mice were inoculated with a lethal dose of the highly virulent isolate, NCTC 13178, either *iv*, *ip*, *in* or *po*. Bacterial loads in blood, spleen, liver, lung, lymph node and brain were determined at day 1, 2 and 3 post infection (data for day 1 and 2 is given in Appendix 2). Following inoculation, growth of *B. pseudomallei* was highest at day 3 in all organs examined. Figure 4.3 and 4.4 show the bacterial loads in various organs at day 3 post infection for *ip*, *in* and *po* routes, in C57BL/6 and BALB/c, respectively. Since all C57BL/6 and BALB/c mice that were challenged by the *iv* route had succumbed to infection by day 3, bacterial loads for day 2 post infection are shown.

Bacterial loads that were comparable to *iv* infection (day 2) were observed at day 3 in C57BL/6 and BALB/c mice that received an *ip* challenge (Figure 4.3 and 4.4, respectively). By day 4 all mice that were inoculated by the *ip* route were moribund and subsequently euthanased. Results for both the *iv* and *ip* routes of infection illustrate that acute, systemic melioidosis can be generated in both C57BL/6 and BALB/c mice using the highly virulent *B. pseudomallei* strain, NCTC 13178, although greater bacterial numbers are required for the more resistant C57BL/6 mouse.

In C57BL/6 mice, bacterial loads were greatly reduced following *in* or *po* inoculation, when compared to *iv* or *ip* infection (Figure 4.3; $in < iv$, $P < 0.05$; $in < ip$, $P < 0.05$; $po < iv$, $P < 0.05$; $po < ip$, $P < 0.05$). While low levels of bacteria were detected in blood of C57BL/6 mice at day 1 and 2 post infection, no bacteria were detected by day 3 in mice that were challenged by the *in* or *po* route (Figure 4.3). The persistence of *B. pseudomallei* in the spleen, liver and lungs however, demonstrate that although C57BL/6 mice are more resistant to NCTC 13178 via the *in* and *po* route of infection, they are not able to clear the infection. In contrast, *in* infection of BALB/c mice with NCTC 13178, results in disseminated infection and bacterial loads that are comparable to BALB/c mice challenged by the *iv* or *ip* route (Figure 4.4; in and iv , $P > 0.05$; in and ip , $P > 0.05$).

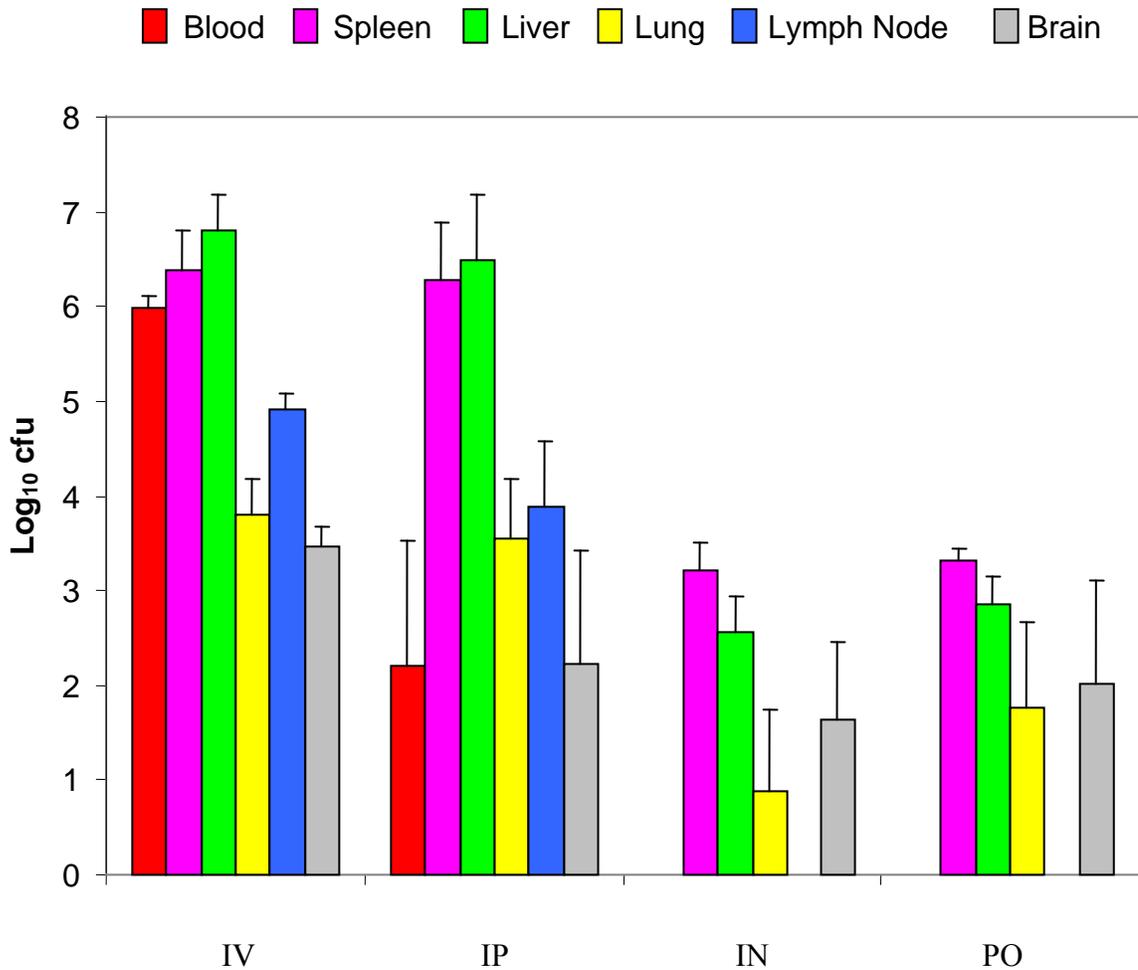


Figure 4.3 Comparison of bacterial loads in tissues of C57BL/6 mice following challenge with virulent *B. pseudomallei* by different routes of infection.

C57BL/6 mice were inoculated with 5×10^5 cfu of NCTC 13178 by either intravenous (iv), intraperitoneal (ip), intranasal (in) or per os (po) route of infection. At days 1, 2 and 3 post infection, three mice per group were euthanased and bacterial loads were determined in blood, spleen, liver, lung, lymph node and brain. Results are expressed as the mean \log_{10} cfu \pm SEM (n=3) at day 3 post-infection. Data for iv infection was obtained at day 2 post-infection.

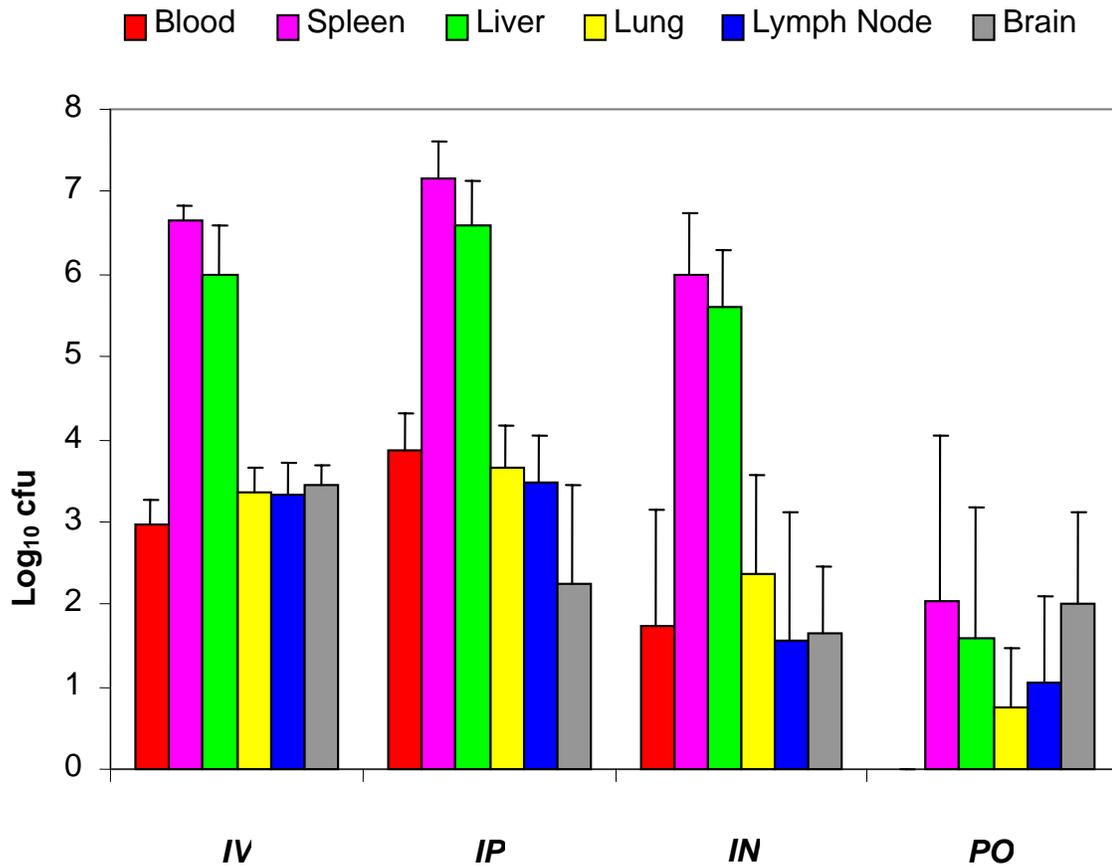


Figure 4.4 Comparison of bacterial loads in tissues of BALB/c mice following challenge with virulent *B. pseudomallei* by different routes of infection.

BALB/c mice were inoculated with 570 cfu of NCTC 13178 by either intravenous (*iv*), intraperitoneal (*ip*), intranasal (*in*) or per os (*po*) route of infection. At days 1, 2 and 3 post infection, three mice per group were euthanased and bacterial loads were determined in blood, spleen, liver, lung, lymph node and brain. Results are expressed as the mean \log_{10} cfu \pm SEM (n=3) at day 3 post-infection. Data for *iv* infection was obtained at day 2 post infection since all mice were dead by day 3.

For *po*, BALB/c mice show greatly reduced loads in all organs, particularly spleen and liver. There was also a high variation in colony counts in all mice (Figure 4.4).

C57BL/6 and BALB/c that had been *iv* challenged with *B. pseudomallei* demonstrated the greatest bacterial loads (Figure 4.3 and 4.4, respectively). Despite BALB/c mice receiving a much lower inoculating dose than C57BL/6 mice (ie. 570 cfu versus 5.7×10^5 cfu), following *iv* infection the levels of bacteria at day 2 in all tissue examined were similar between the two mouse strains.

Bacterial numbers were greatest in spleen and liver for both C57BL/6 and BALB/c mice, regardless of the route of infection used (Figure 4.3 and 4.4, respectively). There were no differences observed in bacterial loads in the C57BL/6 or BALB/c lung following *in* or *po* challenge compared to *iv* or *ip* infection (Figure 4.3: *in* and *iv*, $P > 0.05$; *in* and *ip*, $P > 0.05$; *po* and *iv*, $P > 0.05$; *po* and *ip*, $P > 0.05$; Figure 4.4: *in* and *iv*, $P > 0.05$; *in* and *ip*, $P > 0.05$; *po* and *iv*, $P > 0.05$; *po* and *ip*, $P > 0.05$). Interestingly, bacteria were detected in the brain of C57BL/6 mice following *in* and *po* at day 1 to 3 post infection, despite bacterial loads in blood falling to undetectable levels by day 3 (Figure 4.3). Compared to *iv* and *ip* infection, bacterial loads in brain at day 3 post infection in *in* and *po* C57BL/6 mice were relatively high when compared to spleen and liver. Variations in the colonial morphology of *B. pseudomallei* recovered from brain were observed. Unlike the characteristic wrinkled morphology of isolates from other tissues such as spleen and liver, colonies recovered from the brains of C57BL/6 mice inoculated by the *in* or *po* routes were mucoid in appearance. In comparison, bacteria recovered from brains of C57BL/6 mice that were challenged by the *iv* or *ip* route, demonstrated the characteristic morphology on Ashdown agar. *B. pseudomallei* was also detected in the brain of BALB/c mice following *po*, despite the absence of bacteria in blood at day 3 (Figure 4.4)

4.4 Discussion

The ability of *B. pseudomallei* to cause inflammation in any tissue type contributes to the wide variation in clinical features associated with melioidosis. Clinical manifestations range from acute fulminant septicaemia to a chronic localised infection. Many cases present as either primary or secondary (septicaemic) pneumonia (White 2003) which can lead to a rapid deterioration in respiratory function (Dance 2002). However abscess formation in liver, spleen, skin and prostate also commonly occur following *B. pseudomallei* infection (Dance 2002; White 2003). Compared to Thailand, prostatic abscesses and neurological melioidosis occur more frequently in northern Australia (Currie *et al.* 2000a; Woods *et al.* 1992). The route of infection with *B. pseudomallei* may, at least in part, influence the broad spectrum of clinical presentations associated with melioidosis. *B. pseudomallei* is widely distributed in soil and water in endemic regions. Transmission of the bacterium is thought to occur following subcutaneous inoculation, inhalation or ingestion of contaminated soil or water (Leelarasamee & Bovornkitti 1989). However, for the majority of melioidosis cases, there is typically no obvious causative incident (Dance 2000).

Animal models play an essential role in our understanding of microbial pathogenesis. C57BL/6 and BALB/c mice are used as a model of chronic and acute human melioidosis, respectively (Leakey *et al.* 1998). While the majority of studies using animal models of melioidosis have used the *iv* or *ip* route of inoculation, the relevance of these studies to the natural routes of infection is not always clear. A study by Miller *et al.* (1948b) compared the infectivity of three virulent strains of *B. pseudomallei* by the *ip* route in hamsters with various other portals of entry. They reported increasing minimum lethal doses (MLD's) for the three strains following *ip*, *sc*, *in* and *po* infection, respectively. Later, the high susceptibility of hamsters to *ip* inoculation of *B. pseudomallei*, led Ellison *et al.* (1969) to use this model as a means for recovering the bacterium from soil and surface water. Dannenberg & Scott (1958b) investigated the pathology of melioidosis produced by the *ip* and *in* route in mice, demonstrating the similarities in organ involvement when either portals of entry are used. Following

inhalation of large doses of virulent or avirulent *B. pseudomallei*, lesions with visible bacteria were observed in the lung, spleen and liver of mice (Dannenberg & Scott 1958a,b).

Infectivity, as assessed by determination of the LD₅₀, was investigated for two *B. pseudomallei* strains frequently used in our continued investigations on the immunopathogenesis of melioidosis. Previous studies using the C57BL/6-BALB/c murine model (Leakey *et al.* 1998), demonstrated that NCTC 13178 is a highly virulent isolate, while NCTC 13179 is comparatively avirulent (Ulett 2000). The present study compared the LD₅₀ values for these two *B. pseudomallei* strains following infection by the *in* and *sc* routes. Compared to BALB/c mice, C57BL/6 mice are less susceptible to *B. pseudomallei* infection, regardless of the portal of entry, thus validating the model of differential susceptibility for various routes of infection. However, as demonstrated by others (Leakey *et al.* 1998; Hoppe *et al.* 1999; Liu *et al.* 2002), C57BL/6 mice are not completely resistant to infection by *B. pseudomallei*. Systemic melioidosis can be generated in C57BL/6 mice using different routes of infection, if a high inoculating dose is used. Following *iv* inoculation of BALB/c mice, NCTC 13178 is regarded as a highly virulent strain of *B. pseudomallei* since the LD₅₀ is < 10 cfu. However, if BALB/c mice are inoculated with NCTC 13178 *sc*, the LD₅₀ value increases 100-fold to 1 x 10³ cfu. This value is equivalent to the LD₅₀ of the “less virulent” NCTC 13179 delivered *sc*. The results emphasise that classification of virulence is greatly dependent on the route of infection.

Infection by different routes exposes organisms to different components of the immune system. This may subsequently influence disease outcome. The peripheral lymphoid tissues include lymph nodes, spleen, and the cutaneous and mucosal immune systems. Protective immunity against microbes is mediated by the early responses of the innate immune system of which the principal components are physical barriers, APC, NK cells, $\gamma\delta^+$ T cells, blood proteins and cytokines (Abbas, Lichtman & Pober 2000). The contribution of each of these components varies with the type of lymphoid tissue. For example, high numbers of dendritic cells are present in the epithelia of skin and the

gastrointestinal and respiratory systems. These are the main portals through which natural infection with microbes occurs. Dendritic cells are highly efficient APC that capture and transport antigen to lymph nodes and spleen for presentation to T cells. Similarly, $\gamma\delta^+$ T cells are present in high numbers in epithelial tissues and although their functions are not fully understood, they are believed to contribute to innate immunity (Abbas, Lichtman and Pober 2000). Consequently, the portal of entry of a pathogen has the potential to influence the outcome of an infection.

Therefore we compared the pathogenesis of *B. pseudomallei* NCTC 13178 infection following inoculation by the *iv*, *ip*, *in* and *po* routes. This was investigated by comparing bacterial loads in various tissues of *B. pseudomallei*-infected C57BL/6 and BALB/c mice. A tropism for spleen and liver was demonstrated following infection by each of the four routes examined. It has been previously reported (Leakey *et al.* 1998; Hoppe *et al.* 1999; Gauthier *et al.* 2001) that *B. pseudomallei* multiplies mainly in the spleen, bacterial counts per spleen being higher than those per liver. In the present study, differences between bacterial loads in spleen and liver were minimal and not statistically significant for any route of infection. *B. pseudomallei* could be detected in the tissues of *iv* and *ip* mice earlier and in higher numbers, than in those of *in* and *po* mice, despite receiving equal numbers of bacteria. This reflects the differences in bacterial growth, depending on the route of infection used. Bacterial numbers in mice infected via the *iv* or *ip* route reached $>10^6$ cfu by day 2 post-infection indicating a failure of the innate immune response to control infection, leading to overwhelming sepsis and death. The LPS of gram negative bacteria stimulates the production and release of various cytokines in large quantities and it is this response which is thought to result in the pathologic features associated with septicaemia including multiple organ failure, coagulation defects and shock (Suputtamongkol *et al.* 1992). Hyper-production of cytokines results in systemic inflammation (SIRS and CARS phenomena) that disrupts organ function and leads to death (Cobb *et al.* 2000). The hyper-production of various cytokines and chemokines has been demonstrated in experimental and human cases of acute melioidosis prior to host death (Brown *et al.* 1991; Suputtamongkol *et al.* 1992; Lauw *et al.* 1999; Lauw *et al.* 2000; Simpson *et al.* 2000; Ulett *et al.* 2000a,b).

Bacterial loads in C57BL/6 mice following a lethal dose of NCTC 13178 suggest that this mouse strain is more susceptible to infection by the *iv* versus *in* route. However, the LD₅₀ values do not support this observation. Mortality data obtained in the LD₅₀ experiments for *in* infection of C57BL/6 mice with NCTC 13178 was inconsistent (Appendix 2). Inconsistent MLD values for hamsters infected by the *in* and *po* routes have previously been reported (Miller *et al.* 1948). For example, while some animals became infected after ingestion of relatively small amounts, others appeared capable of readily destroying larger doses of the bacteria (Miller *et al.* 1948). This appears to have occurred in the current study and may be a reflection of poor reproducibility using our technique for *in* infection. In the current study, liquid was dispensed onto the nostrils of mice using a pipette tip in volumes small enough to be inhaled. Natural infection by the respiratory route is more closely resembled by aerosol infection, which is considered an effective and reproducible method for determining virulence of bacteria in animal models (Dannenberg & Scott 1958a; Bracegirdle *et al.* 1994). Future studies to investigate the pathogenesis of *B. pseudomallei* infection in the C57BL/6-BALB/c model following *in* infection could be attempted with a nebuliser. However, the use of this technique would require an appropriate level of containment to eliminate exposure of laboratory workers to *B. pseudomallei*.

Recently, Liu *et al.* (2002) reported that C57BL/6 mice were 100-fold more resistant to *in* infection compared to BALB/c mice. High bacterial loads were demonstrated in the lung, together with spleen of susceptible BALB/c mice when *in* inoculation of *B. pseudomallei* was used. In the present study, we have also demonstrated increased resistance of C57BL/6 mice to two *B. pseudomallei* strains compared to BALB/c mice. However in the current study we concentrated on comparing the pathogenesis within mouse strains using different routes of infection, rather than a comparison between C57BL/6 and BALB/c mice. Bacterial loads in tissues following challenge with a lethal dose of highly virulent NCTC 13178 did not indicate any tropism for the lung following infection by the *in* route. As early as day 1, bacterial loads were greatest in the liver and spleen, not lung, of C57BL/6 and BALB/c mice following *in* challenge (Appendix 2).

This suggests a very early systemic spread of *B. pseudomallei* from the lungs to other organs, although at day 1 post-infection bacterial numbers in blood were less than 10 cfu/ml (Appendix 2).

Bacteria were detected in the brains of all mice following infection by either the *iv*, *ip*, *in* or *po* route. Interestingly though, at day 2 and 3 *B. pseudomallei* was detected in the brain of C57BL/6 mice inoculated *in* or *po*, in the absence of septicaemia. Colonial morphology of *B. pseudomallei* isolated from brains of these mice differed greatly from isolates recovered from other organs. Colonies recovered from the brains of C57BL/6 mice inoculated by the *in* or *po* routes were mucoid in appearance. In comparison, bacteria recovered from brains of C57BL/6 mice that were challenged by the *iv* or *ip* route demonstrated the characteristic wrinkled morphology on Ashdown agar. Variation in colonial morphology of *B. pseudomallei* has been documented previously (Nigg *et al.* 1955). A study by Vorachit *et al.* (1995) demonstrated the ability of *B. pseudomallei* to form biofilms within animal tissue. The production of a glycocalyx facilitates the formation of microcolonies thus providing protection of *B. pseudomallei* from the host defense mechanisms and from antimicrobial therapy (Vorachit *et al.* 1995). A more recent study by Nanagara *et al.* (2000) identified atypical morphology of *B. pseudomallei* strains in human synovial tissue. An immunogold staining technique based on polyclonal antibodies directed against whole cell *B. pseudomallei* antigens, was applied to synovial tissue, then visualised by electron microscopy. Compared to bacteria grown in culture media, decreased surface antigen expression was observed in *B. pseudomallei* colonies from synovial biopsies. The authors suggest this change in antigenic morphology may be an adaptation of *B. pseudomallei* that enables it to evade host immune responses, or allows it to survive within unfavourable environments (Nanagara *et al.* 2000). The variation in colonial morphology on Ashdown agar observed in bacteria isolated from brains of C57BL/6 mice infected by the *in* or *po* route may reflect a change to biofilm formation of *B. pseudomallei* in this tissue. Colonies from brains of C57BL/6 mice challenged *iv* or *ip* routes showed typical wrinkled morphology. In this case, infection in the brain may have been a consequence of the overwhelming septicaemia that spilled over to all organs in these mice.

Neurological abnormalities are documented in animals and humans with melioidosis, and a predilection for the brainstem and spinal cord has been suggested (Currie et al. 2000b). Several reports describe brain abscesses caused by *B. pseudomallei*, many of which are fatal despite treatment (Thin *et al.* 1970; Lee & Chua 1986; Pit *et al.* 1988; Woods *et al.* 1992; Padiglione *et al.* 1997; Lath *et al.* 1998). In many cases of neurological melioidosis, there has been no evidence of direct brain infection suggesting that damage may be a result of immune or toxin-mediated mechanisms (Bartley *et al.* 1999). The current study supports a role for direct brain invasion by *B. pseudomallei* in the pathogenesis of melioidosis. Further investigation using *in* or *po* inoculation in the murine model may be useful in studying the pathogenesis of neurological forms of melioidosis.

In summary, the results of this study have confirmed the effectiveness of C57BL/6 and BALB/c mice as a model for differential susceptibility to *B. pseudomallei*, regardless of the route of infection. The data also emphasises that classification of bacterial virulence is highly dependent on the portal of entry of *B. pseudomallei*. If we are to gain a true understanding of the immunopathogenesis of melioidosis, future studies should consider the use of inoculating techniques that more closely resemble natural *B. pseudomallei* infection

CHAPTER 5

CHEMOKINE AND COLONY STIMULATING FACTOR GENE EXPRESSION IN A MURINE MODEL OF MELIOIDOSIS

5.1 Introduction

Studies on several intracellular bacteria have demonstrated that innate host resistance involves the mobilisation of specific subsets of leucocytes to the inflammatory site (Di Tirro *et al.* 1998; Orme & Cooper 1999). In several models of intracellular infection, chemokines and colony stimulating factors (CSF) have been shown to regulate inflammation (Di Tirro *et al.* 1998; Mohammed *et al.* 1999).

Chemokines are a family of secreted proteins with strong chemotactic properties for particular inflammatory cell types, making them integral components of the initial stages of an immune response. Many chemokines are also involved in activation of the leucocyte populations they attract (Lukacs 1997; De Vries *et al.* 1999). Chemokines are currently grouped into four subfamilies according to the presence or absence of four conserved cysteine (C) residues (Rollins 1997; De Vries *et al.* 1999). CC chemokines, whose first two C residues are adjacent, demonstrate chemotactic activity for monocytes, lymphocytes, eosinophils and basophils, but not neutrophils (Rollins 1997; Vaddi *et al.* 1997). Members of the CC subfamily include RANTES, MCP-1 and MIP-1 α (Wuyts *et al.* 1994).

Separation of two C residues by one amino acid is a distinguishing characteristic of CXC chemokines, which are generally chemotactic for neutrophils. The CXC subfamily is further subdivided into two groups according to the presence or absence of an amino acid group, glutamate-leucine-arginine (ELR), adjacent to the CXC motif. Interleukin 8 (IL-8), LPS-induced CXC chemokine (LIX), KC and MIP-2 are ELR⁺ and preferentially attract neutrophils (Vaddi *et al.* 1997; Teruya-Feldstein *et al.* 1997). On the other hand, the CXC chemokines Mig and IP-10 lack the ELR motif (ELR⁻) and are typically

chemotactic for monocytes and T lymphocytes (Rollins 1997; Teruya-Feldstein *et al.* 1997). The C group, which lacks a cysteine residue at the amino terminal, has only one member: lymphotactin. Lymphotactin is only chemotactic for lymphocytes (Kelner *et al.* 1994). The fourth subfamily includes the CX₃C chemokine, fractalkine (or neurotactin), which is unique as it exists in two forms: attached directly to the cell membrane via a long mucin stalk or as a secreted chemokine once it is cleaved from the mucin stalk (Bazan *et al.* 1997). Fractalkine chemoattracts T lymphocytes, monocytes and NK cells (Rossi *et al.* 1998).

Colony stimulating factors are glycoproteins produced by various cell types throughout the human body. They are necessary for the survival, proliferation and differentiation of haematopoietic progenitor cells of the myeloid and erythroid lineage. Macrophage-CSF enhances the survival and activation of cells of the monocyte lineage, while granulocyte-CSF boosts the activity of neutrophils (Sowter *et al.* 1997). Granulocyte-macrophage CSF has a dual role and is able to augment the accumulation and activation of both neutrophils and macrophages (Sowter *et al.* 1997).

Development of an effective immune response to infection with *B. pseudomallei* is considered to be critical for the survival of the host (Brown *et al.* 1990). In human melioidosis, a correlation between disease severity and serum levels of cytokines and chemokines such as IFN- γ , IL-12 and IL-8 has been demonstrated (Friedland *et al.* 1992; Lauw *et al.* 1999). Studies in patients with melioidosis have also demonstrated that elevated levels of the chemokines interferon- γ -inducible protein 10 (IP-10) and monocyte interferon- γ -inducible protein (Mig) correlate with disease severity and clinical outcome (Lauw *et al.* 2000). However, the kinetics and magnitude of chemokine and CSF responses in acute and chronic *B. pseudomallei* infection are unknown. Therefore in this study reverse transcriptase-polymerisation chain reaction (RT-PCR) and histology were used to assess the production of chemokine and CSF mRNA during infection and to monitor histological changes in the livers and spleens of C57BL/6 and BALB/c mice infected with virulent *B. pseudomallei*.

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

- 1 To determine the kinetics of induction of selected chemokines and CSF (IP-10, Mig, RANTES, MCP-1, KC, MIP-2, G-CSF, M-CSF, GM-CSF) in liver and spleen of C57BL/6 and BALB/c mice following infection with a virulent strain of *B. pseudomallei*;
- 2 To monitor histological changes in liver and spleen of C57BL/6 and BALB/c mice following infection with a virulent strain of *B. pseudomallei*;
- 3 To determine the relationship between bacterial loads and cellular composition of lesions in spleen and liver, and the kinetics of induction of chemokines and CSF.

5.2 Materials and Methods

5.2.1 Bacterial isolate

The highly virulent *B. pseudomallei* strain (NCTC 13178) was used in this study (Chapter 3).

5.2.2 Experimental infection with *B. pseudomallei*

Experiments were performed with C57BL/6 and BALB/c mice (6- to 16-weeks of age) purchased from the James Cook University Small Animal Breeding Unit (Townville, Australia). Animal experiments were performed under the ethics approval number A536 (Chapter 3).

Mice were inoculated *iv* with 25 cfu of *B. pseudomallei* suspended in 200 µl sterile PBS. Control mice received 200 µl sterile PBS only. Following inoculation, groups of five mice were euthanased with CO₂ at day 0, 1, 2, 3, 4, 7 and 14. The liver and spleen were excised and bisected aseptically. Half of each organ was used to determine bacterial load (Chapter 3). The remaining tissue was immediately wrapped in aluminium foil, immersed in liquid nitrogen and stored at -70°C until RNA extraction. Since all BALB/c mice had succumb to infection by day 4 post-infection, samples for BALB/c mice were only obtained at day 0, 1, 2, and 3.

5.2.3 RNA extraction

Total RNA was extracted using the SV Total RNA Isolation System (Promega; Annandale, Australia) with modifications to the manufacturer's protocol. Briefly, 30 mg of frozen spleen or 0.8 g of liver was homogenised in 700 µl of working Lysis Buffer. Half of this mixture was transferred to another sterile eppendorf allowing duplicate samples to be prepared. To each duplicate, 700 µl of Dilution Buffer was added and mixed gently. The eppendorf was placed in a 70°C waterbath for 3 min, then centrifuged for 10 min at 16,000 *g*. Using a sterile pasteur pipette, the cleared lysate solution was transferred to a fresh, sterile eppendorf. To this, 300 µl of 95% (vol/vol) ethanol was added and mixed by gentle pipetting, then 800 µl of this mixture was added to the Spin Basket and centrifuged (14,000 *g*; 1 min.) The eluate was discarded and the Spin Basket washed with 600 µl of Working Wash Solution (14 000*g*; 1 min). Again, the eluate was discarded and 50 µl of the DNase incubation mix was added directly to the membrane inside the Spin Basket and incubated for 15 min at RT. Following incubation, 200 µl of Working Stop Solution was added to the Spin Basket and centrifuged at 14 000 *g* for 1 min. The Spin Basket was washed with 600 µl of Working Wash Solution (14,000 *g*; 1 min) and the eluate discarded. A final wash with 250 µl of Working Wash Solution (14,000 *g*, 2 min) was performed before placing the Spin Basket over the Collection Tube. One hundred microliters of nuclease-free water was added to the membrane and centrifuged at 14,000 *g* for 1 min to elute the RNA. Total RNA was quantified using spectrophotometry at 260 nm and the quality of the RNA was determined by the ration of 260 versus 280 nm. A ratio of greater than 1.8 was considered acceptable.

5.2.4 Reverse transcription

Reverse transcription of 1 µg of total RNA was achieved in a 20 µl reaction mixture containing 0.5 µg Oligo(dT)₁₅ (Promega; Annandale, Australia), 1X First Strand Buffer (50 mmol/L Tris-HCl [pH 8.3], 75 mmol/L KCl, 3 mmol/L MgCl₂; Life Technologies, Australia), 10 mmol/L dithiothreitol (dTT; Invitrogen; Melbourne, Australia), 500 µmol/L deoxynucleoside triphosphate (dNTP) mix (Promega; Annandale, Australia) and

200 U of Superscript II™ reverse transcriptase (Invitrogen; Melbourne, Australia). Residual RNA was depleted with RNase H (Promega; Annandale, Australia).

5.2.5 PCR-assisted amplification

Two microlitres of cDNA were amplified with Taq DNA polymerase (1U; Promega; Annandale, Australia) for 30 cycles (initial step of 95°C for 2 min, then denaturation at 94°C for 50 sec, primer annealing at 60°C for 50 sec, and primer extension at 72°C for 1 min). In preliminary experiments by Ulett *et al.* (2000a), 30 cycles was shown to lie in the linear portion of the curve for the amount of PCR product produced. Sense and antisense primers for the chemokines tested (G-CSF, M-CSF, GM-CSF, IP-10, Mig, RANTES, MCP-1, KC and MIP-2) were from published data (Table 5.1) and were synthesised by Invitrogen (Melbourne, Australia). The optimal MgCl₂ concentration for cytokine-specific primer pairs ranged from 1 mmol/L to 4.5 mmol/L. Primers were used at a final concentration of 1 µmol/L in a 100 µl reaction mixture containing 200 µmol/L dNTP mix (Promega; Annandale, Australia); 1 X PCR buffer (1 mmol/L Tris-HCl [pH 9.0], 5 mmol/L KCl, 0.01% Triton X-100; Promega; Annandale, Australia), and 1 to 4.5 mmol/L MgCl₂ (Promega; Annandale, Australia).

For each cDNA sample, duplicate PCR reactions were prepared using β-actin primers instead of cytokine primers as an internal positive control standard. Negative controls using water instead of cDNA were also included in each run. Non-reverse transcribed total RNA for each sample was subjected to PCR amplification using β-actin primers to confirm that amplification was based solely on cDNA. PCR-assisted mRNA amplification was performed for three separately prepared cDNA samples. PCR products were electrophoresed in 2 to 3% agarose gels stained with 0.5 µg/mL ethidium bromide, and visualised with an ultraviolet transilluminator. A 123-base pair (bp) ladder (Invitrogen; Melbourne, Australia) was used as the DNA marker.

5.2.6 Histology

In parallel experiments, a series of five C57BL/6 and BALB/c mice were inoculated *iv* with 25 cfu of *B. pseudomallei* (NCTC 13178). Control mice received sterile PBS only.

Table 5.1. Chemokine- and colony stimulating factor-specific primer pair sequences used in this study

Chemokine	Oligonucleotide Sequence[*]	Size (bp)[§]	Reference
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^{*}For each primer pair, the sense sequence is shown above the antisense sequence.

[§]Predicted size of the PCR product

At days 1, 3 and 14 (for C57BL/6 mice only) mice were euthanased with CO₂ and their liver and spleen excised for histology. At day 3 only, organs were bisected aseptically and one half was used to determine bacterial load. The remaining tissue was processed for histological examination. Livers and spleens were fixed in 10% neutral-buffered formalin at RT for at least 48 hrs before processing. Tissue was processed on a 15 hr cycle using a Shandon Citadel 2000 Tissue Processor (Shandon Southern Products Ltd; Cheshire, UK). After embedding in paraffin, 4 µm sections were cut and stained with H&E (Appendix1). Histological examination was carried out using light microscopy.

5.3 Results

5.3.1 Bacterial growth kinetics

Bacterial numbers in BALB/c liver and spleen increased rapidly, reaching approximately 1×10^6 cfu by day 3 post-infection, prior to host death. In contrast, bacterial loads in the liver and spleen of C57BL/6 mice were approximately 1000-fold fewer than in BALB/c at day 3 (Figure 5.1). However by day 14, levels of *B. pseudomallei* recovered from C57BL/6 mice were similar to those detected in BALB/c mice at day 3 post-infection ($\approx 10^6$ cfu).

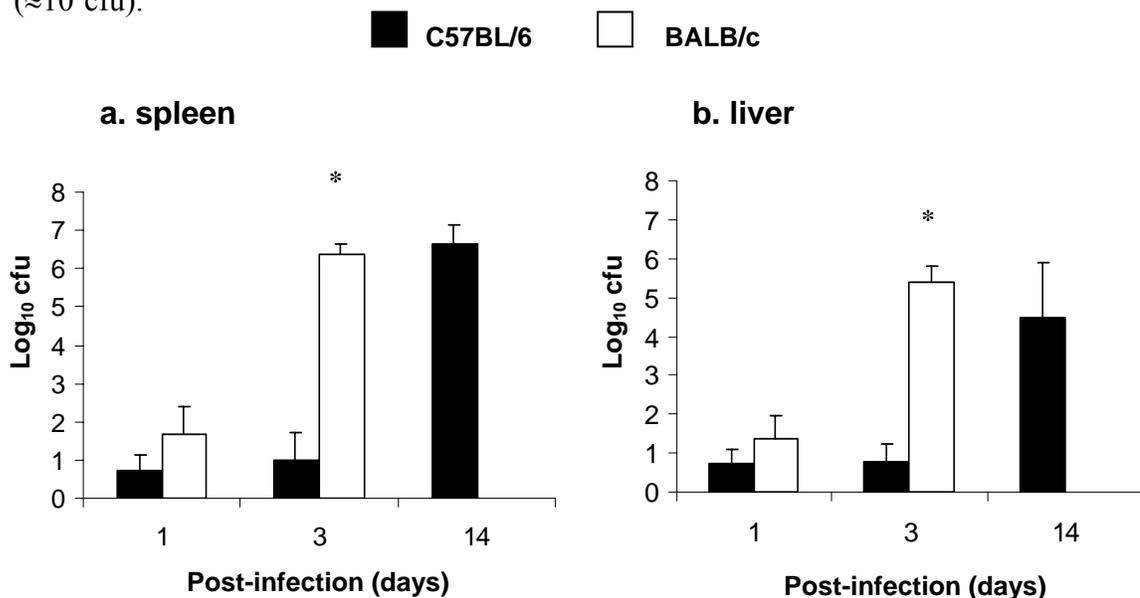


Figure 5.1 Bacterial load in spleen and liver of C57BL/6 and BALB/c mice infected *iv* with 25 cfu of virulent *B. pseudomallei*.

Bacterial load in the (A) spleens and (B) livers of C57BL/6 and BALB/c mice at days 1, 3 and 14 following *iv* inoculation with 25 cfu of virulent *B. pseudomallei*. Results are shown as the mean bacterial load (log₁₀ cfu/mL) for five mice \pm SEM (detection limit of 2 cfu/mL, therefore <2 cfu/mL taken as 0 cfu/mL). *Significantly higher bacterial loads in BALB/c mice at day 3 post-infection was observed (P<0.01; as determined by Student's *t* test).

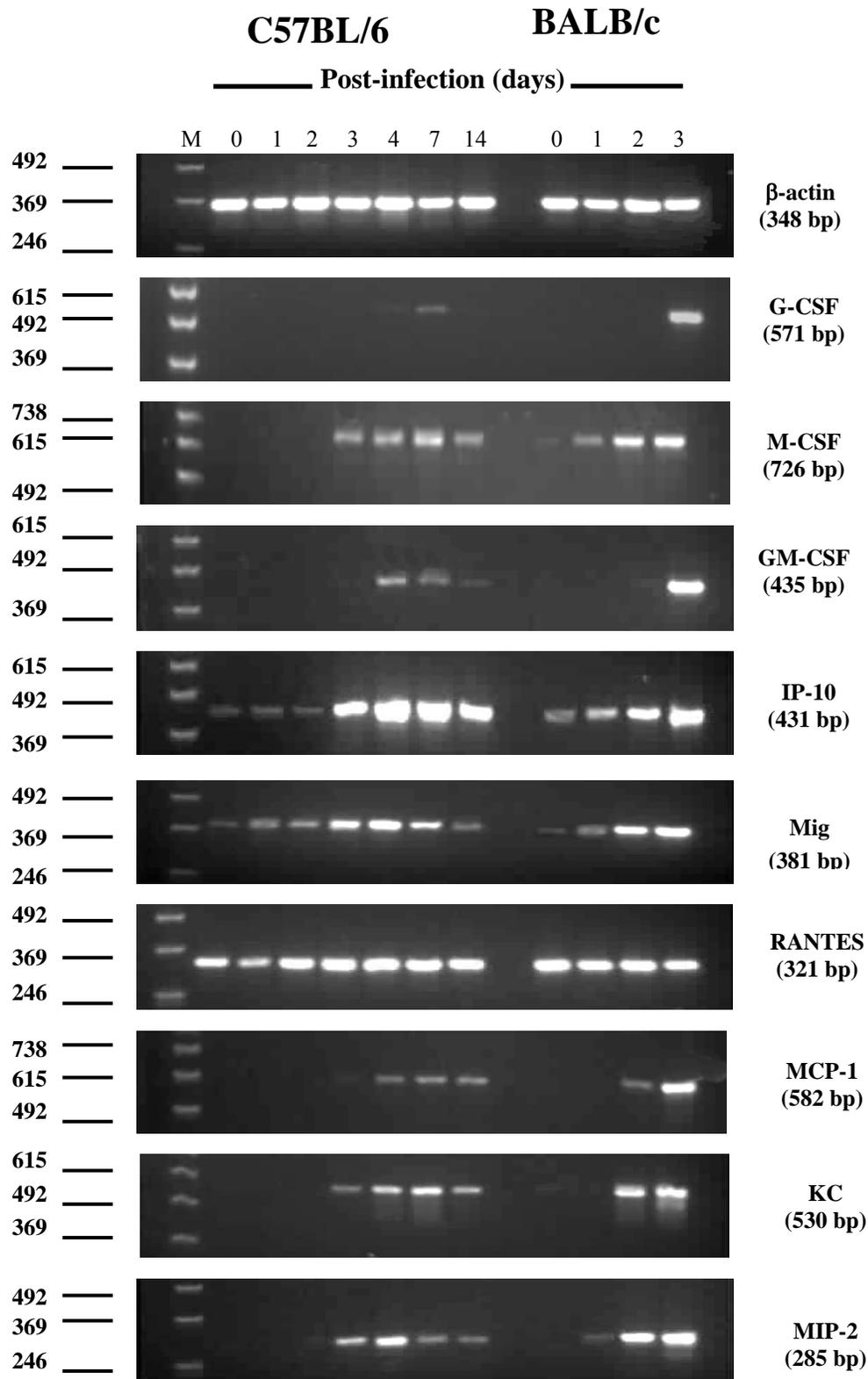


Figure 5.2 Chemokine and CSF mRNA responses in spleen of C57BL/6 and BALB/c mice infected *iv* with 25 cfu of virulent *B. pseudomallei*.

Mice were inoculated *iv* with 25 cfu of virulent *B. pseudomallei*. Control mice received PBS only. At various time intervals following infection (days 0-14), spleens were excised and total RNA was extracted. Three micrograms of total RNA was reverse transcribed and PCR was performed using specific primers. Molecular weight markers (M) are shown in the left-hand lane of each gel. Data shown are representative of three mice at each time point. Similar levels of the house keeping gene, β -actin, were detected in all samples. Maximal expression of mRNA for all chemokines and CSF investigated was demonstrated at day 3 in BALB/c mice and day 4 to 14 in C57BL/6 mice.

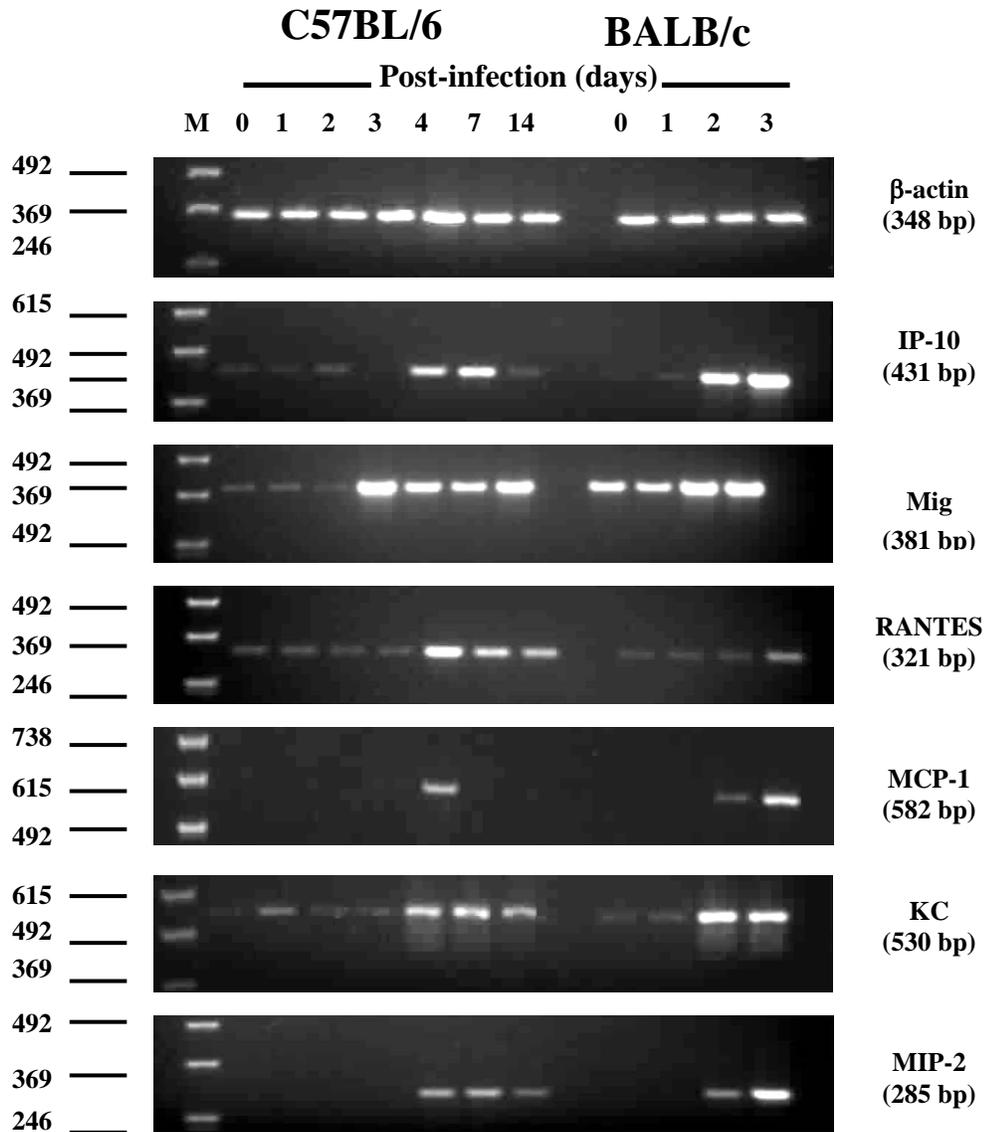


Figure 5.3 Chemokine and CSF mRNA responses in liver of C57BL/6 and BALB/c mice infected *iv* with 25 cfu of virulent *B. pseudomallei*.

Mice were inoculated *iv* with 25 cfu of virulent *B. pseudomallei*. Control mice received PBS only. At various time intervals following infection (days 0-14), livers were excised and total RNA was extracted. Three micrograms of total RNA was reverse transcribed and PCR was performed using specific primers. Molecular weight markers (M) are shown in the left-hand lane of each gel. Data shown are representative of three mice at each time point. Similar levels of the house keeping gene, β -actin, were detected in all samples. Maximal expression of mRNA for all chemokines and CSF investigated was demonstrated at day 3 in BALB/c mice and day 4 to 14 in C57BL/6 mice.

5.3.2 Detection of chemokine and colony stimulating factor mRNA

Patterns of chemokines and CSF induction in the spleen and liver of C57BL/6 and BALB/c mice are illustrated in Figure 5.2 and 5.3, respectively. Similar levels of the housekeeping gene β -actin were detected for all samples (Figure 5.2 & 5.3). These results confirm the semiquantitative nature of the methods used and allow a comparison of chemokine- and CSF-specific mRNA responses during the course of experimental infection. Patterns of chemokine and CSF induction were similar in spleen and liver, although responses were typically greater in spleen (Figure 5.2 & 5.3; data for CSF in liver not shown). Baseline levels of mRNA for the majority of chemokines and CSF in spleen were either not detected or were very low (Figure 5.2). However, constitutive expression of RANTES was observed in both C57BL/6 and BALB/c mice. A slight increase in the expression of RANTES was apparent in spleen of both strains of mice during infection (Figure 5.2). A chemokine- or CSF-specific response to *B. pseudomallei* infection, indicated by an increase in gene transcription, was clearly demonstrated since baseline levels of chemokine and CSF mRNA other than RANTES were minimal. With the exception of RANTES, increased amounts of mRNA for all chemokines and CSF were detected in spleen of BALB/c mice by day 1 and 2 of infection (Figure 5.2). The level of induction of these chemokines and CSF continued to rise over 3 days prior to host death, with greatest expression observed at day 3 (Figure 5.2). In spleen of C57BL/6 mice, increased amounts of mRNA for all chemokines were also observed following infection (Figure 5.2). Increased expression was most pronounced for IP-10 and Mig. In contrast to BALB/c mice, levels of mRNA in spleen of C57BL/6 mice were greatest between day 4 and 7 of infection (Figure 5.2). In spleen of C57BL/6 mice the levels of mRNA for IP-10 remained high for the duration of the assay period (14 days).

The kinetics of induction for chemokine and CSF mRNA differed between the spleen of C57BL/6 and BALB/c mice. While both strains of mice showed evidence of increased induction of IP-10 and Mig at day 1, early increases in mRNA levels of M-CSF and MIP-2 mRNA were demonstrated only in BALB/c mice (Figure 5.2). Similarly at day 2, levels of the chemokines KC, MIP-2 and MCP-1, M-CSF and GM-CSF had increased in spleen of BALB/c but not of C57BL/6 mice (Figure 5.2). At day 3, for spleen of

BALB/c mice, all chemokines and CSF investigated in the present study had increased markedly. In contrast, spleen of C57BL/6 mice demonstrated increased mRNA expression only for IP-10, Mig, RANTES, M-CSF, MIP-2 and KC (Figure 5.2).

Low baseline levels of mRNA for Mig IP-10, KC and RANTES were detected in the liver of both C57BL/6 and BALB/c mice (Figure 5.3). Increased levels of mRNA for G-CSF, M-CSF and GM-CSF were not detected in liver of C57BL/6 mice (data not shown). In contrast, in BALB/c mice slight increases in the level of mRNA for G-CSF, M-CSF and GM-CSF were observed at day 3 post-infection (data not shown). Increased levels of mRNA for IP-10, Mig, RANTES, MCP-1, KC and MIP-2 were detected in liver of both strains of mice following infection (Figure 5.3). However, as for spleen, the kinetics of induction differed between C57BL/6 and BALB/c mice. In liver of BALB/c mice, maximal expression of these chemokines occurred between days 2 and 3 of infection. In C57BL/6 mice, mRNA levels for the majority of chemokines and CSF were maximal between days 4 and 7 of infection, with the exception of Mig (Figure 5.3). A moderate increase in the level of mRNA for Mig persisted in liver of C57BL/6 mice for the duration of the assay period (14 days; Figure 5.3).

In liver of C57BL/6 mice, increased mRNA expression of the neutrophil chemoattractant KC was the earliest detected (day 1; Figure 5.3). Both KC and IP-10 mRNA levels were increased at day 1 in liver of BALB/c mice, while expression of IP-10 mRNA increased by day 2 in liver of C57BL/6. At 2 days post-infection, increased mRNA expression of KC, IP-10, Mig MIP-2 and MCP-1 was demonstrated in BALB/c liver. By day 3 in BALB/c liver, mRNA expression of all chemokines and CSF investigated had increased (Figure 5.3). In contrast, Mig was the only chemokine with increased levels of mRNA in livers of C57BL/6 mice at the same time point (day 3; Figure 5.3).

Patterns of CSF and chemokine expression in liver and spleen reflected bacterial loads (Figure 5.1). Levels of viable *B. pseudomallei* were greatest in the spleen, as were chemokine and CSF responses. In spleen of BALB/c mice, induction of all chemokines and CSF investigated were maximal at day 3 post-infection, correlating with the highest

bacterial numbers (Figure 5.1a & 5.2). In spleen of C57BL/6 mice, while levels of viable *B. pseudomallei* were low initially (days 1 and 3) a dramatic increase was observed by termination of the study at 14 days (Figure 5.1a). Peak bacterial numbers in spleen of C57BL/6 mice (14 days; Figure 5.1a) reflected increasing expression of the majority of chemokines and CSF investigated (7-14 days; Figure 5.2). The exception was with mRNA expression for Mig, which was greatest at day 3 post-infection (Figure 5.2). Similar observations were made for viable *B. pseudomallei* and chemokine/CSF induction in liver of C57BL/6 and BALB/c mice (Figure 5.1b & 5.3). In liver of BALB/c mice, the greatest bacterial load and expression of mRNA for all chemokines and CSF investigated was observed at day 3 post-infection. Increasing bacterial load in liver of C57BL/6 mice between days 3 and 14 (Figure 5.1b) correlated with the period of greatest mRNA expression of chemokines and CSF (Figure 5.3).

5.3.3 Histology

To examine the type of cellular response to *B. pseudomallei* infection, histological assessments of the spleen and liver of C57BL/6 and BALB/c mice euthanased at days 1, 3 and 14 were carried out (Figure 5.4 & 5.5, respectively). Since BALB/c mice succumbed to infection by day 4, tissue from these mice was examined only at days 1 and 3.

No lesions were detected in the spleen of C57BL/6 mice at day 1 (Figure 5.4a) but, by day 3, consolidated, well-defined inflammatory foci were present (Figure 5.4c). Lesions consisted of a central area of suppurative inflammation surrounded by a narrow zone of necrosis containing cellular debris (Figure 5.4e). A zone of macrophages and fibroblasts was adjacent to the necrotic area (Figure 5.4e). By day 14 multiple abscesses were evident in the spleen of C57BL/6 (Figure 5.4g). Compared to control mice, a mild neutrophilic influx was evident in spleen of BALB/c mice at day 1 post-infection (Figure 5.4b) and early inflammatory foci progressed to widespread necrotic lesions by day 3 (Figure 5.4d). Multiple inflammatory foci were dispersed throughout the red and white pulp of BALB/c spleen, with evidence of disseminated intravascular coagulation. Necrotic areas contained remnants of the early neutrophilic influx and were not well

defined (Figure 5.4f). The extent of tissue involvement correlated with the high bacterial loads seen in the liver and spleen of BALB/c mice at day 3 (Figure 5.1).

At day 1 post-infection, small numbers of inflammatory foci were observed in liver of C57BL/6 mice (Figure 5.5a). These lesions contained a mixture of macrophages and neutrophils, in which the number of macrophages present was slightly greater (Figure 5.5a). By day 3, macrophages were the predominant cell type in lesions of C57BL/6 liver (Figure 5.5c) and had surrounded central areas comprising neutrophils, necrotic cells and nuclear debris. Fewer lesions were seen in liver of C57BL/6 compared with BALB/c at day 3 following infection. By day 14 following *B. pseudomallei* infection, an increase in the number of lesions in C57BL/6 liver was observed. However, unlike the terminal stages of infection in BALB/c mice (Figure 5.5d), the inflammatory response in C57BL/6 liver resulted in densely packed accumulations of macrophages and lymphocytes (Figure 5.5e). A rapid inflammatory response consisting predominantly of neutrophils was observed as early as day 1 in liver of BALB/c mice (Figure 5.5B). Increases in mRNA for CXCL1 at day 1 in liver of BALB/c mice (Figure 5.3) correlated with an early neutrophil influx. Macrophages were detected at inflammatory sites by day 3 in BALB/c liver (Figure 5.5d). However, compared to C57BL/6 liver at day 3 (Figure 5.5c), lesions in BALB/c liver were predominantly composed of necrotic tissue with relatively few viable inflammatory cells (Figure 5.5D). Necrosis was extensive in livers of BALB/c mice at day 3 post-infection and numerous thrombi were also present in the blood vessels (Figure 5.5d).

5.4 Discussion

Recruitment, migration and activation of leukocyte subsets are crucial events during an immune response that are regulated by proinflammatory cytokines. Paradoxically, while these cytokines are indispensable for the effective clearance of a pathogen, an excessive inflammatory response is potentially destructive and may contribute to the pathogenesis of disease (Strieter *et al.* 1996).

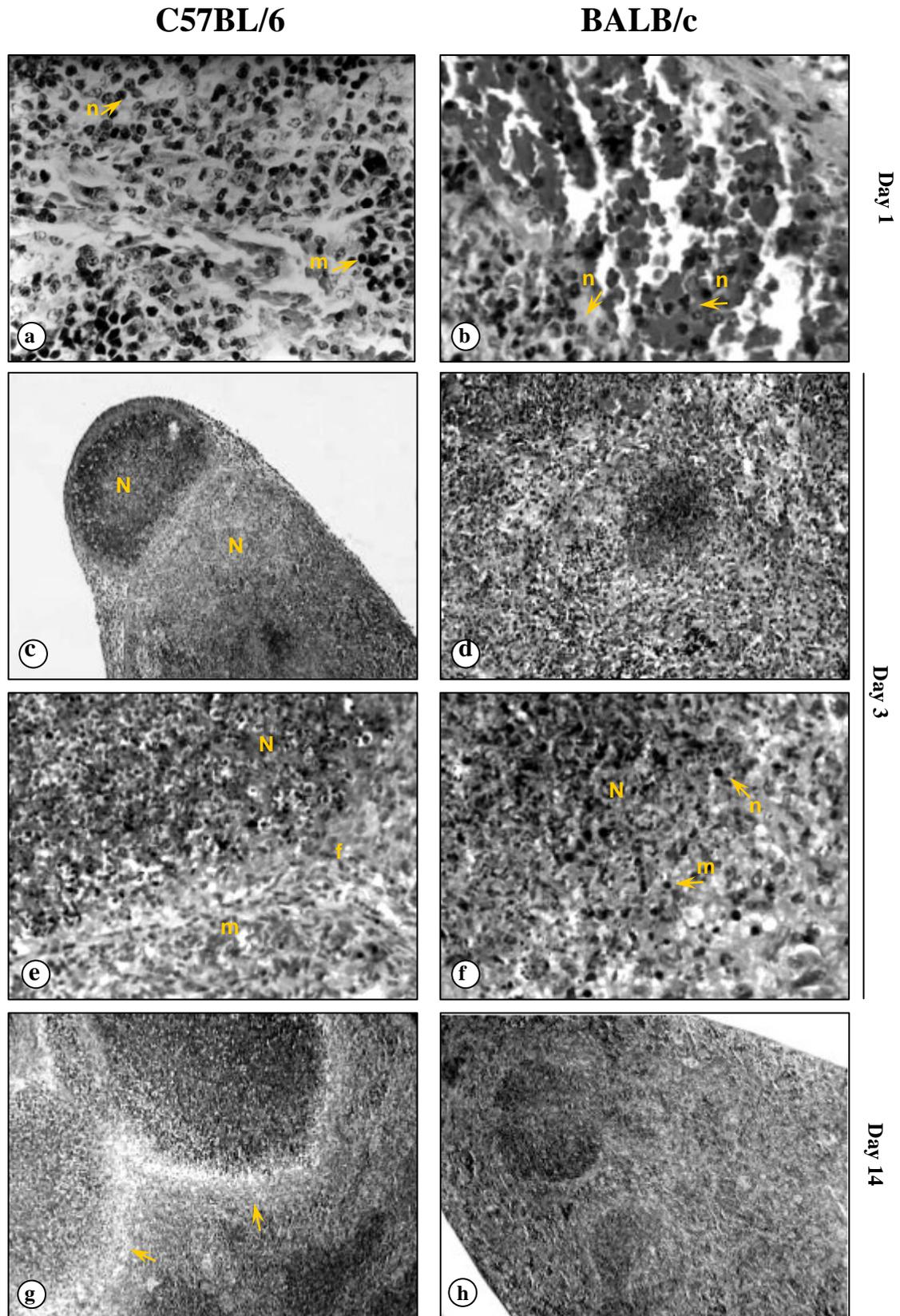


Figure 5.4 Histological examination of spleen of C57BL/6 and BALB/c mice infected *iv* with 25 cfu of virulent *B. pseudomallei*.

Histological examination of spleen of (a,c,e,g) C57BL/6 and (b,d,f) BALB/c mice following inoculation with 25 cfu *Burkholderia pseudomallei*. (h) Control mice received PBS only. Spleen were collected at (a,b) day 1, (c-f) day 3 and (g) day 14 post-infection for histology. (a,b) At day 1, early neutrophil (n) accumulation was evident in BALB/c mice only. (d) Multiple necrotic lesions (N) were observed throughout the spleen by day 3. (f) These lesions were diffuse and contained some macrophages (m) and neutrophils. (c) In C57BL/6 mice, lesions at day 3 consisted of well-defined areas of necrosis. (e) Necrotic tissue and cellular debris were surrounded by fibroblasts (f) and macrophages. (g) By day 14, lesions were larger and often coalesced (arrows).

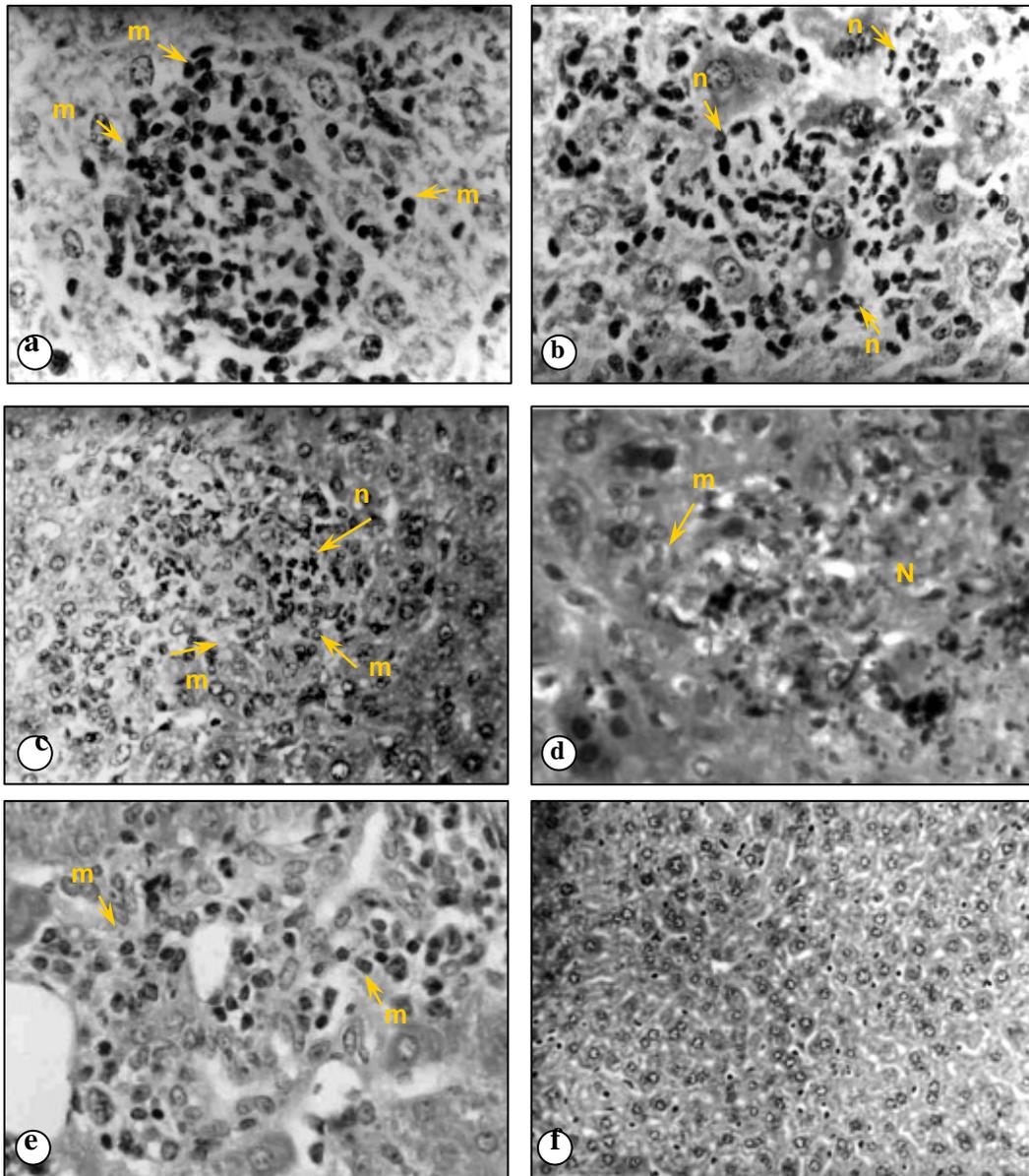
C57BL/6**BALB/c**

Figure 5.5 Histological examination of spleen of C57BL/6 and BALB/c mice infected *iv* with 25 cfu of virulent *B. pseudomallei*.

Histological examination of (a,c,e) C57BL/6 and (b,d) BALB/c liver following inoculation with 25 cfu *Burkholderia pseudomallei*. (f) Control mice received PBS only. Livers were collected at (a,b) day 1, (c,d) day 3 and (e) day 14 postinfection for histology. At day 1, early neutrophil (n) accumulation was evident in (b) BALB/c mice, whereas lesions in (a) C57BL/6 mice contained both neutrophils and macrophages (m). Macrophage accumulation was apparent at day 3 in (d) BALB/c mice, although this was more pronounced in (c) C57BL/6 mice. Extensive necrosis (N) was evident in BALB/c at day 3, preceding death. By day 14 (e), lesions in C57BL/6 mice were comprised predominantly of macrophages and involved a greater area of tissue.

Using animal models of infection with intracellular bacteria such as *M. tuberculosis* and *L. monocytogenes*, investigators have demonstrated the importance of particular types of chemokines and CSF in mediating a successful host immune response (Di Tirro *et al.* 1998; Mohammed *et al.* 1999). In pulmonary tuberculosis for example, macrophages and lymphocytes rather than neutrophils are important for host survival (Sowter *et al.* 1997; Mohammed *et al.* 1999). MCP-1 and MIP-1 α , which are chemotactic for mononuclear cells, are upregulated during infection with *M. tuberculosis* (Mohammed *et al.* 1999). Similarly, the induction of MCP-1 and CSF, which results in a predominantly monocytic infiltrate, is necessary for protection against infection with *L. monocytogenes* (Di Tirro *et al.* 1998; Saunders & Cooper 2000). Both the type and the duration of a chemokine response is critical during infection, as shown by Czuprynski *et al.* (1998) who demonstrated that CXC chemokines contribute to a more severe infection with *L. monocytogenes*. The potential detrimental effects of chemokines is also illustrated in a study by Kasama *et al.* (1994) who show that persistence of neutrophils due to the prolonged expression MIP-2 are responsible for increased tissue destruction.

Studies in patients with melioidosis have demonstrated a link between severity of disease and the levels of cytokines and chemokines in serum, including IFN- γ , TNF- α , IL-6 and IL-8 (Brown *et al.* 1991; Friedland *et al.* 1992; Lauw *et al.* 1999). Interferon- γ , TNF- α and IL-6 have also been implicated as important determinants of disease progression and outcome in murine melioidosis (Ulett *et al.* 2000a; Ulett *et al.* 2000b). Furthermore, induction of mRNA for LIX has been demonstrated in BALB/c mice infected with *B. pseudomallei* (Ulett *et al.* 2002). Lipopolysaccharide-induced CXC chemokine is considered to be a functional homologue of IL-8 in mice because of its potent chemotactic activities for neutrophils (Rovai *et al.* 1998). However, the role of chemokines and CSF during infection with *B. pseudomallei* has not been adequately investigated. Therefore, the aim of this study was to examine the level of expression of a broad range of chemokines and CSF during both the early and later stages of infection in an animal model of human melioidosis.

The present study demonstrated that C57BL/6 and BALB/c mice mount a substantial chemokine response following exposure to a virulent strain of *B. pseudomallei*. Induction of mRNA for neutrophil chemoattractants (KC, MIP-2, G-CSF and GM-CSF) and monocyte/lymphocyte chemoattractants (MCP-1, IP-10, Mig, M-CSF and GM-CSF) occurred in both C57BL/6 and BALB/c mice following infection with *B. pseudomallei*. However, the kinetics and magnitude of mRNA induction differed between the two mouse strains. These differences were associated with contrasting cellular responses to infection and reflected loads in liver and spleen.

While many of the chemokines and CSF responses detected in C57BL/6 mice were similar to those in BALB/c mice, the kinetics of mRNA expression and cellular inflammatory response differed considerably. In C57BL/6 mice, maximal levels of chemokine and CSF expression were observed between days 3 and 7 of the study, coinciding with increasing bacterial loads in the liver and spleen. Low-level increases in mRNA expression for the mononuclear cell chemoattractant Mig in spleen and the neutrophil chemoattractant KC in liver correlated with proportionately smaller inflammatory infiltrates in these organs, comprising predominantly macrophages. An increase in macrophage number and frequency of lesions in C57BL/6 liver at day 3 was associated with a large increase in Mig mRNA expression at the same time point. At day 3 in spleen, a mixture of mRNA for both ELR⁺ and ELR⁻ CXC chemokines was observed, together with M-CSF. Similarly, cellular infiltrates were also mixed and were observed as central regions of necrotic tissue and neutrophils surrounded by mononuclear leucocytes. As abscessation became more widespread in C57BL/6 spleen at day 14, the expression of mRNA for the neutrophil chemoattractants KC, MIP-2, GM-CSF and the mononuclear cell chemoattractant MCP-1 increased. In contrast to spleen, no GM-CSF or MCP-1 mRNA was detected in C57BL/6 liver at day 14. Large increases in mRNA expression of CSF in BALB/c mice at day 3 may reflect the failure of leucocytes to contain *B. pseudomallei* infection at sites of inflammation, and the subsequent high demands this may place on further inflammatory cell recruitment.

In the present study we observed a neutrophil-dominant infiltration in BALB/c compared to C57BL/6 mice during the early stages of *B. pseudomallei* infection. Gram

negative endotoxin is a potent stimulus for neutrophil recruitment in humans, with IL-8 contributing significantly to this cellular influx (Broaddus *et al.* 1994). An early neutrophilic influx and an increase in mRNA expression for KC, MIP-2, Mig and IP-10 in the present study, as well as increases in IFN- γ , TNF- α and IL-12 in previous studies (Ulett *et al.* 2000b), suggest that BALB/c mice are able to mount a strong innate immune response toward *B. pseudomallei* infection. However, the significantly greater bacterial loads in BALB/c mice at day 3, together with their failure to attract mononuclear cells to the same extent as C57BL/6 mice suggests BALB/c mice may not be able to mount an appropriate cellular response to *B. pseudomallei*. A study by Jones *et al.* (1996) demonstrated the ability of *B. pseudomallei* to resist killing by neutrophil defensins, thus facilitating the escape of bacteria from neutrophils. Our results, together with those of Jones *et al.* (1996), indicate that an early neutrophilic influx is not sufficient to eradicate *B. pseudomallei* from tissues of BALB/c mice. In C57BL/6 mice, the infiltration of macrophages within the first 3 days of infection may serve to adequately contain *B. pseudomallei* for a longer period than in BALB/c mice, allowing generation of an adaptive immune response. This is reflected in the significantly lower bacterial loads in the spleen and liver of C57BL/6 in the first 3 days post-infection. In the present study and others (Ulett *et al.* 2000a; Ulett *et al.* 2000b), the hyperproduction of proinflammatory cytokines such as IL-6 and TNF- α as well as CC and CXC chemokines and CSF in BALB/c tissues at day 3 may be an attempt to bring the *B. pseudomallei* infection under control. However, increased concentrations of proinflammatory cytokines and chemokines, combined with high levels of bacterial toxins are likely to contribute to tissue destruction, triggering multiple organ system failure. These results are similar to the course of infection seen in humans with acute melioidosis.

The comparatively low numbers of macrophages at sites of infection in spleen and liver of BALB/c mice by day 3 implies an inability of the chemokine response to attract mononuclear leucocytes in these tissues. Consequently, bacterial growth and replication proceeds, leading to bacteremia (Leakey *et al.* 1998). There are several possibilities that may explain the apparent inability of BALB/c mice to attract macrophages to sites of inflammation. While a broad range of chemoattractants were investigated in this study,

contributions from additional CXC and CC chemokines must be examined. Critical factors in determining accumulation of a particular leucocyte subset are considered to be both the ratio of local and systemic chemokine concentrations, and the interaction of particular chemotactic groups in a precise sequence (Gutierrez-Ramos *et al.* 2000; Call *et al.* 2001). Moreover, the role of chemokine receptors in *B. pseudomallei* infection has not yet been studied. Since chemokine action is dictated by a spectrum of chemokine receptor expression (Mantovani *et al.* 1998; Tsai *et al.* 2000; Tateda *et al.* 2001) it is possible that a decrease in CC or ELR⁺ CXC chemokine receptor expression contributes to the lack of macrophage recruitment in BALB/c mice. Finally, studies involving *L. pneumophila* and *M. tuberculosis* have indicated an immunomodulatory role for neutrophils through the production of cytokines such as IL-12 and TNF- α (Pedrosa *et al.* 2000; Tateda *et al.* 2001). Increased expression of both CXC and CC chemokines has been reported during infection with *M. tuberculosis*, despite observations that the predominant cell types observed at sites of infection were macrophages and lymphocytes (Rhoades *et al.* 1995). The authors suggested that despite the production of neutrophil chemoattractants such as MIP-2, the chemotactic properties of these CXC chemokines might be modulated or neutralised in the presence of other chemokines or cytokines (Rhoades *et al.* 1995). A cross-regulatory role in chemokine production has been demonstrated for a number of cytokines including IL-2, IL-4, IFN- γ and IL-10 (Kasama *et al.* 1994; Loetscher *et al.* 1996; Mohammed *et al.* 1999). Hence, it is likely that cytokines also play an important role in regulating chemokine and CSF production during infection with *B. pseudomallei*.

A recent study by Saukkonen *et al.* (2002) identified strain- and host-specific differences in the induction of CC chemokines during *M. tuberculosis* infection. Following infection of alveolar macrophages with virulent *M. tuberculosis* significantly less MIP-1 α was induced when compared to infection with an avirulent strain. Intracellular growth of *M. tuberculosis* within alveolar macrophages was inhibited up to 3-fold in the presence of the CC chemokines RANTES and MIP-1 β . In HIV-infected individuals, production of these chemokines is significantly reduced, as is TNF- α . The authors therefore postulate that deficiencies in the ability to produce RANTES and MIP-1 β may lead to

defects in the acquired immune response, as well as in the antimicrobial killing of intracellular *M. tuberculosis* (Saukkonen *et al.* 2002). Further investigations into the role of neutrophils, chemokines and their receptors in the early stages of *B. pseudomallei* infection are certainly warranted.

In summary, the present study demonstrated disparate expressions of mRNA for several chemokines and haematopoietic cytokines in a murine model of melioidosis. These discrepancies are associated with differences in bacterial growth and the composition of cellular infiltrate and may underlie the highly susceptible phenotype of BALB/c mice to *B. pseudomallei* infection. Since RT-PCR provides only semi-quantitative analysis of gene expression, future studies that incorporate newer quantitative techniques such as real-time PCR and microarray analysis, may be more suitable.

CHAPTER 6

DEMONSTRATION OF CELL-MEDIATED IMMUNE RESPONSES IN A MURINE MODEL OF MELIOIDOSIS

6.1 Introduction

Protection from infections with facultative intracellular bacteria such as *M. tuberculosis*, *L. monocytogenes* and *L. pneumophila*, have been shown to be mediated largely by CMI responses of the host (Schiabbe *et al.* 1999). The successful elimination of the pathogen depends mainly on efficient interactions between infected macrophages and antigen-specific T cells (Raupach & Kaufmann 2001). Macrophages play a central role in the innate immune response to bacterial infection. These mononuclear phagocytes are normally present in tissues, as resident macrophages that are susceptible to infection. In the presence of an inflammatory stimulus, macrophages develop into activated cells and increase their secretory and phagocytic activities. IFN- γ is the principal cytokine for activation of macrophages, thereby increasing their capacity to kill intracellular pathogens. One of the mechanisms utilised by activated macrophages in the elimination of intracellular bacteria is the production of toxic, free radicals. Reactive nitrogen intermediates (RNI) released from stimulated macrophages play a pivotal role in the killing of intracellular *B. pseudomallei* (Miyagi, Kawakami & Saito 1997). Nitric oxide (NO) is produced from the enzymatic action of inducible nitric oxide synthases (iNOS) and, in the presence of oxygen, is rapidly converted to NO₂⁻. The colorimetric Griess assay is one of the commonly used assays to estimate the NO secretory activity of macrophages in culture by determining NO₂⁻ concentrations in supernatants.

The adaptive immune response is detected several days after the innate immune response and is involved in bacterial clearance and the development of long-term immunity. Clearance of intracellular bacteria such as *L. monocytogenes* and *L. pneumophila*, is predominantly mediated by CD4⁺ T lymphocytes (Andersen &

Smedegaard 2000; Mittrucker, Kohler & Kaufmann 2000). These lymphocytes recognise antigens presented by APC and can be further classified as T_H1-type or T_H2-type subpopulations according to the cytokines they secrete. T_H1-type cells produce IL-2 and IFN- γ , which are important for CMI responses to intracellular pathogens. T_H2-type cells are mostly involved in stimulating B lymphocytes and produce anti-inflammatory cytokines such as IL-4 and IL-10, which exacerbate infections with intracellular pathogens. For clearance of intracellular infections, it is important that T_H1-type responses predominate. Cytokine depletion studies carried out by Santanirand *et al.* (1999) demonstrated the absolute dependence on IFN- γ of resistance to acute infection with *B. pseudomallei*. The same group demonstrated that both NK cells and CD8⁺ T cells are the dominant source of IFN- γ (Lertmemongkolchai *et al.* 2001). However, recent studies suggest that a mixed population of T_H cells is involved in host protection against *B. pseudomallei* infection (Ulett *et al.* 2000a; Liu *et al.* 2002). Currently there is very little data on the development of adaptive CMI responses in melioidosis. The purpose of this component of the project was to provide basic data regarding the generation of CMI responses during *B. pseudomallei* infection using the C57BL/6-BALB/c murine model.

The generation of an adaptive CMI response following infection with *B. pseudomallei* was investigated using delayed-type hypersensitivity (DTH) and lymphocyte proliferation assays. DTH is a CMI response observed *in vivo* that is mediated by antigen-specific T cells. Upon first exposure, antigen is phagocytosed and processed by APC then presented to antigen-specific CD4⁺ T cells. An adaptive immune response is generated and includes the development of a population of antigen-specific memory T cells that persist in the host. These memory T cells are able to respond rapidly if the host is subsequently exposed to the same antigen (Abbas, Lichtman & Pober 2000). To invoke a DTH response, the antigen used for initial “priming” is injected subcutaneously, whereby it is phagocytosed and processed by APC. Memory T cells that specifically recognise this antigen become activated and secrete cytokines and chemokines that act on the vascular endothelium and initiate the recruitment of phagocytes and plasma to the site of injection. This response is observed as visible

swelling at the site of injection, peaking at approximately 48 hrs following antigen challenge. In contrast, the inflammatory reaction referred to as immediate hypersensitivity can be measured as early as 3 hrs post-challenge and is mediated by IgE antibody and mast cells, rather than T cells (Abbas, Lichtman & Pober 2000). T cell proliferation in response to a specific antigen can be measured by stimulating cultures of purified PBML or splenic MNL with test antigen for 4 to 7 days (Abbas, Lichtman & Pober 2000). Four hours before harvesting, thymidine labeled with the radioactive isotope tritium ($[^3\text{H}]$ -thymidine) is added to the cultures. While normal, non-dividing lymphocytes do not take up thymidine, proliferating cells do since they are actively synthesizing DNA. Therefore, the radioactivity incorporated into DNA of cells provides a measure of the degree of T cell proliferation. The ratio of the radioactivity in stimulated cultures to the radioactivity in unstimulated controls (stimulation index, SI) gives an indication of the response to a particular antigen.

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

- 4 To compare NO production in response to *B. pseudomallei* in peritoneal macrophages from C57BL/6 versus BALB/c mice
- 5 To demonstrate the generation of *B. pseudomallei*-specific T cells in C57BL/6 and BALB/c mice by measuring (i) DTH response, (ii) lymphocyte proliferation and (iii) IFN- γ and IL-4 production

6.2 Materials and Methods

6.2.1 Nitrite production by peritoneal macrophages

To elicit macrophages from C57BL/6 mice (n=5) and BALB/c (n=5), 2.5 ml of Brewer's thioglycollate medium (BD, NSW, Australia) was injected *ip*. After 3 days, peritoneal cells (PEC) were obtained from the abdominal cavity by repeated lavage with 10 ml of ice-cold lavage medium (10 U/ml heparin in PBS). Plasticware, media and cells were kept on ice at all times. PEC from each mouse strain were pooled and a total volume of

50 ml of cell-suspension was collected for both C57BL/6 and BALB/c mice. To each 50 ml suspension, 2.5 ml of heat-inactivated foetal calf serum (HIFCS) was added and the contents of the tubes were mixed. PEC were then centrifuged (500 *g*, 10 min, 4°C) and resuspended in 5.2 ml of erythrocyte-lysing buffer (Qiagen, VIC, Australia) and left at RT for 3 min. The suspension was then reconstituted to the original volume (52.5 ml) with 5% HIFCS in ice-cold PBS and centrifuged (500 *g*, 10 min, 4°C). The supernatant was discarded and this wash step was repeated. Cell counts were performed and cells were resuspended to a concentration of 2×10^6 cells/ml in culture media (Appendix 1). PEC were dispensed into 24 well plates in 1 ml aliquots and incubated at 37°C in 5% CO₂ overnight. Monolayers were washed twice with PBS at RT to remove nonadherent cells then replenished with 0.5 ml of culture media. The adherent monolayers contained approximately 3×10^5 macrophages per well with >95% purity. Macrophage purity was assessed using a non-specific esterase staining kit (Sigma, NSW, Australia).

Priming with recombinant murine IFN- γ (rIFN- γ ; 100 U/ml; BD, NSW, Australia) was carried out first by adding the cytokine (50 μ l) to appropriate wells then incubating plates for 30 min at 37°C in 5% CO₂. Additional stimulants were added (50 μ l) to wells where appropriate. Wells containing peritoneal macrophages were either (i) left unstimulated, or stimulated with (ii) BpLy1 alone (1 μ g/ml), (iii) BpLy1 + rIFN- γ , (iv) *Pseudomonas aeruginosa*-LPS (100 ng/ml) alone, (v) *P.aeruginosa*-LPS + rIFN- γ or (vi) rIFN- γ alone. Nitrite (NO₂⁻) accumulation in the cell culture supernatants was measured by the Griess assay at 4, 24, 48, 72 and 96 hrs following macrophage stimulation. A standard curve was generated using five-fold dilutions of sodium nitrite (NaNO₂; 10 mM). Briefly, 50 μ l of sample or standard was added to individual wells of a 96-well plate, followed by 50 μ l of Griess reagent (Appendix 1; 1% sulphanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H₃PO₄). After incubation for 10 min at RT, the absorbance was read at 540 nm on a microplate reader (Multiskan EX; Labsystems).

6.2.2 Delayed-type hypersensitivity

6.2.2.1 Effect of dose and time

The effect of immunising dose and the length of time between immunisation and assessment of DTH responses were investigated using BALB/c and C57BL/6 mice. Mice (n=30) were divided into three groups. Immunisations were performed in 200 μ l volumes, delivered *iv*. One group was immunised with a low-dose (70 cfu; equivalent to $\sim 0.008 \times LD_{50}$ in BALB/c mice, $1 \times 10^{-5} \times LD_{50}$ in C57BL/6 mice) of *B. pseudomallei* NCTC 13179 and another with a high-dose (7×10^3 cfu; equivalent to $\sim 0.8 \times LD_{50}$ in BALB/c mice, $1 \times 10^{-3} \times LD_{50}$ in C57BL/6 mice) of NCTC 13179. The third group were control mice that received PBS only. DTH responses were assessed in 5 mice per group at day 14 and day 28 following immunisation. To assess DTH, 5 μ g/40 μ l of BpLy1 was injected *sc* into the left hind footpad of each mouse. An equal volume of PBS was injected *sc* into the right hind footpad. Thicknesses of left and right footpads were measured with digital calipers at 0 and 48 hrs post-footpad challenge. Change in footpad thickness was calculated by subtracting mean measurements (n=5 per foot) at 0 hrs from footpad measurements at 48 hrs after challenge. Change in footpad thickness was expressed in mm \pm SEM. Univariate ANOVA was used to assess differences in footpad thickness of low- and high-dose immunised mice challenged with BpLy1 compared to controls. Responses obtained 14 days after *B. pseudomallei* exposure were also compared to responses at 28 days.

6.2.2.2 Specificity of DTH response

The specificity of the DTH response in C57BL/6 and BALB/c mice was assessed using three different immunisation groups. One group of mice (n=45) were inoculated *iv* with $0.1 \times LD_{50}$ of NCTC 13179 (C57BL/6= 6×10^5 cfu; BALB/c=900 cfu). A second group of mice (n=45) were immunised *iv* with $0.1 \times LD_{50}$ of *B. cepacia* (C57BL/6= 2.15×10^7 cfu; BALB/c= 4.3×10^6 cfu). The third group were control mice (n=45) that received PBS only. At day 7 post-immunisation, 5 mice per group were euthanased and bacterial loads were assessed in liver and spleen to ensure sterility. At day 14 post-immunisation,

40 μ l of PBS was injected *sc* into the right hind footpad of all mice. In each of the three immunisation groups, half the mice (n=20) had BpLy1 (5 μ g/40 μ l) injected into the left hind footpad. The remaining half (n=20) were challenged with CepLy (5 μ g/40 μ l; Chapter 3) in the left hind footpad. Thickness of right and left footpads was measured with a digital gauge at 0 and 48 hrs post-footpad challenge. Change in footpad thickness was expressed in mm \pm SEM. Univariate ANOVA was used to assess differences in footpad thickness of immunised mice challenged with BpLy1 compared to controls.

6.2.3 Lymphocyte proliferation assays

6.2.3.1 Effect of dose and time

The effect of immunising dose and the length of time between immunisation, and assessment of lymphocyte proliferation was investigated using BALB/c and C57BL/6 mice. Mice (n=90) were divided into three groups. Immunisations were performed in 200 μ l volumes, delivered *iv*. One group was immunised with a 0.01 x LD₅₀ (C57BL/6=6 x 10⁴ cfu; BALB/c=90 cfu) of *B. pseudomallei* NCTC 13179 and another with 0.1 x LD₅₀ (C57BL/6=6 x 10⁵ cfu; BALB/c=900 cfu) of NCTC 13179. The third group were control mice that received PBS only. At day 6 post-immunisation, 5 mice per group were euthanased and bacterial loads were assessed in liver and spleen to ensure sterility. At day 7, 14 and 28 post-immunisation, mice (n=10) were euthanased and spleens removed aseptically. Splenic MNL were isolated using protocols outlined previously (Chapter 3). MNL were resuspended in culture medium (Appendix 1) and dispensed into 96-well round-bottom plates at 1 x 10⁵ cells/well. Cell cultures were stimulated with BpLy1 (1 μ g/ml), concanavalin A (ConA; 2 μ g/ml; Sigma) or left unstimulated as negative controls. All tests were performed in triplicate. [³H]-Thymidine incorporation into proliferating lymphocytes was measured at 24 hr intervals between 96 to 168 hrs (Amersham-Pharmacia Biotech; 1.25 μ Ci/mL for 4 hrs). Results were expressed as mean maximum SI \pm SEM and the response of *B. pseudomallei*-specific lymphocytes were compared with controls by one-way ANOVA.

6.2.3.2 Specificity of proliferative response

Mice (n=10) were inoculated *iv* with 0.01 x LD₅₀ of *B. pseudomallei* NCTC 13179 and splenic MNL were isolated 28 days post-immunisation using protocols outlined previously (Chapter 3). MNL were resuspended in culture medium (Appendix 1) and dispensed into 96-well round-bottom plates at 1 x 10⁵ cells/well. Cell cultures were stimulated with BpLy1 (1 µg/ml), CepLy (1 µg/ml), *P. aeruginosa*-LPS (100 ng/ml), ConA (2 µg/ml; Sigma) or left unstimulated as negative controls. In parallel wells, antigens were treated with polymyxin B (10 µg/ml) to neutralise LPS, prior to addition to cultures. Equal volumes of antigens and polymyxin B were mixed and incubated on ice for 30 min. Aliquots (10 µl/well) of this mixture were then dispensed into the appropriate wells. All tests were performed in triplicate. [³H]-Thymidine incorporation into proliferating lymphocytes was measured at 24 hr intervals between 96 to 168 hrs (Amersham-Pharmacia Biotech; 1.25 µCi/ml for 4 hrs). Results were expressed as mean maximum SI ± SEM and analysed by one-way ANOVA.

6.2.4 Assessment of IFN-γ and IL-4 production

A preliminary study was performed to assess the use of ELISPOT to measure IFN-γ and IL-4 production in *B. pseudomallei*-exposed mice. C57BL/6 (n=20) and BALB/c (n=20) mice were divided into four equal groups. Group 1 were naïve mice that were not injected, while mice in the second group were injected *iv* with 200 µl of PBS. In group three, mice were injected *iv* with 0.01 x LD₅₀ of *B. pseudomallei* NCTC 13179. The fourth group of mice were injected *sc* with BpLy1 (5 µg/40 µl). Fourteen days following injection, mice were euthanased, spleens removed and splenic MNL recovered using protocols described previously (Chapter 3). To quantitate antigen-stimulated MNL producing IFN-γ and IL-4, ELISPOT assay kits (551083 & 551017; Pharmingen-BD) were used according to the manufacturers' instructions. MNL were suspended in culture medium (Appendix 1) and added in 100 µl volumes at 2.5 x 10⁴ cells/well for IFN-γ assays or 1 x 10⁵ cells/well for IL-4 assays. Wells were either left unstimulated or were stimulated (10 µl/well) with BpLy1 (1 µg/ml), BpLy1 + polymyxin B (10 µg/ml), ConA (2 µg/ml) and plates were incubated for 24 hrs at 37°C in 5%CO₂. Spots were enumerated using an Immunospot® Series I Analyzer with Immunospot® Software

(CTL Analyzers LLC, Cleveland, OH). Data collection was kindly performed by Barbara Holt, Telethon Institute of Child Health and Research, Perth, Western Australia. Results were analysed using one-way ANOVA.

6.3 Results

6.3.1 Comparison of nitrite production by peritoneal macrophages

NO₂⁻ production was greatest in peritoneal macrophages primed with rIFN- γ prior to stimulation with BpLy1 or *P.aeruginosa*-LPS (Figure 6.1). In contrast, NO₂⁻ levels were low in macrophage cultures stimulated with BpLy1, *P.aeruginosa*-LPS or rIFN- γ alone. No differences in NO₂⁻ levels were observed between peritoneal macrophages obtained from C57BL/6 and BALB/c mice. Similarly, NO₂⁻ production for both mouse strains was comparable in IFN- γ -primed peritoneal macrophage cultures stimulated with BpLy1 or *P.aeruginosa*-LPS (Figure 6.1).

6.3.2 DTH response to *B. pseudomallei* antigens

6.3.2.1 Effect of immunising dose and time

As illustrated in Figure 6.2, footpad swelling in response to BpLy1 was significantly greater in *B. pseudomallei*-immunised mice compared to control mice (P<0.05). The DTH response to BpLy1 was greatest when a high-dose of *B. pseudomallei* was used to immunise C57BL/6 (Fig 6.2a; P<0.05) and BALB/c (Figure 6.2b; P<0.01) mice. For C57BL/6 mice, no difference was observed for footpad swelling in mice immunised with a low-dose of *B. pseudomallei* compared to control mice. For C57BL/6 and BALB/c mice, DTH responses were typically greater when assessed 28 days after immunisation, compared to 14 days. However, these differences were not statistically significant.

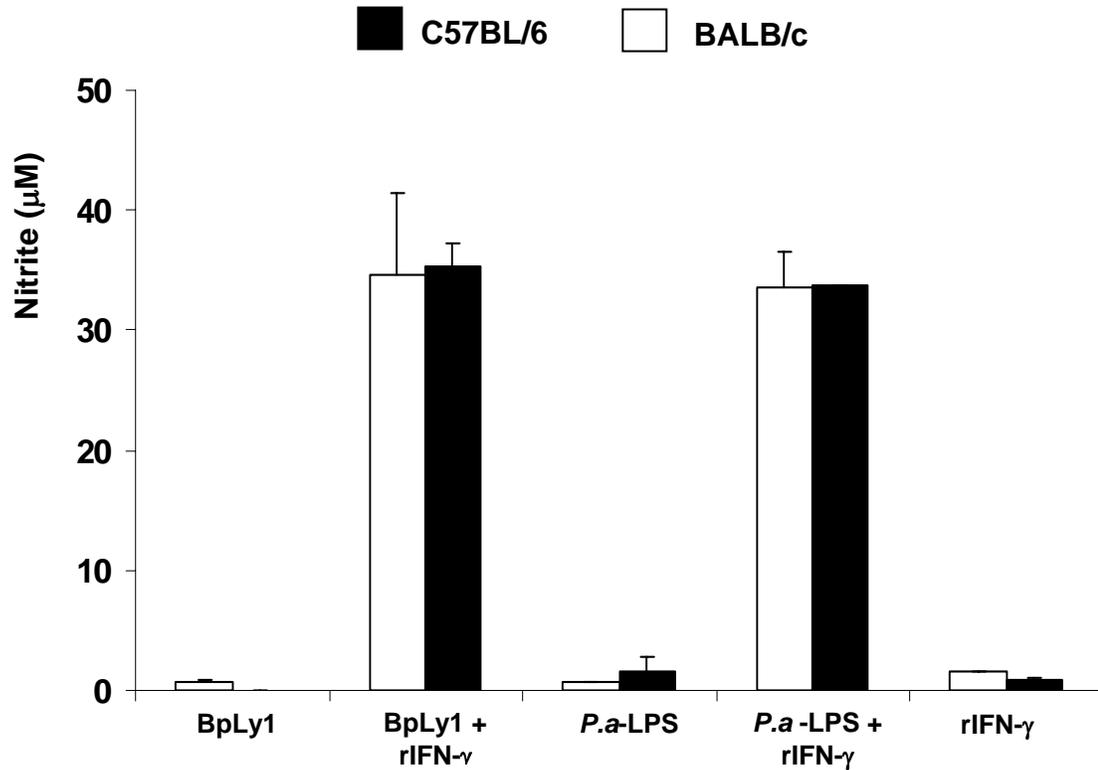


Figure 6.1 Nitrite production in C57BL/6 and BALB/c peritoneal macrophage cultures.

Peritoneal macrophages from C57BL/6 and BALB/c mice were stimulated with BpLy1, BpLy1 + rIFN- γ , *P.aeruginosa*-LPS (*P.a.*-LPS) alone, *P.a.*-LPS + rIFN- γ or rIFN- γ alone. Nitrite levels in culture supernatants were determined using the Griess assay and results obtained at 72 hrs of culture are shown. High levels of NO₂⁻ were observed in IFN- γ -primed peritoneal macrophages of C57BL/6 and BALB/c mice stimulated with BpLy1 ($P < 0.01$) or *P.aeruginosa*-LPS ($P < 0.01$).

6.3.2.2 Specificity of DTH response

The DTH response to *B. pseudomallei* antigens was not shown to be specific (Figure 6.3). In C57BL/6 mice, significant increases in footpad swelling were observed in *B. cepacia*- ($P < 0.01$) and *B. pseudomallei*-immunised ($P < 0.01$) mice compared to controls. In *B. cepacia*-immunised C57BL/6 mice, footpad swelling was significantly greater in response to CepLy than BpLy1 ($P < 0.05$). Footpad swelling was significantly greater in *B. pseudomallei*-immunised BALB/c mice compared to controls ($P < 0.05$).

However, no significant differences were observed in the DTH response to BpLy1 and CepLy in BALB/c mice.

6.3.3 Lymphocyte proliferation

6.3.3.1 Effect of immunising dose and time

Compared to controls, proliferative responses to BpLy1 were greater in C57BL/6 and BALB/c mice immunised with *B. pseudomallei* (Figure 6.4). For C57BL/6 mice, proliferation was greatest in 0.1 x LD₅₀-immunised mice whose lymphocytes were harvested 14 days after exposure to *B. pseudomallei* (Figure 6.4a). The greatest lymphocyte proliferation in BALB/c mice was observed at 28 days, where responses in 0.01x LD₅₀- and 0.1 x LD₅₀-immunised mice were comparable (Figure 6.4b). Compared to C57BL/6 mice, lymphocyte proliferation at day 28 was significantly greater in *B. pseudomallei*-immunised BALB/c mice (P<0.01). Immunisation doses greater than 0.1 x LD₅₀ were not cleared by mice and resulted in abscesses in the spleen. Lymphocyte proliferation assays were not performed on cells from infected spleen.

6.3.3.2 Specificity

The specificity of lymphocyte proliferation to *B. pseudomallei* antigens was assessed by comparing responses to BpLy1 and CepLy. *B. pseudomallei*-immunised C57BL/6 (P<0.05; Figure 6.5a) and BALB/c (P<0.05; Figure 6.5b) mice demonstrated significantly greater proliferation in response to BpLy1 compared to controls. Lymphocytes from immunised C57BL/6 mice demonstrated significantly higher proliferation in response to stimulation with BpLy1 compared to CepLy (P<0.05; Figure 6.5a). No differences in proliferation were observed in BALB/c lymphocyte cultures stimulated with BpLy1 or CepLy (P>0.05; Figure 6.5b). Proliferative responses of C57BL/6 mice were comparable to BALB/c mice. To determine the influence of LPS on lymphocyte proliferative responses, antigens were pretreated with polymyxin B prior to addition to cultures. Figure 6.6 illustrates the proliferative responses of MNL stimulated with BpLy1, CepLy or *P.aeruginosa*-LPS following neutralisation of LPS with polymyxin B. Low proliferative responses in cultures to which *P.aeruginosa*-LPS was

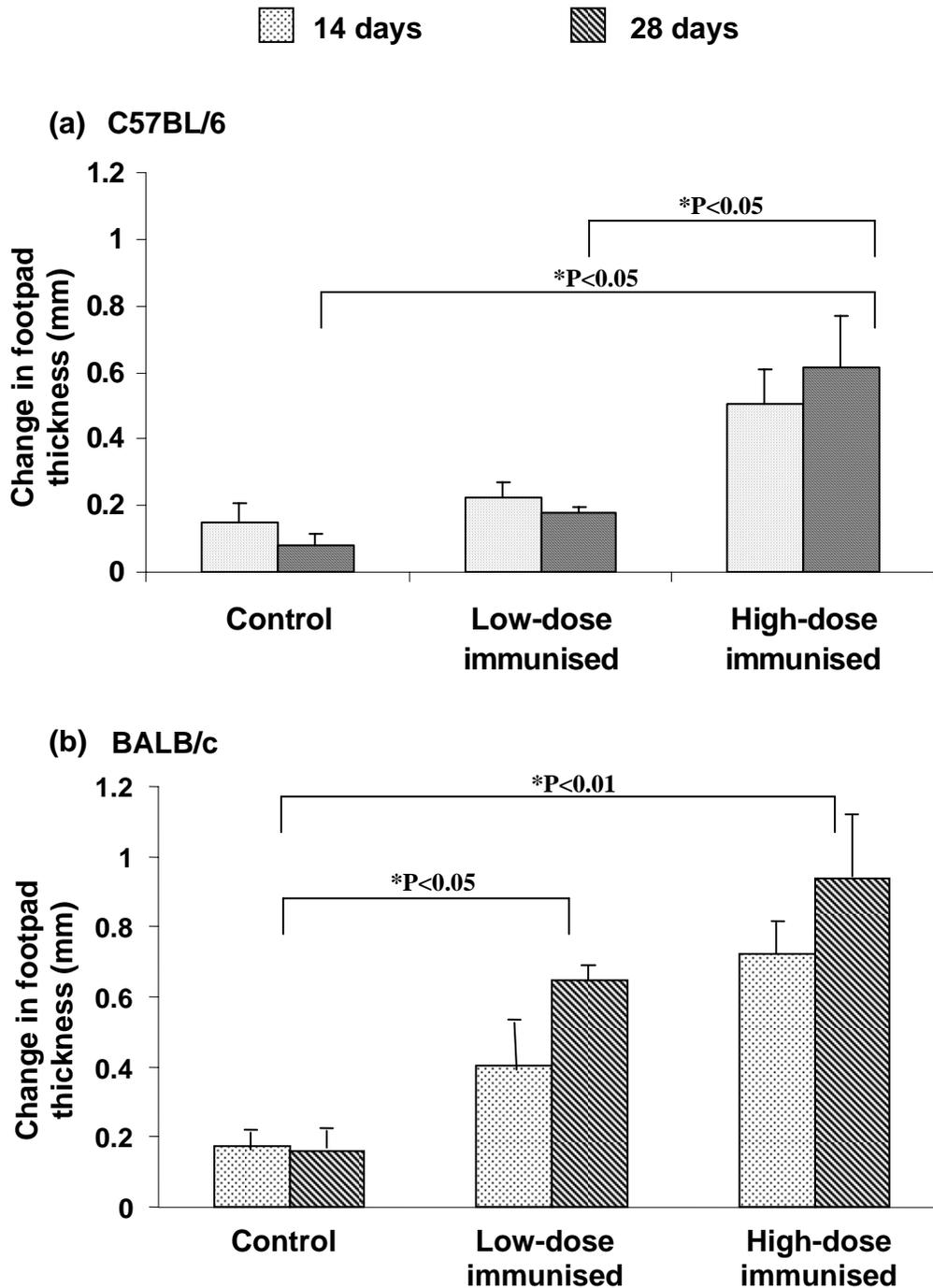


Figure 6.2 DTH response to BpLy1 in (a) C57BL/6 and (b) BALB/c mice.

Mice were immunised *iv* with a low-dose (70 cfu) or high-dose (7×10^3 cfu) of *B. pseudomallei* NCTC 13179. Control mice received PBS only. DTH response to BpLy1 was assessed in mice (n=5) at 14 days and 28 days post-infection by *sc* injecting 40 μ l of PBS into the right hind footpad and 5 μ g/40 μ l of BpLy1 into the left hind footpad. Footpad thicknesses were measured at 0 and 48 hrs and the change in thickness in response to BpLy1 was calculated by subtracting non-specific swelling to PBS, in the right footpad. DTH responses were significantly greater in C57BL/6 ($P<0.05$) and BALB/c ($P<0.01$) mice immunised with a high-dose of *B. pseudomallei* compared to control mice. Footpad swelling in mice challenged 28 days after immunisation was greater than mice challenged after 14 days. However, this difference was not significant ($P>0.05$). Results are expressed as change in footpad thickness \pm SEM.

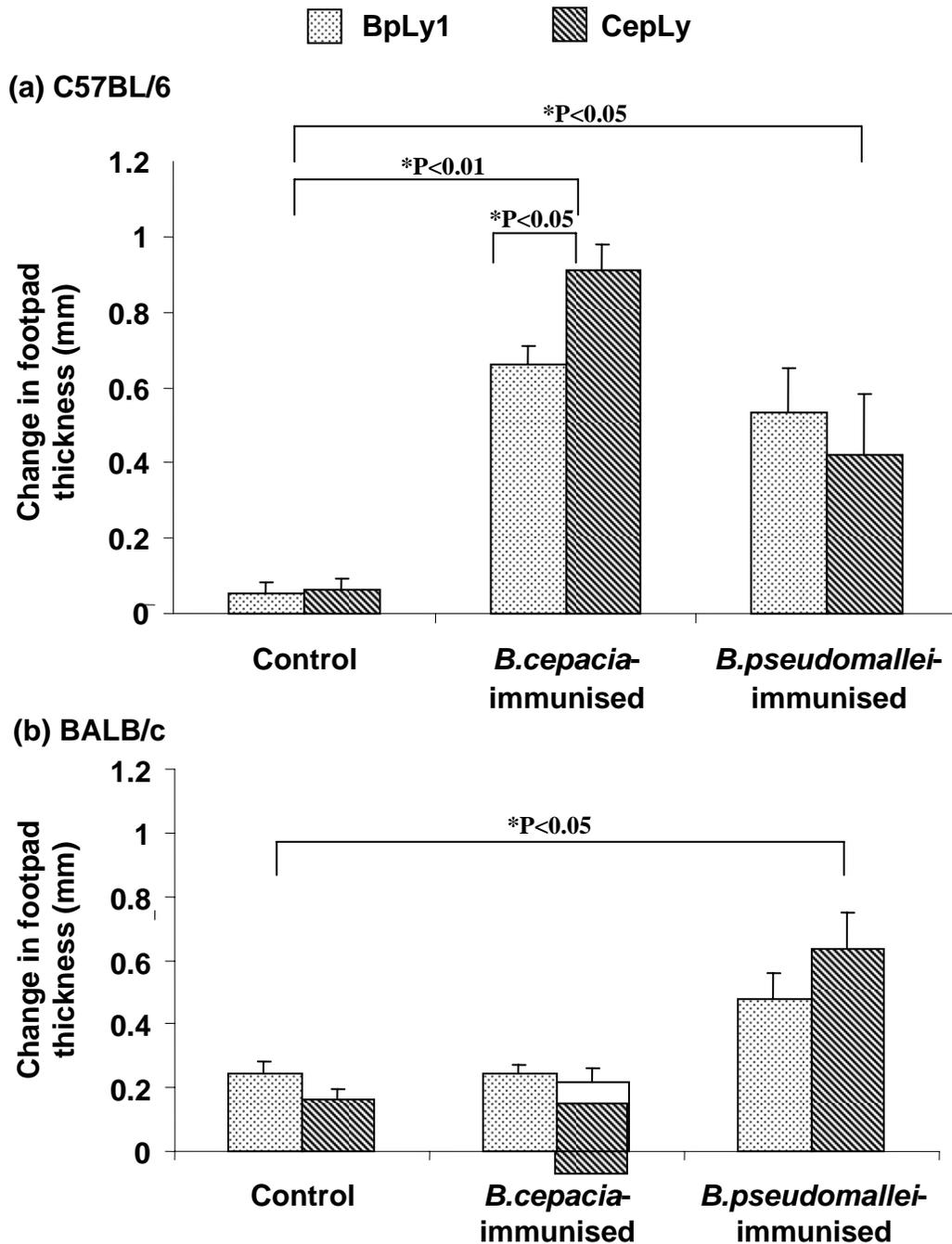


Figure 6.3 DTH response to BpLy1 and CepLy in (a) C57BL/6 and (b) BALB/c mice. Mice were immunised *iv* with 0.1 x LD₅₀ of *B. cepacia* or 0.1 x LD₅₀ of *B. pseudomallei* NCTC 13179. Control mice received PBS only. DTH response to BpLy1 or CepLy was assessed in mice (n=5) at day 28 post-infection by *sc* injecting 40 µl of PBS into the right hind footpad and 5 µg/40 µl of BpLy1 or CepLy into the left hind footpad. Footpad thicknesses were measured at 0 and 48 hrs. Results are expressed as change in footpad thickness (mm) ± SEM. In C57BL/6 mice, significant increases in footpad swelling were observed in *B. cepacia*- (P<0.01) and *B. pseudomallei*-immunised (P<0.05) mice compared to controls. For C57BL/6 mice immunised with *B. cepacia*, a stronger DTH response to CepLy was observed when compared to BpLy1 (P<0.05). Footpad swelling was significantly greater in *B. pseudomallei*-immunised BALB/c mice compared to controls (P<0.05). However, no significant differences were observed in the DTH responses to BpLy1 and CepLy in BALB/c mice.

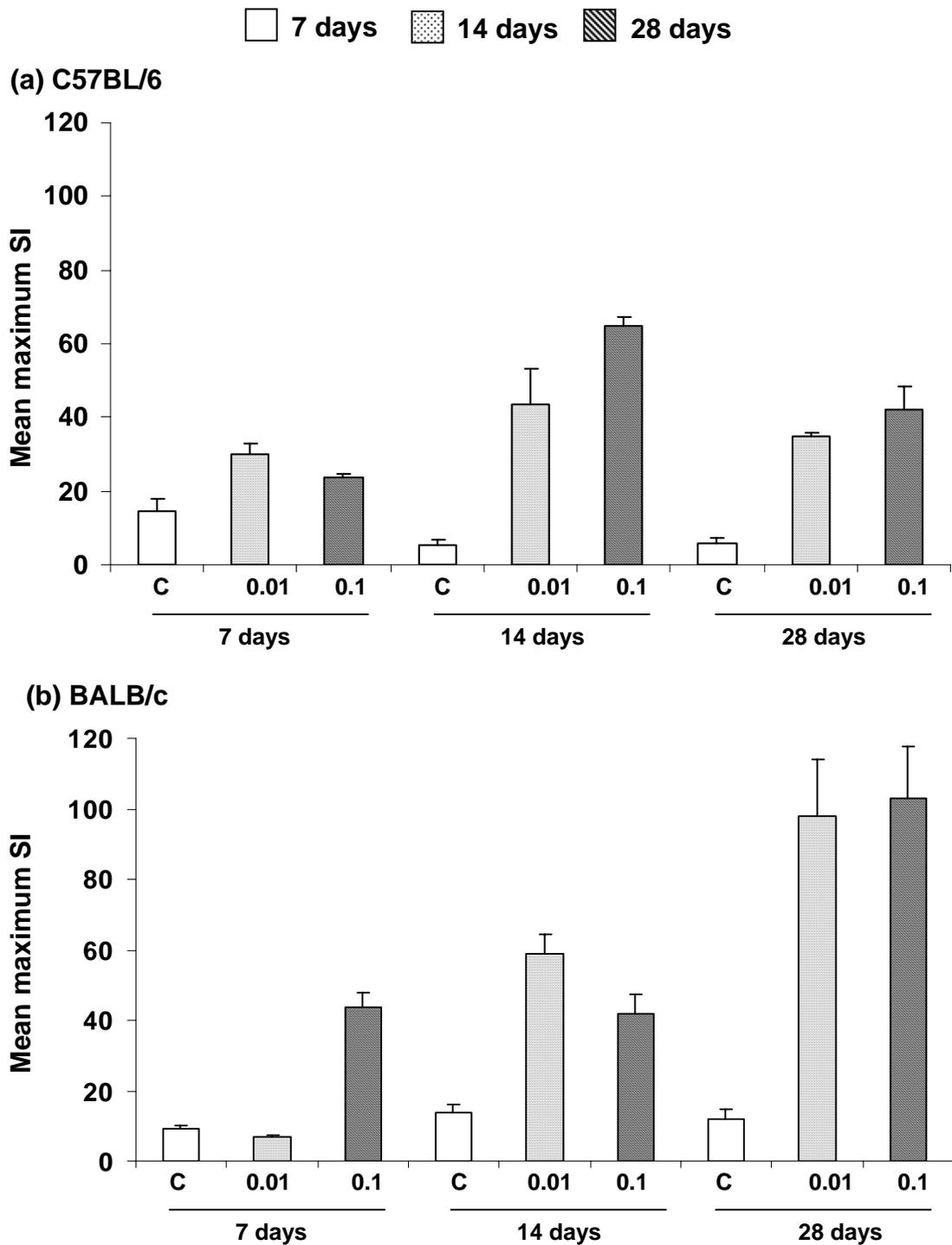


Figure 6.4 Effect of immunising dose and time on lymphocyte proliferation in (a) C57BL/6 and (b) BALB/c mice.

Mice were immunised *iv* with 0.01 x LD₅₀ or 0.1 x LD₅₀ of *B. pseudomallei* NCTC 13179. Control mice that received PBS only. At 7, 14 and 21 days post-immunisation, mice (n=5) were euthanased and MNL from spleen were isolated and cultured in the presence or absence of BpLy1. Proliferation was measured from 96 to 168 hrs of culture using [³H]-thymidine incorporation. Results are expressed as the mean maximum SI ± SEM. For C57BL/6 mice, proliferation was greatest in 0.1 x LD₅₀-immunised mice whose MNL were harvested 14 days after exposure to *B. pseudomallei*. The greatest lymphocyte proliferation in BALB/c mice was observed at 28 days, where responses in 0.01x LD₅₀- and 0.1 x LD₅₀-immunised mice were comparable.

added indicate that neutralisation occurred (Figure 6.6). Compared to control mice, significant proliferation was observed in lymphocytes from C57BL/6 ($P < 0.01$) and BALB/c ($P < 0.05$) mice that were immunised with *B. pseudomallei*. However, for immunised mice differences in lymphocyte proliferation were not significantly different between cultures stimulated with LPS-neutralised BpLy1 or with LPS-neutralised CepLy (Figure 6.6). No differences were observed in the proliferative response of C57BL/6 versus BALB/c mice.

6.3.4 IFN- γ and IL-4 production

Increased numbers of IFN- γ -producing lymphocytes were detected in all wells stimulated with ConA (Figure 6.7). However, low numbers of IFN- γ -producing lymphocytes were detected in cultures that were stimulated with BpLy1, or with LPS-neutralised BpLy1. No differences were observed between IFN- γ production in cultures from control mice compared to *B. pseudomallei*- or BpLy1-immunised C57BL/6 (Figure 6.7a) or BALB/c (Figure 6.7b) mice. Similarly, there were no significant differences between the two mouse strains in the numbers of IFN- γ -producing cells ($P > 0.05$).

Increased numbers of IL-4-producing lymphocytes were detected in all wells stimulated with ConA (Figure 6.8). For C57BL/6 mice, IL-4-producing cells were more numerous in BpLy and LPS-neutralised BpLy1 stimulated cultures derived from *B. pseudomallei*-immunised mice compared to naïve ($P < 0.05$), PBS controls ($P < 0.05$) or BpLy1-immunised mice ($P < 0.05$; Figure 6.8a). In contrast, no differences were observed in IL-4 production in MNL cultures from control or immunised BALB/c mice (Figure 6.8b). *B. pseudomallei*-immunised C57BL/6 mice had significantly higher numbers of IL-4-producing lymphocytes compared to *B. pseudomallei*-immunised BALB/c mice ($P < 0.05$). Although not significant, IL-4 production appeared to be higher in MNL cultures stimulated with LPS-neutralised BpLy1 versus BpLy1 alone.

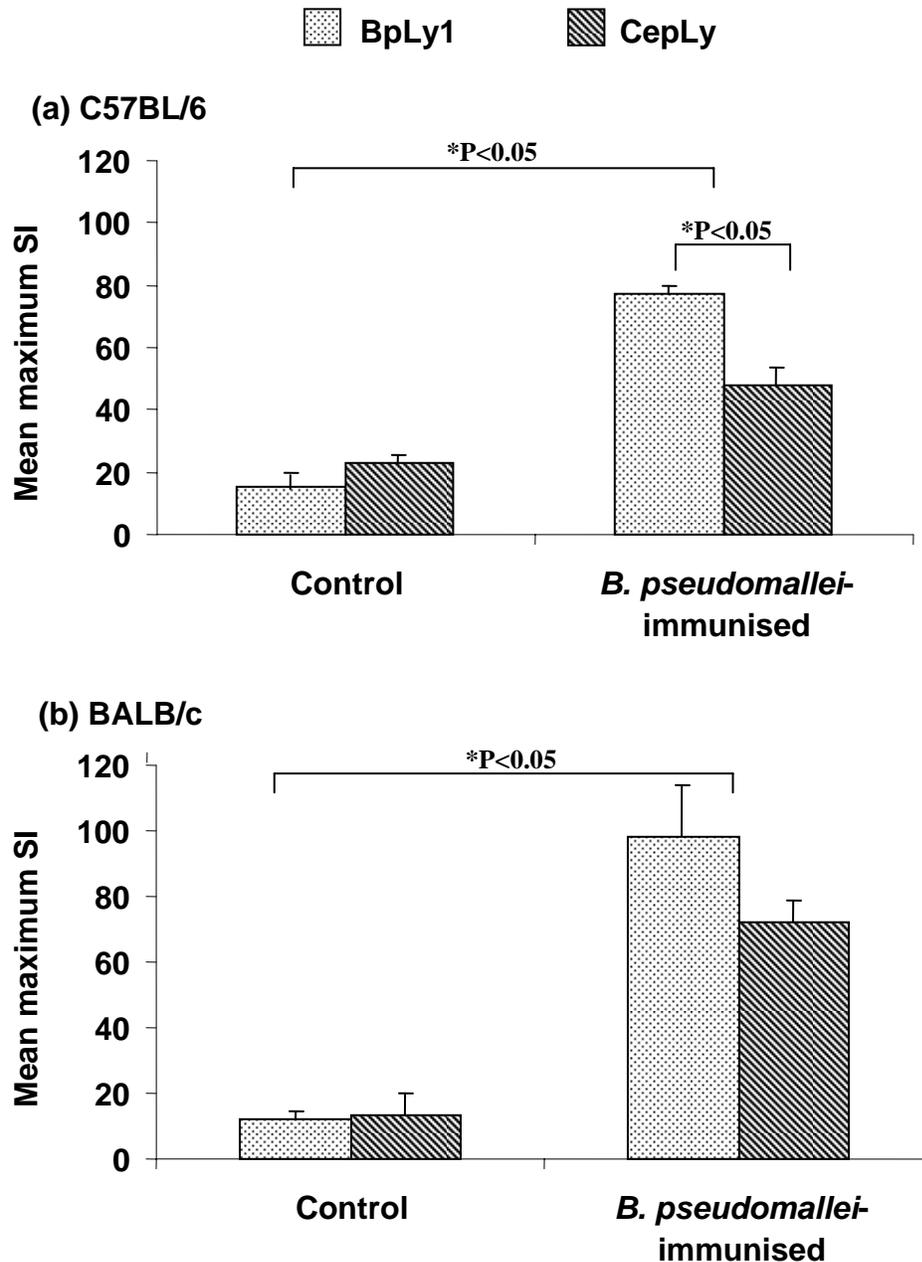
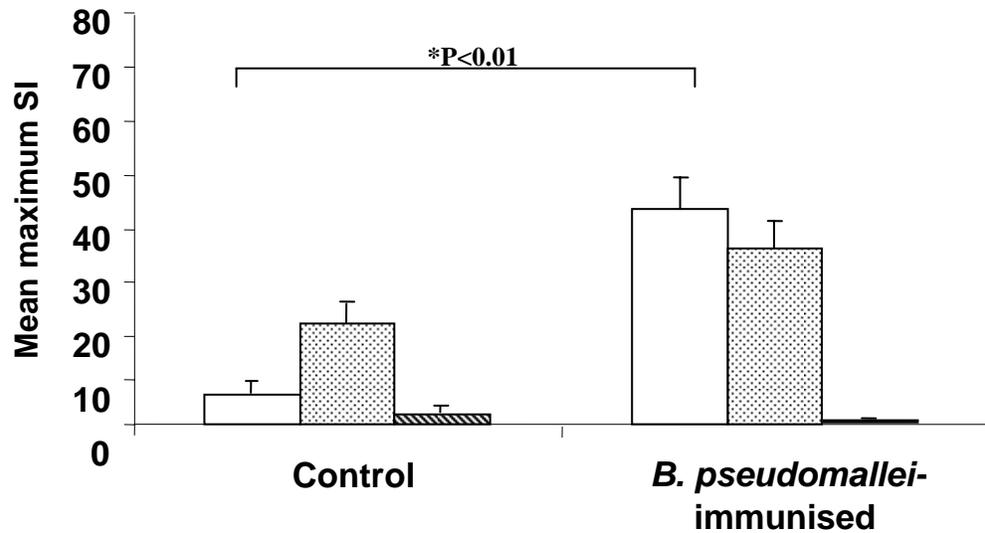


Figure 6.5 Lymphocyte proliferation in (a) C57BL/6 and (b) BALB/c mice in response to BpLy1 and CepLy.

Mice were immunised *iv* with 0.1 x LD₅₀ of *B. pseudomallei* NCTC 13179. Control mice received PBS only. At 28 days post-immunisation, mice (n=5) were euthanased and MNL were isolated and cultured in the presence or absence of BpLy1 or CepLy. Proliferation was measured from 96 to 168 hrs of culture using [³H]-thymidine incorporation. Results are expressed as the mean maximum SI ± SEM. Compared to controls, lymphocyte proliferation was significantly greater in *B. pseudomallei*-immunised C57BL/6 (P<0.05) and BALB/c (P<0.05) mice. Proliferation of lymphocytes from immunised C57BL/6 mice was higher in cultures stimulated with BpLy1 than CepLy (P<0.05).

□ BpLy1 ▨ CepLy ▩ *P. aeruginosa*-LPS

(a) C57BL/6



(b) BALB/c

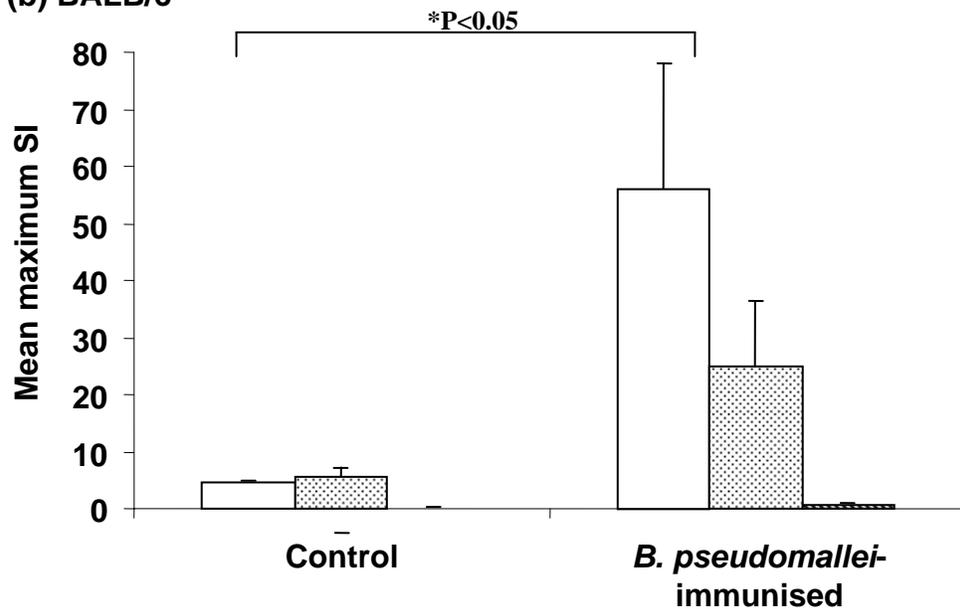


Figure 6.6 Lymphocyte proliferation in (a) C57BL/6 and (b) BALB/c mice following neutralisation of LPS in BpLy1, CepLy and *P. aeruginosa*-LPS. MNL from control and *B. pseudomallei*-immunised mice were stimulated with BpLy1, CepLy or *P.aeruginosa*-LPS that had been pretreated with polymyxin B. Proliferation was measured from 96 to 168 hrs of culture using [³H]-thymidine incorporation. Results are expressed as the mean maximum SI ± SEM. Compared to controls, lymphocyte proliferation was significantly greater in *B. pseudomallei*-immunised C57BL/6 (P<0.01) and BALB/c (P<0.05) mice. There were no differences in lymphocyte proliferation between cultures stimulated with LPS-neutralised BpLy1 or with LPS-neutralised CepLy (P>0.05).

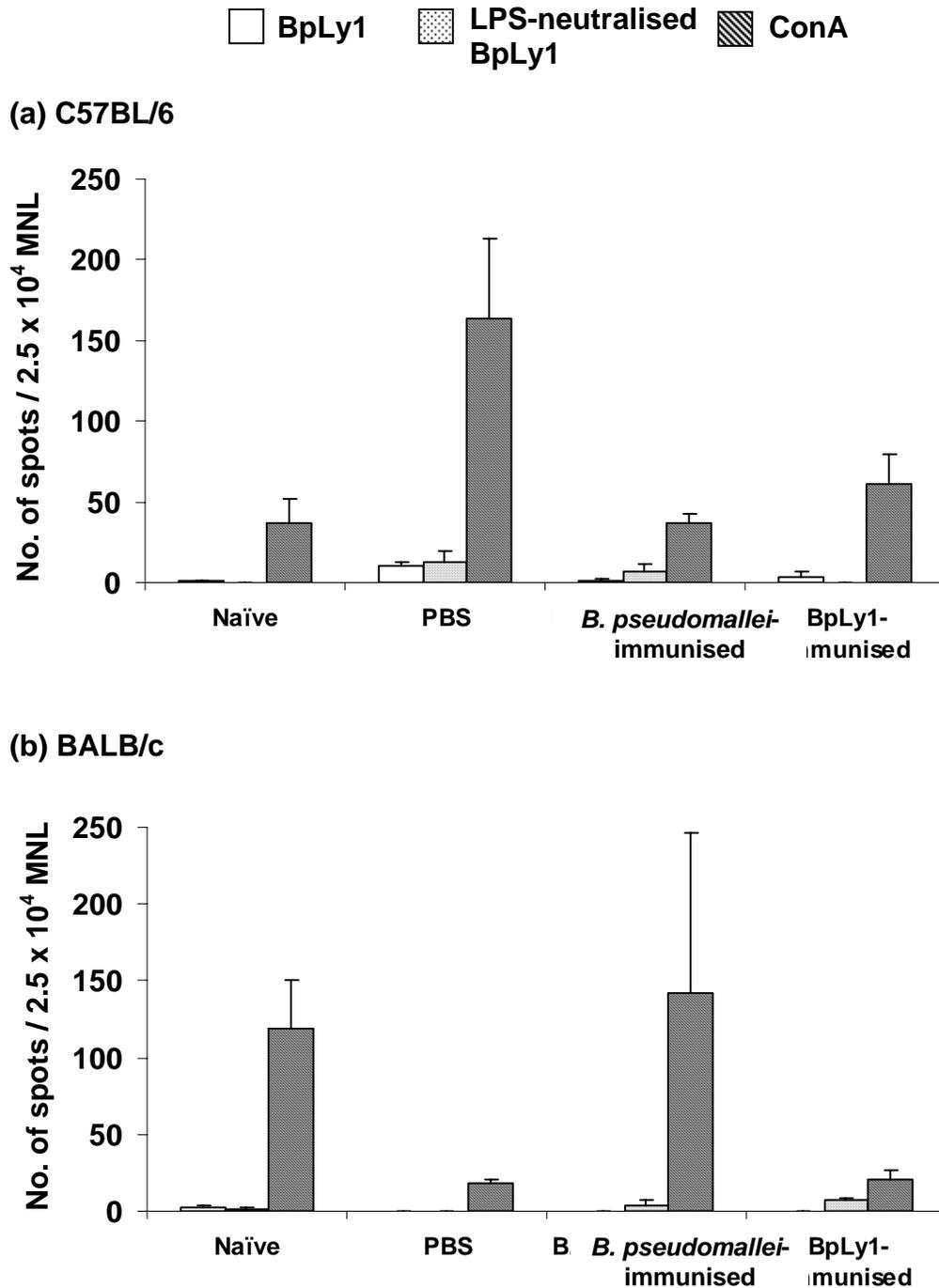


Figure 6.7 IFN- γ -producing lymphocytes from (a) C57BL/6 and (b) BALB/c mice. MNL were isolated from spleen of naïve, PBS-immunised, *B. pseudomallei*-immunised or BpLy1 immunised mice (n=5 per group) and cultures were stimulated with BpLy1, LPS-neutralised BpLy1 or ConA. After 24 hrs of culture, numbers of IFN- γ -producing lymphocytes were determined using ELISPOT assay. Numbers of IFN- γ -producing cells were increased in all wells stimulated with ConA. No significant increases were observed in BpLy1 stimulated wells for C57BL/6 or BALB/c mice. Similarly, responses in lymphocyte cultures from control mice were comparable to immunised mice.

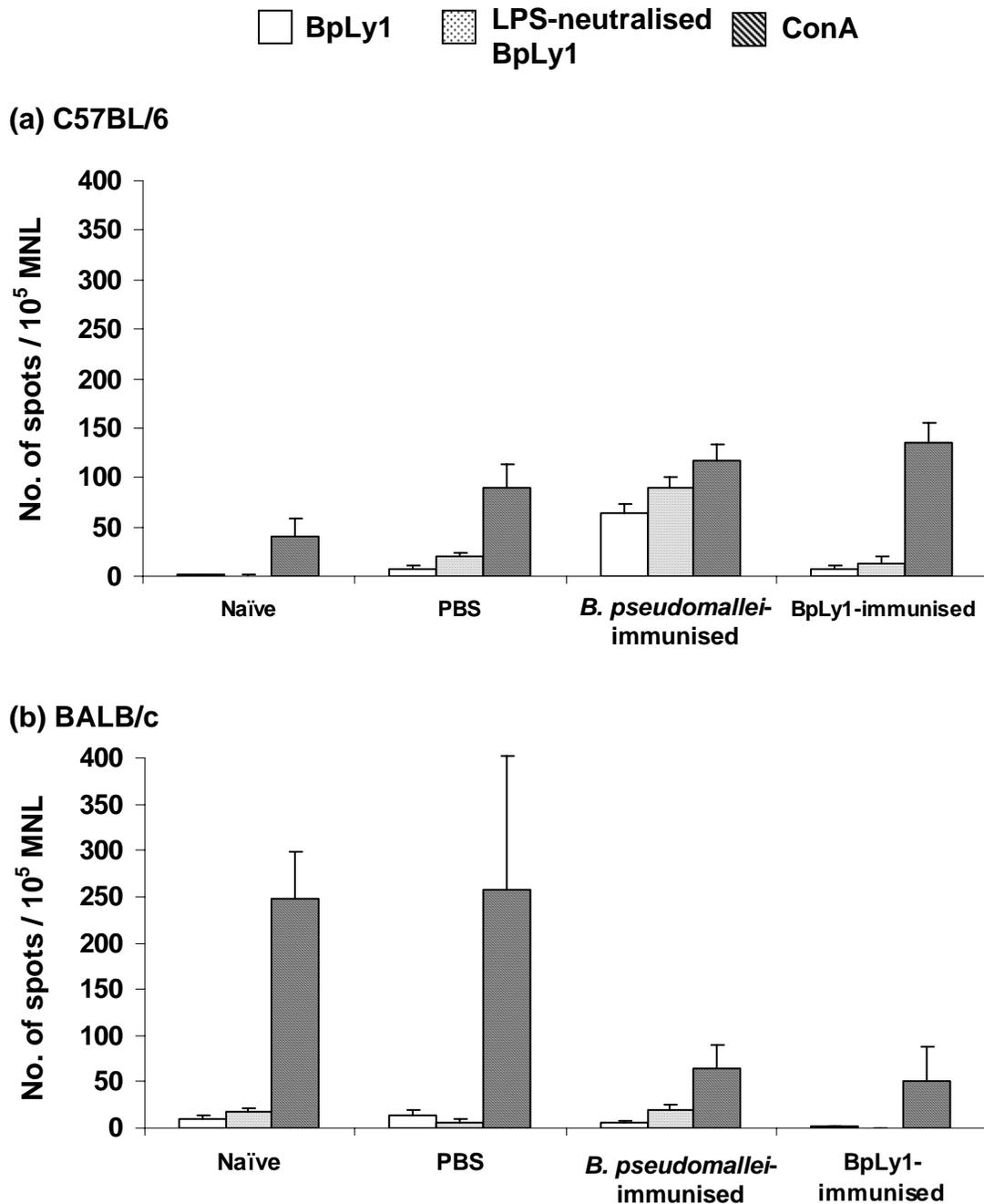


Figure 6.8 IL-4-producing lymphocytes from (a) C57BL/6 and (b) BALB/c mice. MNL were isolated from spleen of naïve, PBS-immunised, *B. pseudomallei*-immunised or BpLy1 immunised mice (n=5 per group) and cultures were stimulated with BpLy1, LPS-neutralised BpLy1 or ConA. After 24 hrs of culture, numbers of IL-4-producing lymphocytes were determined using ELISPOT assay. Numbers of IL-4-producing cells were increased in all wells stimulated with ConA. IL-4 production was significantly greater in lymphocyte cultures from *B. pseudomallei*-immunised C57BL/6 mice stimulated with LPS-neutralised BpLy1 compared to control mice (P<0.05). This was not observed in lymphocyte cultures from *B. pseudomallei*-immunised BALB/c mice.

6.4 Discussion

The interaction between macrophages and pathogens is vital in determining the outcome of infection. The inability of a host to activate its macrophages to a state of heightened microbial resistance increases its susceptibility to infection by intracellular pathogens. A number of intracellular pathogens possess various mechanisms that enable them to evade or resist killing by macrophages. Survival mechanisms include inhibition of phagolysosome formation, escape into the cytoplasm, resistance or inactivation of ROI or lysosomal enzymes, or failure to trigger a complete respiratory burst (Schaible *et al.* 1999). In the present study, NO production was estimated by measuring the by-product, NO_2^- in supernatants of peritoneal macrophage cultures. NO is a freely diffusible radical gas that has potent antimicrobial and antiparasitic effects. In macrophages, NO is generated during the oxidation of L-arginine by the enzyme, iNOS. Maximal expression of iNOS in murine macrophages occurs following stimulation with IFN- γ plus bacterial LPS. However, IFN- γ can also induce low levels of NO in the absence of LPS (Nathan & Hibbs 1991). NO has been shown to influence cytokine secretion by T cells and may modulate inflammation by inducing apoptosis in regulatory cells (Liew 1995; Allione *et al.* 1999). Clearance of *L. major* and *M. tuberculosis* from infected murine tissues is dependent on the generation of NO from mouse macrophages (Liew *et al.* 1990; Flesch & Kaufmann 1991). Ehlers *et al.* (1999) demonstrated a regulatory role for NO in granuloma formation in *M. avium*-infected mice. The modulatory effects of NO on cytokine secretion affect the number, size and cellular composition of granulomas. In *M. avium*-infected mice, NO contributes to the reduction of immunopathology, such that when NO production is inhibited, infection with mycobacterium is exacerbated resulting in high bacterial loads in organs and widespread necrosis (Ehlers *et al.* 1999). In contrast, high NO production by macrophages can have an “immunosuppressive” effect in infections with pathogens such as *L. monocytogenes* and *S. typhimurium* (Gregory *et al.* 1993; Schwacha & Eisenstein 1997).

In the present study the NO-secretory ability of C57BL/6 and BALB/c peritoneal macrophages after stimulation with *B. pseudomallei* antigens was compared. NO was produced only following priming of peritoneal macrophages with IFN- γ , prior to stimulation with BpLy1. Similar amounts of NO were produced by peritoneal macrophages from C57BL/6 and BALB/c mice under these conditions. To assess the contribution of LPS in stimulating NO production, *P. aeruginosa*-LPS was included since purified LPS from *B. pseudomallei* or *B. cepacia* were not commercially available at the time the study was undertaken. Previously, *B. pseudomallei* was regarded as a member of the genus *Pseudomonas* until reclassification in 1992 into the new genus, *Burkholderia* (Yabuuchi *et al.* 1992). NO levels in response to BpLy1 were equivalent to levels in cultures stimulated with *P. aeruginosa*-LPS suggesting that LPS from *B. pseudomallei* may play an important role in stimulating NO production *in vivo*.

Despite the similarity in NO production observed in C57BL/6 and BALB/c macrophages in the current investigation, a previous study demonstrated that peritoneal macrophages from C57BL/6 mice are more efficient at killing *B. pseudomallei*, when compared to BALB/c macrophages (Ulett, Ketheesan & Hirst 1998). However, in that study NO levels were not determined (Ulett, Ketheesan & Hirst 1998). Bactericidal activity of macrophages toward *B. pseudomallei in vitro* is stimulated only in the presence of lymphocytes (Ulett, Ketheesan & Hirst 1998). Macrophages activated by IFN- γ are capable of inhibiting the intracellular growth of *B. pseudomallei* (Utaiincharoen *et al.* 2001). Their bactericidal activity against the bacterium is predominantly mediated by RNI (Miyagi *et al.* 1997). Intracellular growth of *B. pseudomallei* is suppressed following IFN- γ activation of macrophages and increased expression of iNOS (Utaiincharoen *et al.* 2001). However, *B. pseudomallei* has been shown to invade and replicate within both unstimulated and stimulated macrophages, without activating iNOS (Miyagi, Kawakami & Saito 1997; Utaiincharoen *et al.* 2001). When infected, or exposed to microbial products of gram negative bacteria such as LPS, macrophages produce IFN- β . IFN- β modulates the ability of macrophages to synthesize iNOS via the upregulation of interferon regulatory factor-1 (IRF-1), a transcriptional activator of the iNOS gene. Protection from *L. monocytogenes* or *M. avium* infection has been generated

in mice using recombinant IFN- β therapy (Fujiki & Tanaka 1988; Denis 1991). Since *B. pseudomallei* stimulates minimal production of IFN- β , iNOS expression is decreased and the intracellular growth of the bacterium is not controlled (Utainscharoen *et al.* 2003). The comparable NO production observed in the current study, combined with the contrasting microbicidal efficiency of C57BL/6 and BALB/c macrophages (Ulett, Ketheesan & Hirst 1998) suggest that there may be a difference in the priming of macrophages between the two mouse strains following infection with *B. pseudomallei*. This is supported by the findings of Barnes *et al.* (2001; Chapter 5) who demonstrated that although macrophages are present at sites of infection in BALB/c mice in the early stages of *B. pseudomallei* infection, their numbers are significantly lower compared to C57BL/6 mice as the infection proceeds. A failure of macrophages to become stimulated during the initial stages of *B. pseudomallei* infection may underlie the susceptible phenotype of BALB/c mice since an effective innate, and consequently an adaptive immune response would not be initiated.

The ability of *S. typhimurium* to persist intracellularly varies with the type of macrophage. Bacterial survival is greater in splenic versus peritoneal macrophages, correlating with high bacterial numbers in this organ during *S. typhimurium* infection (Buchmeier & Heffron 1989). The functional differences in resident macrophages in different tissue may contribute to the reported organotropism of *B. pseudomallei* for spleen and liver (Leakey *et al.* 1998; Hoppe *et al.* 1999; Liu *et al.* 2002). The current study was a preliminary investigation of the contribution of NO production to the resistant and susceptible phenotypes of C57BL/6 and BALB/c mice respectively. Future studies at James Cook University will continue to compare the functional abilities of macrophages derived from various tissues within these mouse strains. Such studies will include flow cytometric analysis of macrophage activation in response to *B. pseudomallei* through the investigation of cell surface marker expression and cytokine production.

Host protective immunity to intracellular pathogens such as *M. tuberculosis* and *L. monocytogenes* involves an adaptive CMI response. *B. pseudomallei* is a facultative

intracellular bacterium that can survive and replicate within phagocytic cells of the host (Pruksachartvuthi, Aswapokee & Thankerngpol 1990; Jones *et al.* 1996; Harley *et al.* 1998). Although it is widely accepted that CMI responses are important in *B. pseudomallei* infection, there is little evidence of this role in the literature. Recently, we published the first evidence of the involvement of adaptive CMI responses in human melioidosis (Chapter 9; Ketheesan *et al.* 2002). In this chapter, we carried out preliminary experiments to demonstrate the generation of adaptive CMI responses during *B. pseudomallei* infection in experimental melioidosis and to compare the responses of susceptible BALB/c mice, with comparatively resistant C57BL/6 mice.

The DTH and lymphocyte proliferation assays were used to demonstrate the presence of *B. pseudomallei*-specific lymphocytes in mice previously exposed to the bacterium. Greater DTH and lymphocyte proliferation to BpLy1 was observed in *B. pseudomallei*-immunised C57BL/6 and BALB/c mice compared to controls, indicating the generation of specific lymphocytes that recognised *B. pseudomallei* antigens. However, for the DTH assays both C57BL/6 and BALB/c mice responded equally well to BpLy1 and CepLy, demonstrating cross-reactivity between the antigen preparations. This was also observed for lymphocyte proliferation assays when LPS-neutralised BpLy1 was used. Cross-reactivity is not surprising considering the high degree of homology between the two *Burkholderia* species (Dharakul *et al.* 1999) and the antigen preparations used as lysates. Most members of the genus *Burkholderia*, including *B. cepacia*, are primarily plant pathogens. However, *B. cepacia* is also emerging as an important respiratory pathogen, particularly in immunocompromised individual such as those with cystic fibrosis. Therefore, as well as being opportunistic human pathogens, phylogenetic analysis also demonstrates that *B. cepacia* and *B. pseudomallei* share considerable homology (Dharakul *et al.* 1999). *B. cepacia* was used in the present study to assess the specificity of DTH and lymphocyte proliferation responses of mice towards *B. pseudomallei* antigens.

Interestingly, *B. cepacia*-immunised C57BL/6 mice subsequently challenged with CepLy, had a significantly increased DTH response compared to challenge with BpLy1.

This may reflect the generation of specific anti-*B. cepacia* lymphocytes. This response was not observed in *B. pseudomallei*-immunised C57BL/6 mice later challenged with BpLy1. One theoretical explanation for this observation is that a greater proportion of specific lymphocytes generated during *B. pseudomallei* infection are specific for secreted *B. pseudomallei* antigens, rather than structural antigens. In contrast, following *B. cepacia* infection the majority of specific lymphocytes may target structural antigens. A high similarity between the structural antigens of *B. pseudomallei* and *B. cepacia* may explain the lack of specificity of the DTH response in *B. pseudomallei*-immunised mice when compared to CepLy. A DTH response was not observed in *B. cepacia*-immunised BALB/c mice. Compared to *B. pseudomallei*, BALB/c and C57BL/6 mice are both resistant to the *B. cepacia* strain used. It is possible that the immunising dose of *B. cepacia* used in BALB/c mice was insufficient to drive an adaptive immune response.

We found that increasing the immunisation dose caused an increase in the DTH response and lymphocyte proliferation observed in C57BL/6 and BALB/c mice. However there were limits to this effect. The lower limit was observed in the DTH response of C57BL/6 mice where no significant difference was found between control mice and the low-dose immunisation group. This may be a reflection of the more resistant phenotype of the C57BL/6 mouse towards *B. pseudomallei*, such that below a certain threshold dose, *B. pseudomallei* is successfully eliminated before infection is established or an adaptive immune response is generated. Consequently, a DTH response is not observed. At immunising doses above the upper threshold, the innate immune responses are unable to control *B. pseudomallei* multiplication and dissemination of infection occurs. In these mice, death occurs before an effective adaptive immune response is generated. A similar situation has been described for *M. tuberculosis* infection (Orme, Anderson & Boom 1993). When infection is initiated with low numbers of *M. tuberculosis*, monocytes function successfully as APC and a small number of CD4⁺ T cells become activated. Consequently, the CD4⁺ T cells undergo clonal expansion and secrete cytokines that inhibit further bacterial growth and initiate granuloma formation. A memory state is also formed, which mediates a DTH response (Orme, Anderson & Boom 1993). Infection with a large number of *M. tuberculosis* generates a correspondingly large number of

sensitised T cells. There is also an increase in cytotoxicity of T cells that leads to the lysis and destruction of the infected monocyte. This contributes to inflammatory conditions and may promote further dissemination (Orme, Anderson & Boom 1993). Based on the findings of DTH and lymphocyte proliferation assays in the present study, 0.1 x LD₅₀ of *B. pseudomallei* NCTC 13179 should be used as the immunising dose in subsequent investigations of adaptive CMI responses in *B. pseudomallei* infection. Responses should be assessed 14 days after *B. pseudomallei* exposure, a time-frame that is sufficient to allow generation of specific lymphocytes.

IFN- γ , produced by NK and T cells, is a potent macrophage activator and is important for driving a T_H1-type, or CMI response. In contrast, IL-4 is a key B cell activator and an initiator of a T_H2-type, or humoral response. In the present preliminary study we compared the number of IFN- γ and IL-4 producing lymphocytes in cultures derived from controls or mice immunised with live *B. pseudomallei* versus BpLy1. No significant increases in IFN- γ production were observed between control and immunised mice, or between C57BL/6 and BALB/c mice. In contrast, numbers of IL-4-producing lymphocytes were increased in cultures derived from *B. pseudomallei*-immunised C57BL/6 mice, compared to the other three treatment groups. A previous study demonstrated the upregulation of mRNA for both T_H1 and T_H2 cytokines during experimental infection with *B. pseudomallei* (Ulett *et al.* 2002). Stimulation of cultured lymphocytes with a *B. pseudomallei* antigens rather than live bacteria may explain the predominance of an IL-4 (or T_H2-type) response in the *in vitro* system. Secreted bacterial antigens that are absent in the *B. pseudomallei* lysate (BpLy1), may be important in generating a T_H1-type response. Therefore, stimulation of *B. pseudomallei*-specific lymphocytes with BpLy1 *in vitro* may tip the cytokine response towards a T_H2-type response. Interestingly, lymphocytes from *B. pseudomallei*-immunised BALB/c mice did not demonstrate this increase in IL-4. It was expected that following immunisation of mice with BpLy1 an increase in cytokine-secreting activity would be observed in MNL cultures stimulated with BpLy1, indicating the presence of lymphocytes that are specific for BpLy1 antigens. However, this was not observed and may reflect a failure of an adaptive immune response generated in mice that were

immunised with a single dose of 5 µg of BpLy1. Further work is necessary to determine the threshold dose of BpLy1, delivered *sc* that is required to generate an adaptive immune response.

The results of data presented in the current chapter have demonstrated a role for CMI responses in melioidosis. These studies provided basic data on which to base future investigations into the generation of adaptive CMI responses during *B. pseudomallei* infection.

CHAPTER 7

ADOPTIVE TRANSFER OF RESISTANCE TO *B. pseudomallei* IN A MURINE MODEL OF MELIOIDOSIS

7.1 Introduction

Collaboration between innate and adaptive immune responses to an invading pathogen promotes the control of a primary infection and the generation of long-term protective immunity. Protective immunity is mediated by antigen-specific lymphocytes and is rapidly activated after re-exposure to a pathogen, greatly limiting the extent of infection. DTH and acquired cellular resistance are manifestations of CMI responses to intracellular pathogens (Tsukada *et al.* 1991). As mentioned previously, CMI responses are believed to play an important role in providing protection against infection with *B. pseudomallei*, since it is a facultative intracellular bacterium. In the previous chapter, we demonstrated the generation of *B. pseudomallei*-specific lymphocytes in C57BL/6 and BALB/c mice that had been previously immunised with the bacterium. In the work described in this chapter we investigated the ability of these antigen-specific cells to provide resistance in naïve mice using adoptive transfer assays. These experiments were performed using C57BL/6 mice due to their relative innate resistance to *B. pseudomallei* infection.

Adoptive transfer of lymphocytes has been used to investigate protective immune responses against infection with several intracellular pathogens including *M. tuberculosis* and *L. monocytogenes* (Mielke, Ehlers & Hahn 1988; Orme 1988; Tsukada *et al.* 1991; Silva *et al.* 1999). Development of protective immunity toward *M. tuberculosis* and *L. monocytogenes* is dependent on the involvement of several T cell subsets including CD4⁺, CD8⁺ and $\gamma\delta$ ⁺ T cells (Mielke, Ehlers & Hahn 1988; Boom 1996). Protection generated by non-lethal infection with *L. monocytogenes* can be adoptively transferred to naïve immunocompetent mice with CD4⁺ alone, CD8⁺ alone or both CD4⁺ and CD8⁺ T cells (Kaufmann *et al.* 1985; Orme 1989). Similarly, splenic T

cells from mice immunised with *M. tuberculosis* protect naïve recipients from a subsequent, normally lethal challenge (Orme 1988). Often adoptive transfer experiments use lymphocytes isolated from spleen since, following systemic infection, this lymphoid organ is an important site for activation and expansion of antigen-specific T cells. Using live and heat-killed *M. leprae* for immunisation, Graham & Navalkar (1984) demonstrated that adoptive immunity was expressed equally well in non-irradiated and sublethally irradiated recipient mice.

Therefore, MNL from *B. pseudomallei*-immunised C57BL/6 mice were used in this study and the specific aims for the work described in this chapter were:

1. To assess the ability to transfer a DTH reaction to naïve recipients challenged *sc* with BpLy1
2. To assess the ability to transfer protection to naïve recipients challenged *iv* with live *B. pseudomallei*

7.2 Materials and Methods

7.2.1 Primary immunisation

C57BL/6 mice were divided into two immunisation groups. The first group (AT-Bp; n=66) were *iv* inoculated with 0.1 x LD₅₀ of *B. pseudomallei* NCTC 13179. Control mice (AT-PBS; n=46) received 200 µl of PBS, delivered *iv*. At day 10 post-immunisation, three AT-Bp mice were euthanased and bacterial loads were assessed in spleen to ensure sterility. Adoptive transfer assays were performed at day 14 post-immunisation.

7.2.2 Isolation and purification of splenic MNL

At day 14 post-immunisation, AT-Bp and AT-PBS mice were euthanased and spleen were removed aseptically. Splenocytes were recovered from spleen using protocols described previously (Chapter 3). MNL were isolated by overlaying the splenocyte suspension on Ficoll-Paque and centrifuging (500 g; 20 min). MNL were washed twice in transport media (Appendix 1). MNL from immunised mice were transferred to two groups of naïve mice (n=25). A third group of naïve mice were included that received 200 µl *iv* of PBS only. Two parallel adoptive transfer assays were performed using either

MNL or purified T cells. For the MNL experiments, cell concentration was adjusted to enable transfer of 5×10^6 cells in 200 μ l of PBS per mouse. For experiments with purified T cells, 1×10^8 MNL were passed through a mouse T cell recovery column (Cedarlane, Ontario, Canada) as per the manufacturers' instructions. T cells were suspended in PBS and cell concentration was adjusted to enable *iv* adoptive transfer of 4×10^6 purified T cells in 200 μ l of PBS per mouse. Mice were allowed to recover for 24 hrs then 5 mice per group were used to assess DTH response to BpLy1. Of the remaining mice, half were challenged *iv* with 1 x LD₅₀ of *B. pseudomallei* NCTC 13178 and half with 200 μ l of PBS. Spleen weights and bacterial load (Chapter 3), serum antibody levels and mouse survival were measured to determine the degree of protection afforded by adoptive transfer of either unfractionated MNL or purified T cells.

7.2.3 DTH responses

DTH response was assessed in AT-Bp, AT-PBS and PBS groups (n=5), 24 hrs after adoptive transfer. Right and left hind footpads were measured at 0 hrs, prior to footpad challenge. Sterile PBS (40 μ l) was *sc* injected into the right hind footpad of each mouse. The left hind footpad was *sc* injected with 5 μ g/40 μ l of BpLy1. Footpad thickness was recorded at 0 and 48 hrs using digital calipers. Change in footpad thickness was calculated by subtracting mean measurements (n=5 per foot) at 0 hrs from footpad measurements at 48 hrs after challenge. Results were presented as mean change in footpad thickness (mm) \pm SEM.

7.2.4 Host survival and bacterial loads

Following challenge with the highly virulent *B. pseudomallei* strain, NCTC 13178, survival of n=5 mice per group was monitored for 10 days. In parallel experiments, n=3 mice per group were euthanased, spleen removed and weighed. Spleen indices were calculated by dividing weights of spleen from infected mice by the average spleen weight of naive C57BL/6 mice (n=10) of the same gender and age. Bacterial loads in spleen were determined using methods described previously (Chapter 3).

7.2.5 TNF- α levels

TNF- α levels were determined at day 2 post-challenge in serum from recipients of purified T cells that were challenged with NCTC 13178. A commercially available

ChemiKine™ Mouse Tumor Necrosis Factor- α EIA kit (Chemicon International; California, USA) was used according to the manufacturers' instructions.

7.2.6 Serum IgG_{2a} and IgG₁ levels

Serum was collected from AT-Bp, AT-PBS and PBS mice that survived to day 10 following challenge with NCTC 13178. An indirect ELISA was used to measure IgG_{2a} and IgG₁ levels in serum. For comparison, antibody levels were also determined in serum from naïve C57BL/6 mice, as well as C57BL/6 mice that were *iv* infected with 1 x LD₅₀ NCTC 13178. Immulon-4 (Nalge Nunc International; Illinois, USA) 96-well plates were coated with BpLy1 (10 μ g/well/100 μ l PBS) for 48 hrs at 4°C in a humidified chamber. Plates were washed four times in wash buffer (Appendix 1) using a microtitre plate washer (Wellwash 4 MK2; Labsystems, Finland). Blocking buffer (Appendix 1) was then added to each well (200 μ l/well) and incubated for 2 hrs at RT in a humidified chamber. After 4 washes, mouse serum diluted 1:8 in blocking buffer was added (100 μ l/well) to wells in triplicate. Positive control serum was obtained from C57BL/6 mice (n=10) that received two consecutive doses of 0.01 x LD₅₀ of *B. pseudomallei* NCTC 13179. Pooled serum from these mice had been previously shown to contain high levels of IgG_{2a} and IgG₁. Positive control serum, diluted 1:8, was added in triplicate and plates were incubated for 2 hrs at 37°C in a humidified chamber. Plates were washed four times and HRP-conjugated goat anti-mouse IgG_{2a} (1:1000; R19-15; BD Pharmingen) or goat anti-mouse IgG₁ (1:1500; X56; BD Pharmingen) diluted in PBS was added (100 μ l/well) for 2 hrs at 37°C. After washing, ABTS (Sigma) was added (100 μ l/well) and colour developed for 25 min. Absorbance at 414 nm with a reference wavelength of 492 nm, was measured on a microtiter plate reader (Multiskan EX355 v1.0, Labsystems, Finland). Readings were corrected for background and the positive cut-off was set at the mean absorbance of pooled normal mouse serum plus three standard deviations.

7.3 Results

7.3.1 DTH responses

As illustrated in Figure 7.1a, a significant DTH response was observed following adoptive transfer of unfractionated MNL from *B. pseudomallei*-immunised mice to naïve

mice (AT-Bp) compared to AT-PBS ($P < 0.05$) and PBS only mice ($P < 0.01$). Although a similar trend was also observed when purified T cells were adoptively transferred, differences between AT-Bp, AT-PBS and PBS only groups were not statistically significant ($P > 0.05$; Figure 7.1b).

7.3.2 Host survival and bacterial loads

Resistance of the three treatment groups were compared following challenge with 1 x LD₅₀ of highly virulent *B. pseudomallei* NCTC 13178. Naïve mice that received purified T cells from AT-Bp and AT-PBS mice died within 3 days following challenge with highly virulent NCTC 13178. Therefore, spleen indices, bacterial loads and antibody levels were determined at day 2 post-challenge in these mice. No differences were observed in the spleen indices (Figure 7.2a) or bacterial loads (Figure 7.3a) of AT-Bp, AT-PBS or PBS only mice when unfractionated MNL were adoptively transferred. Similarly, following adoptive transfer of purified T cells no differences were observed for either spleen indices (Figure 7.2b) or bacterial loads (Figure 7.3b) between control and immunised mice.

Survival rates were monitored for ten days following challenge with NCTC 13178 (Figure 7.4). By ten days post-challenge, no differences were observed in the overall number of deaths between AT-Bp, AT-PBS and PBS controls that received either unfractionated MNL (Figure 7.4a) or purified T cells (Figure 7.4b). Adoptive transfer of unfractionated MNL from *B. pseudomallei*-immunised mice did not enhance resistance to subsequent challenge with NCTC 13178, as indicated by the comparable survival rates of AT-Bp and control PBS only mice (Figure 7.4a). This correlates with spleen weights (Figure 7.2a) and bacterial loads (Figure 7.3a) between these two groups. Interestingly, adoptive transfer of purified T cells in AT-Bp and AT-PBS groups led to rapid death of all mice ($n=5$) within 72 hrs of challenge with NCTC 13178 (Figure 7.4b). In contrast, the mortality rate for control PBS only mice was more gradual with the majority of deaths occurring 3 days post-challenge (Figure 7.4b). No deaths were recorded by day 10 in purified T cell recipients that received a PBS challenge rather than NCTC 13178.

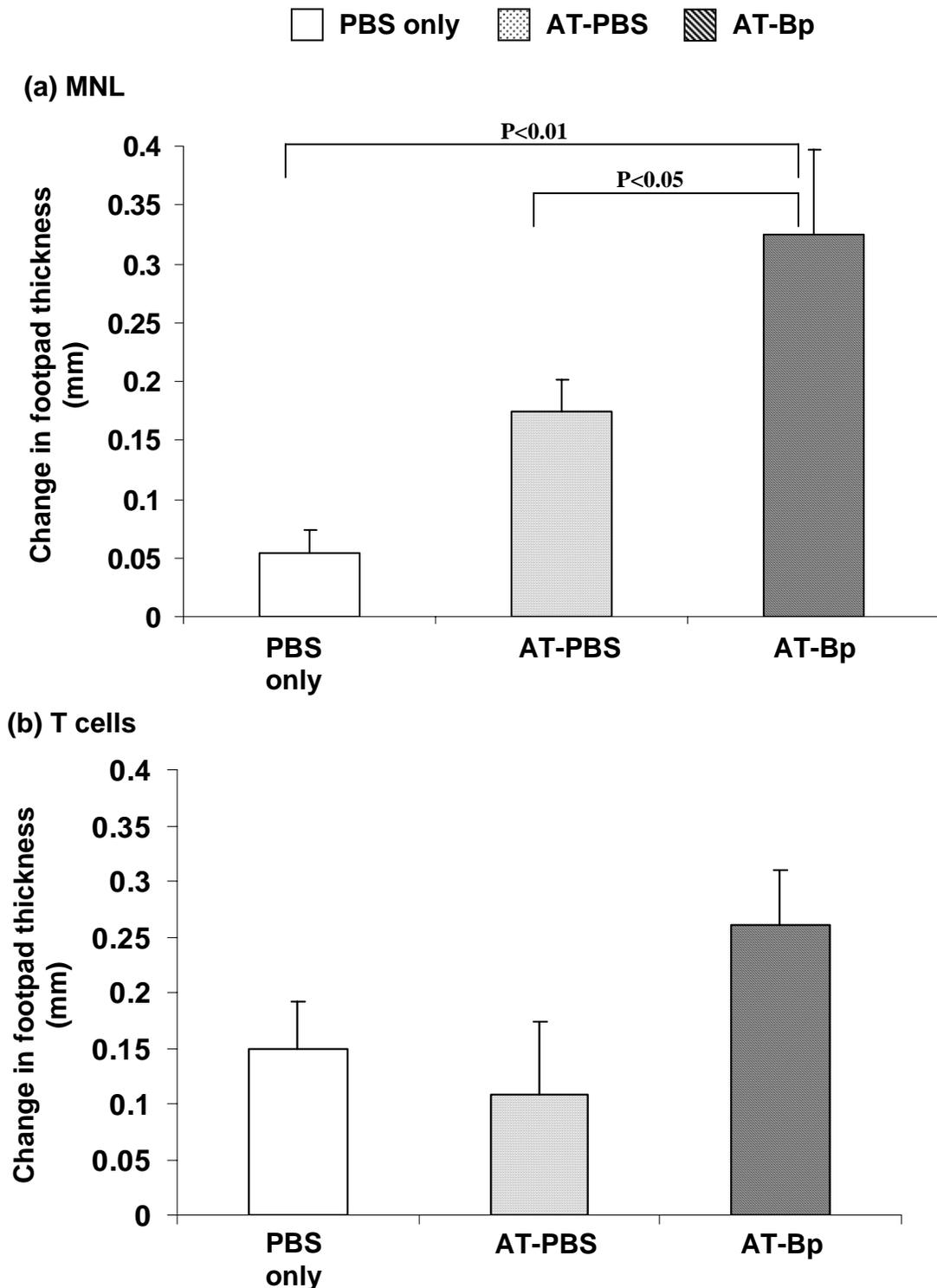


Figure 7.1 DTH response following adoptive transfer of (a) unfractionated MNL and (b) purified T cells.

Mice were *iv* immunised with 0.1 x LD₅₀ of *B. pseudomallei* NCTC 13179 (AT-Bp) or PBS (AT-PBS). After 2 weeks, (a) MNL or (b) purified T cells from these mice were adoptively transferred to naïve mice. A third group of naïve mice received 200 µl of PBS (PBS only) without MNL or T cells. DTH responses were assessed 24 hrs after adoptive transfer by *sc* injection of BpLy1 into the left hind footpad, and PBS into the right hind footpad (n=5). Footpad thickness was measured at 0 and 48 hrs after footpad challenge. Results are expressed as the change in footpad thickness (mm) ± SEM. A significant increase in footpad thickness was observed in mice that received unfractionated MNL from AT-Bp mice, compared to AT-PBS (P<0.05) and PBS only mice (P<0.01). Although a similar trend was observed when purified T cells were transferred, this difference was not statistically significant (P>0.05).

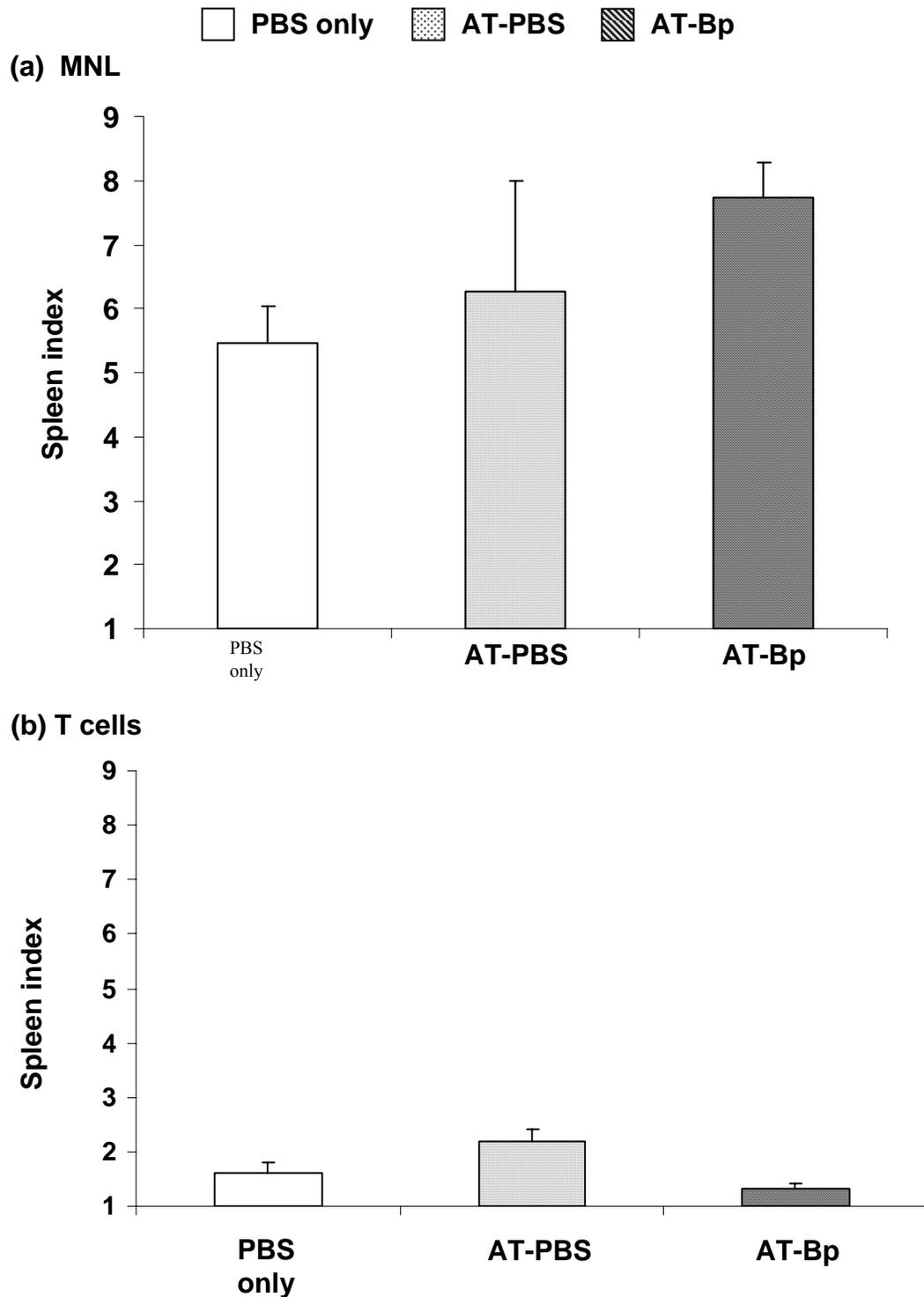


Figure 7.2 Spleen indices of mice following adoptive transfer of (a) unfractionated MNL or (b) purified T cells, subsequently challenged with NCTC 13178.

Mice were *iv* immunised with 0.1 x LD₅₀ of *B. pseudomallei* NCTC 13179 (AT-Bp) or PBS (AT-PBS). After 2 weeks, (a) MNL or (b) purified T cells from these mice were adoptively transferred to naïve mice. A third group of naïve mice received 200 µl of PBS (PBS only) without MNL or T cells. Mice were challenged with 1 x LD₅₀ *B. pseudomallei* NCTC 13178, 24 hrs after adoptive transfer. At day 10 following challenge, mice were euthanased and spleen removed. The weight of each spleen was divided by the mean weight of spleen from naïve mice (n=10) to give the spleen index. Results are expressed as the mean spleen indices of five mice ± SEM. No significant differences were observed between the spleen indices of AT-Bp, AT-PBS or PBS only mice when either unfractionated MNL or purified T cells were adoptively transferred.

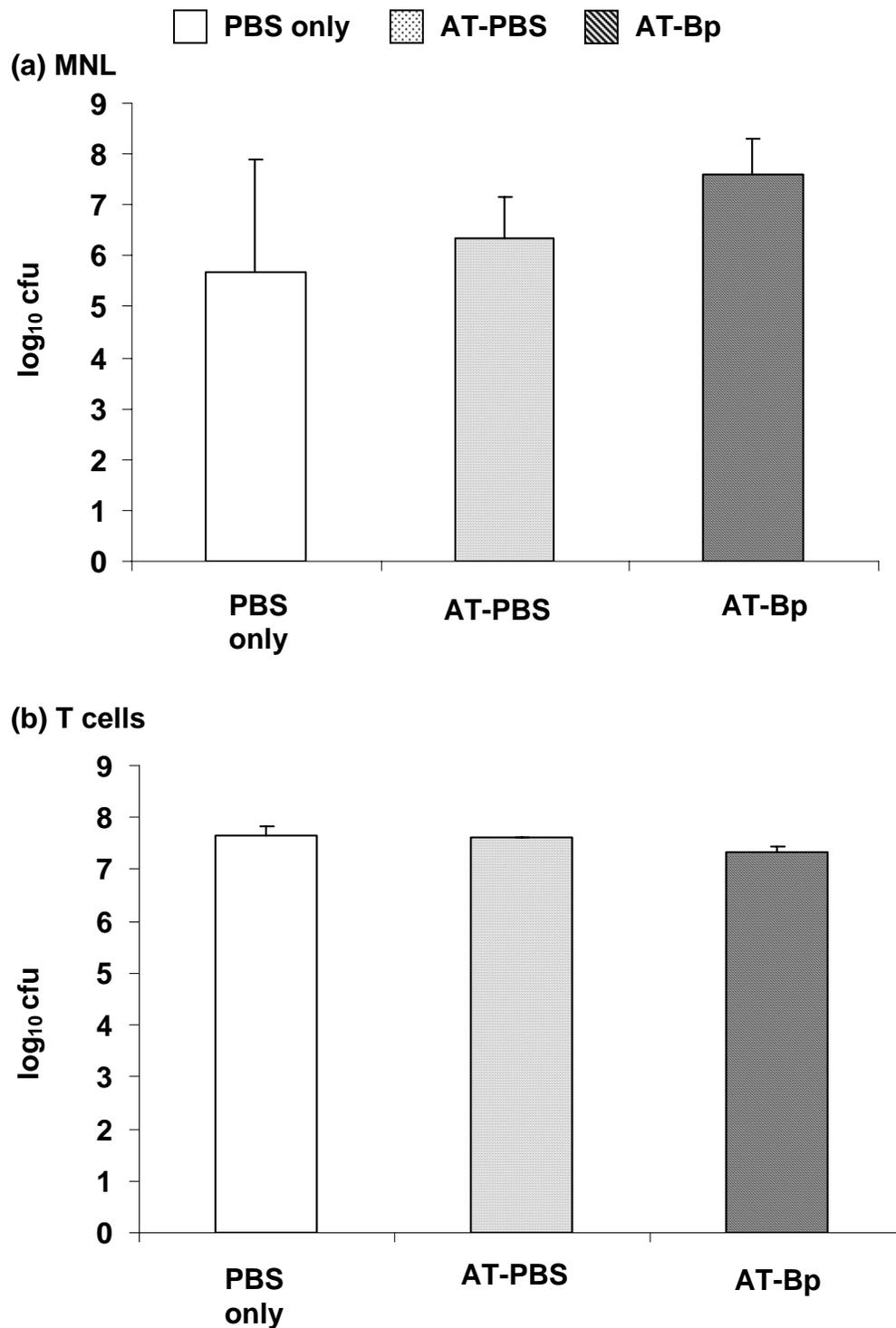


Figure 7.3 Bacterial loads in spleen of adoptive transfer mice following challenge with NCTC 13178.

Mice were *iv* immunised with 0.1 x LD₅₀ of *B. pseudomallei* NCTC 13179 (AT-Bp) or PBS (AT-PBS). After 2 weeks, (a) MNL or (b) purified T cells from these mice were adoptively transferred to naïve mice. A third group of naïve mice received 200 µl of PBS (PBS only) without MNL or T cells. Mice were challenged with 1 x LD₅₀ *B. pseudomallei* NCTC 13178, 24 hrs after adoptive transfer. At day 10 following challenge, mice were euthanased (n=5 per group) and spleen removed. Bacterial loads in spleen were determined by plating serial dilutions of homogenate onto Ashdown agar. Results are expressed as the mean bacterial load (log₁₀ cfu/spleen) for five mice ± SEM. No significant differences were observed between the bacterial loads in spleen of AT-Bp, AT-PBS or PBS only mice when either unfractionated MNL or purified T cells were adoptively transferred. ¹³³

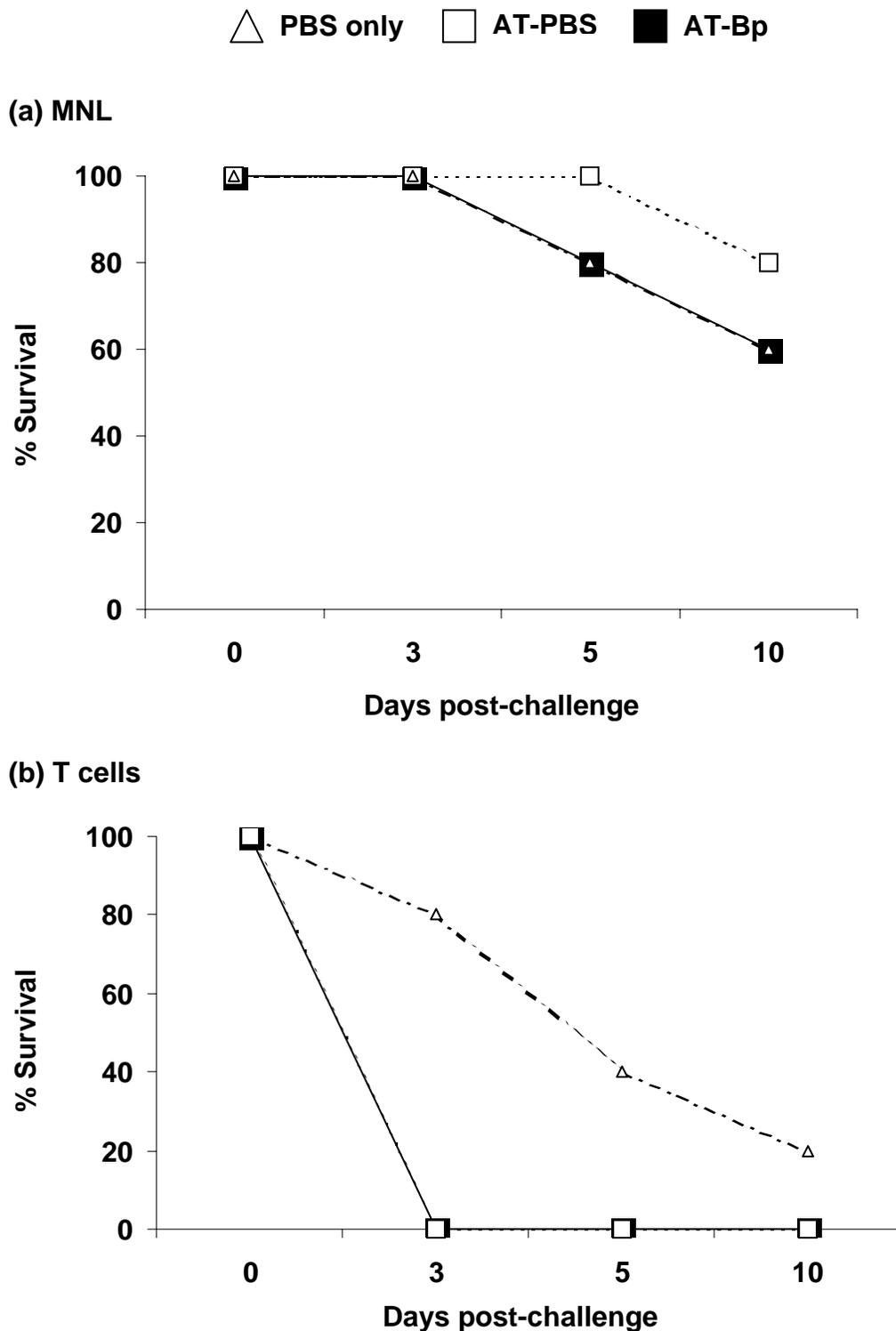


Figure 7.4 Survival of adoptive transfer mice following challenge with NCTC 13178. Mice were *iv* immunised with 0.1 x LD₅₀ of *B. pseudomallei* NCTC 13179 (AT-Bp) or PBS (AT-PBS). After 2 weeks, (a) MNL or (b) purified T cells from these mice were adoptively transferred to naïve mice. A third group of naïve mice received 200 μ l of PBS (PBS only) without MNL or T cells. Mice were challenged with 1 x LD₅₀ *B. pseudomallei* NCTC 13178, 24 hrs after adoptive transfer and monitored for 10 days to assess survival rates. No significant differences were observed in overall survival between AT-BP, AT-PBS or PBS only mice following adoptive transfer of unfractionated MNL. In contrast,¹³⁴ rapid death within 3 days of challenge occurred in AT-Bp and AT-PBS groups when purified T cells were adoptively transferred.

7.3.3 TNF- α levels

TNF- α levels were compared between AT-Bp, AT-PBS and PBS only mice that received purified T cells and were subsequently challenged with NCTC 13178. Serum was collected at 1 and 2 days post-challenge. Increasing levels of TNF- α levels were detected in AT-Bp, AT-PBS and PBS only mice (Figure 7.5). However, no significant differences between treatment groups were found at either time-point.

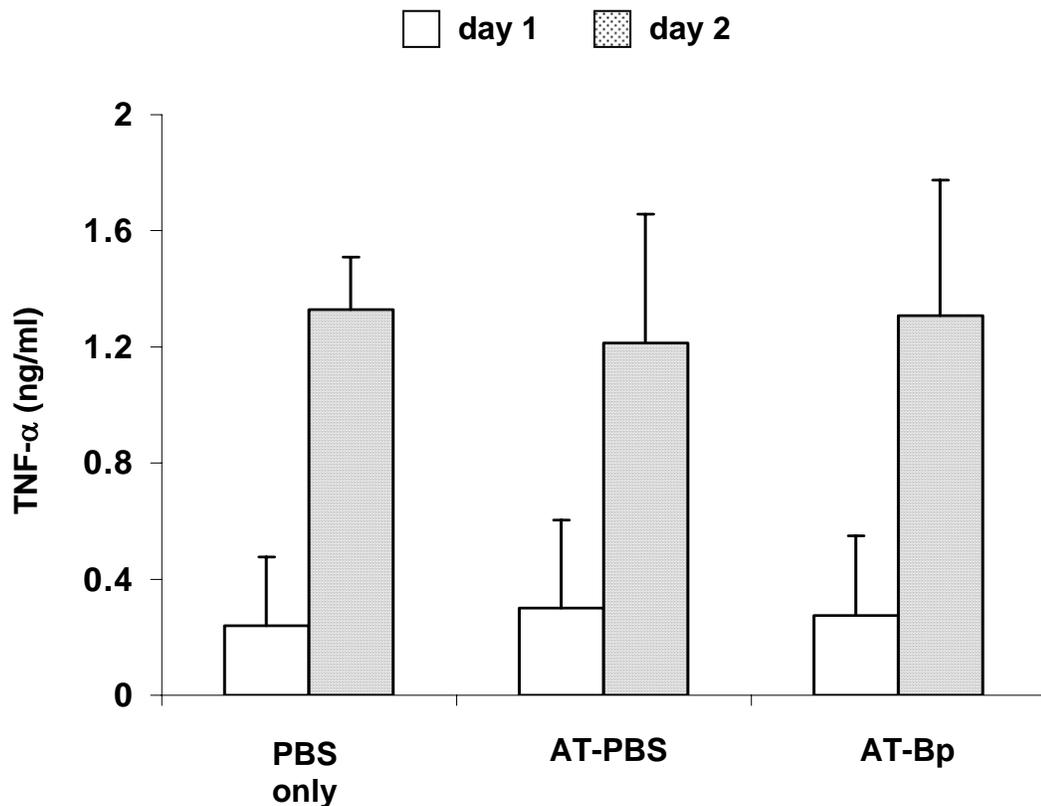


Figure 7.5 TNF- α levels in serum of purified T cell-recipient mice following challenge with NCTC 13178.

Mice were *iv* immunised with 0.1 x LD₅₀ of *B. pseudomallei* NCTC 13179 (AT-Bp) or PBS (AT-PBS). After 2 weeks, T cells purified from spleen of these mice were adoptively transferred to naïve mice. A third group of naïve mice received 200 μ l of PBS (PBS only) without T cells. Mice were challenged with 1 x LD₅₀ *B. pseudomallei* NCTC 13178, 24 hrs after adoptive transfer and TNF- α levels were assessed in serum at day 1 and day 2 post-challenge using EIA. Increased levels of TNF- α were detected in all treatment groups.

7.3.4 IgG₁ and IgG_{2a} levels

For mice into which MNL were adoptively transferred, antibody levels in serum were assessed at day 10 post-challenge with NCTC 13178 (Figure 7.6). Compared to IgG₁, serum levels of IgG_{2a} were significantly greater in PBS only mice and mice with a primary *B. pseudomallei* infection (Figure 7.6). IgG_{2a} levels were lower in recipients of MNL compared to controls. No IgG₁ was detected in AT-Bp mice. Serum from day 2 post-challenge was used to determine antibody levels for purified T cell adoptive transfer mice. IgG_{2a} and IgG₁ were not detected in AT-Bp, AT-PBS or PBS only mice at this time-point (data not shown).

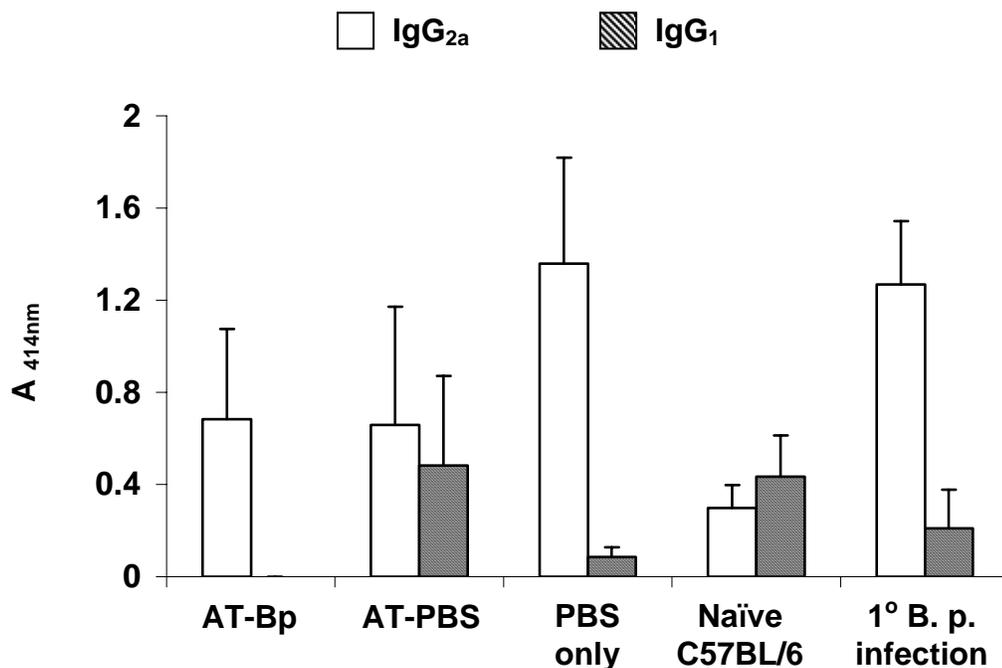


Figure 7.6 Antibody levels in serum from unfractionated MNL-recipient mice following challenge with NCTC 13178.

Mice were *iv* immunised with 0.1 x LD₅₀ of *B. pseudomallei* NCTC 13179 (AT-Bp) or PBS (AT-PBS). After 2 weeks, MNL from spleen of these mice were adoptively transferred to naïve mice. A third group of naïve mice received 200 µl of PBS (PBS only) without MNL. Mice were challenged with 1 x LD₅₀ *B. pseudomallei* NCTC 13178, 24 hrs after adoptive transfer an indirect ELISA was used to measure IgG_{2a} and IgG₁ levels in serum at day 10 in mice that received unfractionated MNL. For comparison, antibody levels in serum from uninfected mice and mice that were *iv* inoculated with 1 x LD₅₀ NCTC 13178 (1° B.p. infection; day 10 post-infection) are given. Compared to IgG₁, high levels of IgG_{2a} were observed following *B. pseudomallei* challenge. Compared to control mice, IgG_{2a} levels were lower in MNL recipients. However, this was not statistically significant (P>0.05).

7.4 Discussion

As described in Chapter 6, *B. pseudomallei*-specific lymphocytes are generated in BALB/c and C57BL/6 mice following exposure to the bacterium. In the data provided in this chapter we have demonstrated for the first time that these antigen-specific lymphocytes can be adoptively transferred to naïve recipients where they mediate a DTH response to BpLy1. However, the presence of the *B. pseudomallei*-specific lymphocytes is not sufficient to afford protection of recipient mice to a subsequent challenge with highly virulent *B. pseudomallei* as indicated by the comparable bacterial loads in spleen and deaths in *B. pseudomallei*-immunised versus control mice.

Compared to unfractionated MNL, adoptive transfer of purified T cells derived from spleen of *B. pseudomallei*-immunised and PBS-immunised mice resulted in a decreased DTH response. The successful transfer of DTH to particular antigens also requires the presence of antibodies (Crowle *et al.* 1975). For such antigens, the transferred splenic lymphocytes usually consist of a mixture of T and B cells, among which are cells that produce T-independent antibodies that sensitize the recipients' monocytes.

Paradoxically, although the macrophages “sensitised” with T-independent antibodies can complicate the interpretation of adoptive and passive transfers of DTH, it is necessary for these transfers (Crowle *et al.* 1975). There has been very little recent research conducted into the mechanism of DTH development. However, a reliance on antibodies for a DTH response to bacterial antigens contained in BpLy1 might explain the decreased DTH response that was observed in the current experiments when purified T cells were adoptively transferred.

In the current experiments, transfer of protection was assessed by several parameters including comparison of weights and bacterial loads in spleen and recipient survival over 10 days following challenge with highly virulent *B. pseudomallei*. In contrast to adoptive transfer mice that received MNL, rapid death of all mice that received purified T cells was observed within 3 days of challenge. Such a response is observed in acute *B. pseudomallei* infection in BALB/c and C57BL/6 mice where death occurs as a result of

bacterial dissemination and overwhelming septicemia (Leakey *et al.* 1998; Hoppe *et al.* 1999). Hyper-production of cytokines such as IFN- γ and TNF- α are associated with the acute form of melioidosis, contributing to immunopathology (Brown *et al.* 1991; Suputtamongkol *et al.* 1992; Ulett *et al.* 2000a). To investigate the influence of cytokine hyper-production on the rapid death of purified T cell recipients, we assayed levels of the pro-inflammatory cytokine, TNF- α , in serum of mice. However, no differences in TNF- α production was observed between immunised and control mice at day 2 post-challenge. It is likely that other pro-inflammatory cytokines such as IFN- γ , IL-1 β and IL-6, which were not assessed in the current study, contributed to the fatal outcome observed in the purified T cell recipients. Adoptive transfer of purified T cells appeared to increase the susceptibility of naïve C57BL/6 mice to *B. pseudomallei* infection. This did not appear to be a direct toxic effect of the transferred T cells, since no deaths were recorded in adoptive transfer mice that were challenged with PBS rather than highly virulent *B. pseudomallei*.

Bacterial loads in spleen were comparable between recipients of both MNL and purified T cells, despite the measurements being taken at day 10 versus day 2 post-challenge, respectively. In contrast, although spleen weights were greatly increased in MNL recipients, weights were comparatively low in purified T cell recipients. These discrepancies reflect the pathological differences observed in this lymphoid organ. Compared to day 2 where spleen appeared 'normal' in size and gross morphology, splenomegaly and multiple, protruding abscesses up to 4 mm in diameter were visible at day 10 post-challenge. Spleen abscessation may reflect an attempt by the host immune response to contain *B. pseudomallei* infection. Such 'focussing' of infection is supported by observations of organotropism of *B. pseudomallei* for spleen and liver (Leakey *et al.* 1998; Hoppe *et al.* 1999; Gauthier *et al.* 2000). In contrast, dissemination of large numbers of *B. pseudomallei* in purified T cell recipients would have contributed to the rapid death observed in these mice.

In the mouse, IgG_{2a} isotype switching is promoted by IFN- γ , whereas IgG₁ isotype switching is induced by IL-4. Therefore IgG_{2a} is associated with T_H1-type responses and

promotes opsonisation and phagocytosis of microbes. During a primary infection with *B. pseudomallei*, levels of IgG_{2a} were significantly higher than IgG₁. Adoptive transfer of MNL appeared to dampen the recipient antibody response to subsequent challenge with *B. pseudomallei*. This may reflect the development of a predominantly CMI response in the recipient mice. It is possible that the low levels of antibody that were detected are generated in response to T-independent antigens of *B. pseudomallei* by B cells in the MNL suspension. In contrast, no detectable levels of antibody were observed in recipients of purified T cells. However, the significance of this finding is questionable since IgG_{2a} and IgG₁ was also negative in serum from PBS only mice. Also, since these mice had succumb to infection by day 3, serum was collected at day 2 post-challenge which is likely to have been too early in the immune response to detect antibody production.

Previous studies suggest that a DTH response occurs independently of a protective immune response (Pais *et al.* 1998). Investigations of infection with mycobacteria indicate that recognition of the mycobacterial antigens by the hosts' immune system develops sequentially (Andersen 1997). Secreted mycobacterial antigens are the predominant targets during the initial stages of infection then later, as bacilli are killed by the host immune response structural antigens are exposed and recognised (Andersen 1997). Therefore the outcome of a natural infection depends on the balanced activation and regulation of distinct cell types responding to different classes of antigen. Silva *et al.* (1999) compared the development of protection following vaccination of mice with bacillus Calmette-Guérin (BCG) or a DNA-vaccine based on mycobacterium heat-shock protein (hsp 65). Following BCG-immunisation, antigen-specific CD4⁺ T cells were the predominant cell type and were associated with an increased IL-4, rather than IFN- γ production. Protection was not successfully transferred using these cells. In contrast, immunisation with hsp 65 DNA vaccine resulted in the generation of activated, high-IFN- γ producing CD8⁺ T cells. The cytotoxic CD8⁺ T cells were able to transfer protection to naïve mice (Silva *et al.* 1999).

The strength of antigen-specific IFN- γ producing T cells is also important for protection from *Yersinia enterocolitica* infection (Autenrieth *et al.* 1994). Similarly, early observations by Mielke *et al.* (1988) suggested that during recall of established immunity to *L. monocytogenes* a DTH response is entirely dependent on CD4⁺ T cells. However, CD8⁺ T cells, with cooperation from CD4⁺ T cells, are ultimately responsible for bacterial clearance (Mielke, Ehlers & Hahn 1988; Goossens, Marchal & Milon 1992). This is supported by the high ratio of CD8⁺ to CD4⁺ T cells at sites of *L. monocytogenes* infection (Goossens, Jouin & Milon 1991). Such observations may partly explain the failure to transfer protection to *B. pseudomallei* in the present study, despite a significant DTH response. Do the BpLy1 antigens recognised in the DTH assay have any relevance to the antigens that are recognised by the T cells responsible for development of protective immunity during *B. pseudomallei* infection? Certainly, IFN- γ production from CD8⁺ and NK cells is known to be important for survival of *B. pseudomallei* infection (Santanirand *et al.* 1999; Lertmemongkolchai *et al.* 2001). However, little is known regarding the predominant cell types that are responsible for providing protection against infection with *B. pseudomallei*.

In summary, the results of the current chapter illustrated that DTH can be transferred to naïve recipients through *B. pseudomallei*-specific MNL. However, the presence of these cells was not sufficient to provide protection against a subsequent challenge. It is possible that the large challenge dose of NCTC 13178 used may have masked a partially protective response in the adoptive transfer mice and this requires investigation by using a lower dose in future adoptive transfer experiments.

The lack of protection observed in the adoptive transfer mice prompted the question as to whether exposure to a single small dose of *B. pseudomallei* via the *iv* route is sufficient to generate a protective immune response in the donor mice. Consequently, alternative immunisation protocols for inducing protection were considered.

CHAPTER 8

GENERATION OF RESISTANCE IN SUSCEPTIBLE BALB/c MICE

8.1 Introduction

CMI responses are essential for the control of infections with intracellular pathogens. The induction of a humoral immune response can lead to a chronic, or progressive and fatal disease since a strong antibody response also down-regulates the CMI response required for elimination of the pathogen (Surcel *et al.* 1994; Power, Wei & Bretscher 1998). Power *et al.* (1998) proposed that APC have the potential to induce both T_H1-type and T_H2-type responses. However, the dose of antigen that is encountered by the APC governs the type of response that predominates in a particular situation. For a variety of antigens, the administered dose is crucial for determining the type of immunity that is induced. For example, low doses of mycobacterial antigen favour a cell-mediated, T_H1-type response while higher doses favour mixed T_H1/T_H2 responses (Power, Wei & Bretscher 1998). Similarly, a decline in CMI responses corresponding with upregulated antibody production is associated with infection with increasing numbers of *L. major* (Menon & Bretscher 1996).

The results outlined in the previous chapter illustrated that DTH can be transferred to naïve recipients through *B. pseudomallei*-specific MNL. However, the presence of these cells was not sufficient to provide protection against a subsequent challenge. This supports previous intracellular bacterial studies that suggest DTH and a protective, adaptive immune response occur independently (Mitsuyama, Nomoto & Takeya 1982; Orme 1988; Tsukada *et al.* 1991). It appears that exposure to a single small dose of *B. pseudomallei* via the *iv* route is insufficient to generate a protective host immune response. Therefore, alternative methods of immunisation were attempted in the present study.

CMI rather than humoral responses are important for protection against *L. major*. BALB/c mice are innately susceptible to, and produce antibodies following infection with the intracellular parasite, *L. major*. Bretscher *et al.* (1992) demonstrated that repeated *sc* injection of low numbers of *L. major* resulted in “low zone immune deviation” where the antibody response of mice is decreased, compared to control mice, in response to subsequent lethal challenge with *L. major*. Decreased antibody levels correlated with an increased DTH response and host resistance to infection. The purpose of the current study was to increase the resistance of BALB/c mice to *B. pseudomallei* by exposing them to repetitive low-doses of the bacterium by employing the immunisation strategy of Bretscher *et al.* (1992).

Studies of adaptive immunity to several intracellular pathogens, such as *M. tuberculosis* and *L. monocytogenes*, have demonstrated that a protective immune response is generated only in the presence of the living organism, since it is predominantly mediated by secreted antigens (Mitsyamo, Nomoto & Takeya 1982; Koga *et al.* 1987; Orme 1988). While immunisation with heat-killed *M. tuberculosis* cells generates strong DTH responses to PPD, it does not result in specific resistance to subsequent infection (Orme 1988). In contrast, DTH is lower following immunisation with live *M. tuberculosis* but protective T cells are generated. Similarly, infection with *L. monocytogenes* leads to the development of both DTH and adaptive CMI responses that are protective. Only DTH is expressed following immunisation with heat-killed *L. monocytogenes* (Mitsyamo, Nomoto & Takeya 1982).

Therefore, the specific aims for the work described in this chapter were:

1. To identify a suitable dose of *B. pseudomallei* NCTC 13179 to induce resistance toward *B. pseudomallei* in BALB/c mice
2. To generate resistance to *B. pseudomallei* in BALB/c mice using repetitive low-dose immunisation with live *B. pseudomallei*
3. To compare the resistance generated in BALB/c mice following immunisation with live *B. pseudomallei* versus *B. pseudomallei* antigens (BpLy1)

8.2 Materials and Methods

8.2.1 Determination of effective immunisation dose

A preliminary experiment was performed to identify a suitable dose of *B. pseudomallei* to be used as an immunising dose for subsequent low-dose resistance experiments.

BALB/c mice were divided into 8 groups of 25 mice per group and injected *sc* with live *B. pseudomallei* NCTC 13179. Table 8.1 below illustrates the infective dose received by each group.

Table 8.1 Doses of NCTC 13179 used to *sc* infect BALB/c mice.

	Group No.							
	1	2	3	4	5	6	7	8
Infective dose (cfu/mouse)	Control (PBS)	60	290	590	2350	3500	5860	58600

Appropriate numbers of *B. pseudomallei* were suspended in PBS and 40 µl was injected *sc* into the left hind footpad of each mouse. Footpad swelling was measured at 24 hr intervals from day 0 to day 10 post-infection. At day 1, 3, 5, 10 and 20 post-infection, n=3 mice per group were euthanased. Spleen weights and bacterial loads in spleen were measured at each time point. Mortality data was recorded for 10 days following infection.

8.2.2 Immunisation schedule and lethal challenge

In an attempt to induce protection in BALB/c mice, the immunising dose of live *B. pseudomallei* identified in the previous experiment was used. To compare the effect of live versus sonicated *B. pseudomallei* antigens, a second group of mice were included that were *sc* immunised with BpLy1. These mice received immunising doses of 5 µg/40 µl of BpLy1. Control mice that received PBS only were also included.

At day 0, mice were immunised with live NCTC 13179, BpLy1 or PBS. Appropriate boosters were then given at day 10 and day 30. At day 45 after primary immunisation, each treatment group was divided into two. Half were used to assess either DTH or lymphocyte proliferation in response to BpLy1 using protocols described previously. The remaining mice were challenged *iv* with 4 x LD₅₀ (~40 cfu) of highly virulent *B. pseudomallei* NCTC 13178. Following challenge, mice were assessed for mortality, bacterial loads in spleen (Chapter 3) and antibody levels in serum (Chapter 7.2.6).

8.2.3 DTH response

The DTH response of low-dose resistance BALB/c mice to BpLy1 was determined using methods outlined in Chapter 6.2.2. Briefly, 5 µg of BpLy1 in 40 µl of PBS was injected *sc* into the left hind footpad and 40 µl of PBS into the right hind footpad. Footpad thickness was measured at 0 and 48 hrs post-challenge and results were expressed as change in footpad thickness (mm) ± SEM. Differences in footpad swelling were compared with one-way ANOVA.

8.2.4 Lymphocyte proliferation assays

MNL were isolated from spleen using methods described in Chapter 3. MNL were resuspended in culture medium (Appendix 1) and dispensed into 96-well round-bottom plates at 1 x 10⁵ cells/well. Cell cultures were stimulated with BpLy1 (1 µg/ml), ConA (2 µg/ml; Sigma) or left unstimulated as negative controls. All tests were performed in triplicate. Lymphocyte proliferation was measured using [³H]-thymidine incorporation at 24 hr intervals between 96 and 168 hrs (Amersham-Pharmacia Biotech; 1.25 µCi/mL for 4 hrs). Results were expressed as mean maximum SI ± SEM and the response of *B. pseudomallei*-specific lymphocytes was compared with controls by one-way ANOVA.

8.3 Results

8.3.1 Immunisation dose

The results of footpad swelling, spleen indices, bacterial loads in spleen and mortality data for the various infective doses inoculated into BALB/c mice are given in Appendix 3 (Figure A3.1, A3.2, A3.3 and A3.4, respectively). An immunising dose of 290 cfu

(Group 3) was selected for use in subsequent low-dose immunisation experiments. Subcutaneous inoculation of this dose resulted in significant footpad swelling at 48 hrs post-immunisation (Figure 8.1a). Significant increases were also observed in spleen weight (Figure 8.1b) and bacterial loads (Figure 8.1c) by day 5 in Group 3 mice. However, bacteria were cleared to undetectable levels by day 20 post-infection, correlating with a decrease in spleen weight (Figure 8.1c and 8.1b, respectively). A mortality rate of 11.1% was recorded for BALB/c mice infected with 290 cfu of NCTC 13179 (Figure A3.4).

8.3.2 DTH

There was a significant DTH response to BpLy1 at 48 hrs in BALB/c mice immunised with live *B. pseudomallei*, compared to PBS control mice (Figure 8.2). Although increased swelling was also observed in BpLy1-immunised mice, this response was not significantly greater than controls. Similarly, the difference in DTH response between mice immunised with live *B. pseudomallei* or with BpLy1 was not significant (Figure 8.2).

8.3.3 Lymphocyte proliferation

Compared to controls, significant lymphocyte proliferation in response to BpLy1 was observed in cultures derived from *B. pseudomallei*-immunised and BpLy1-immunised BALB/c mice (Figure 8.3; $P < 0.05$ and $P < 0.05$, respectively). However, the difference in proliferation of lymphocytes from *B. pseudomallei*-immunised versus BpLy1-immunised mice was not significant.

8.3.4 Host survival and bacterial loads

The mortality rates resulting from a lethal challenge with highly virulent *B. pseudomallei* NCTC 13178 are illustrated in Figure 8.4. There was 80% mortality in PBS control mice by day 3 post-infection, which remained constant until completion of the study at day 10.

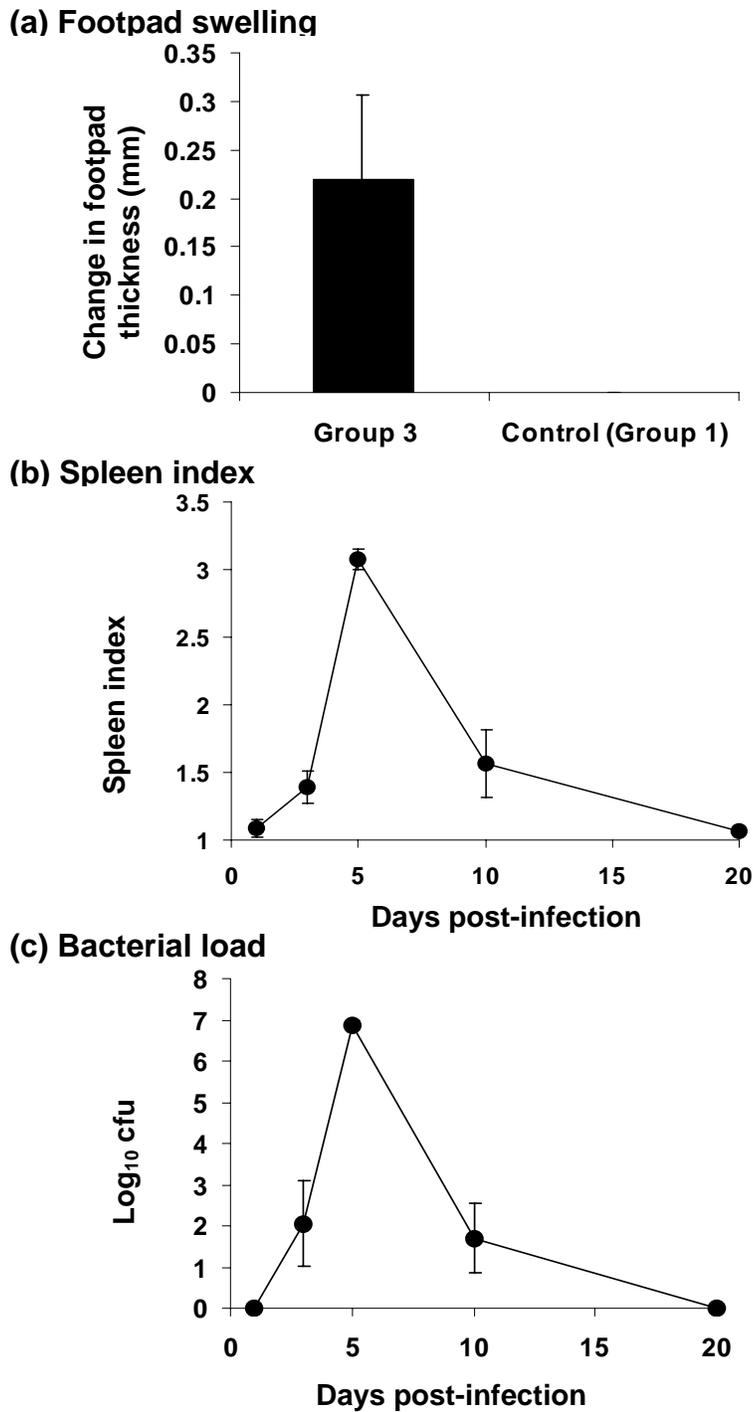


Figure 8.1 Identification of a suitable immunising dose for BALB/c mice as assessed by (a) footpad swelling, (b) spleen index and (c) bacterial loads in spleen.

BALB/c mice were *sc* inoculated with various doses of *B. pseudomallei* NCTC 13179 to identify a dose that could be used for subsequent immunisation experiments. Group 3 mice, which received 290 cfu, were selected based on a significant footpad swelling at 48 hrs post-infection and a significant increase in spleen weight and bacterial load by day 5, that was subsequently cleared by day 20 post-infection. Results are expressed as mean response \pm SEM.

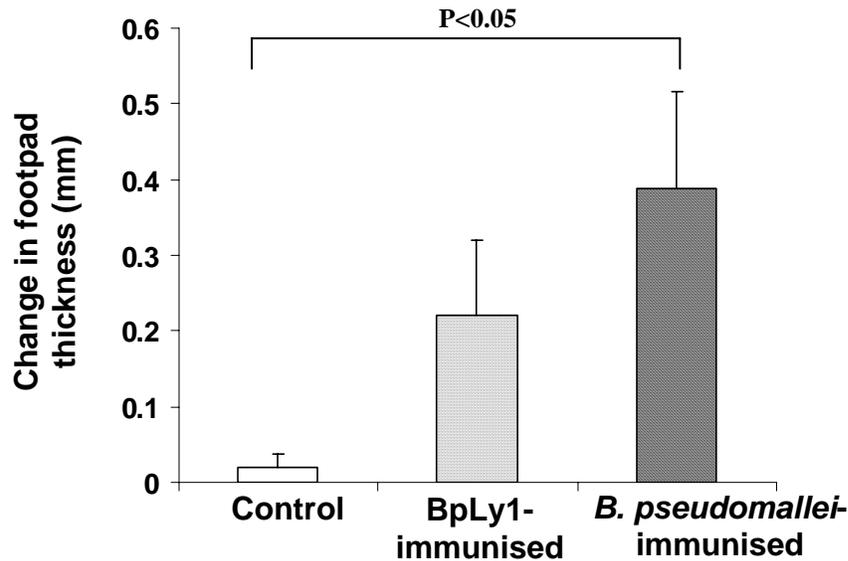


Figure 8.2 DTH response to BpLy1 in low-dose immunised BALB/c mice.

BALB/c mice were immunised *sc* with three low-doses of live *B. pseudomallei* (3 x 290 cfu) or BpLy1 (3 x 5 µg) at day 0, 10 and 30. Control mice received repetitive doses of PBS. At day 45 after initial immunisation, DTH responses were assessed by *sc* injection of BpLy1 into the left hind footpad and PBS into the right hind footpad (n=5). Footpad thickness was measured at 0 and 48 hrs after footpad challenge. Results are expressed as the change in footpad thickness (mm) ± SEM. Compared to controls, significantly greater swelling was observed in mice immunised with live *B. pseudomallei* (P<0.05). Differences in footpad swelling between *B. pseudomallei*-immunised and BpLy1-immunised mice were not significant (P>0.05).

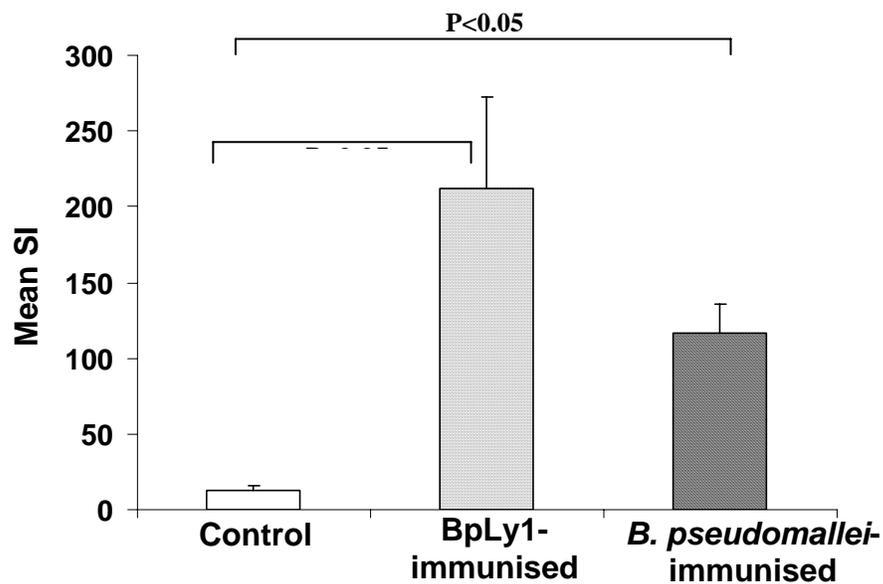


Figure 8.3 Lymphocyte proliferation in response to BpLy1 in cultures from low-dose immunised BALB/c mice.

Splenic MNL derived from *B. pseudomallei*-immunised, BpLy1-immunised or control mice were cultured in the presence of BpLy1. Proliferation was measured at 96 to 168 hrs of culture using [³H]-thymidine incorporation. Results are expressed as the mean maximum SI ± SEM. Compared to controls, proliferation was significantly higher in MNL cultures derived from mice immunised with live *B. pseudomallei* or BpLy1.

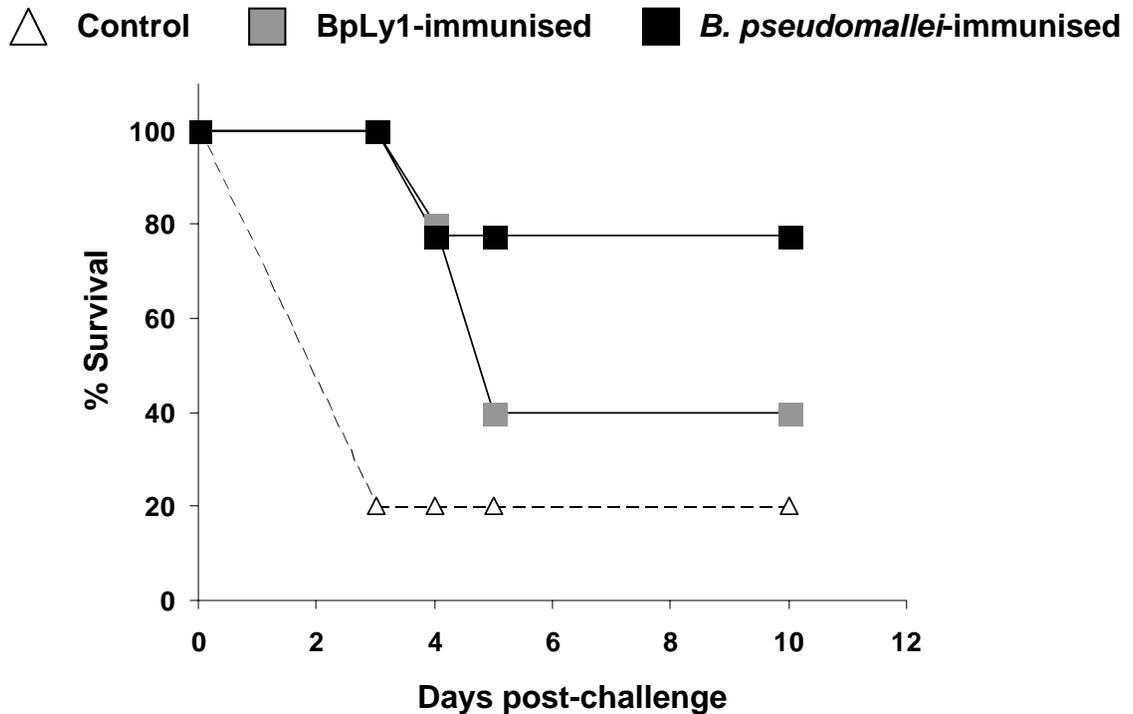
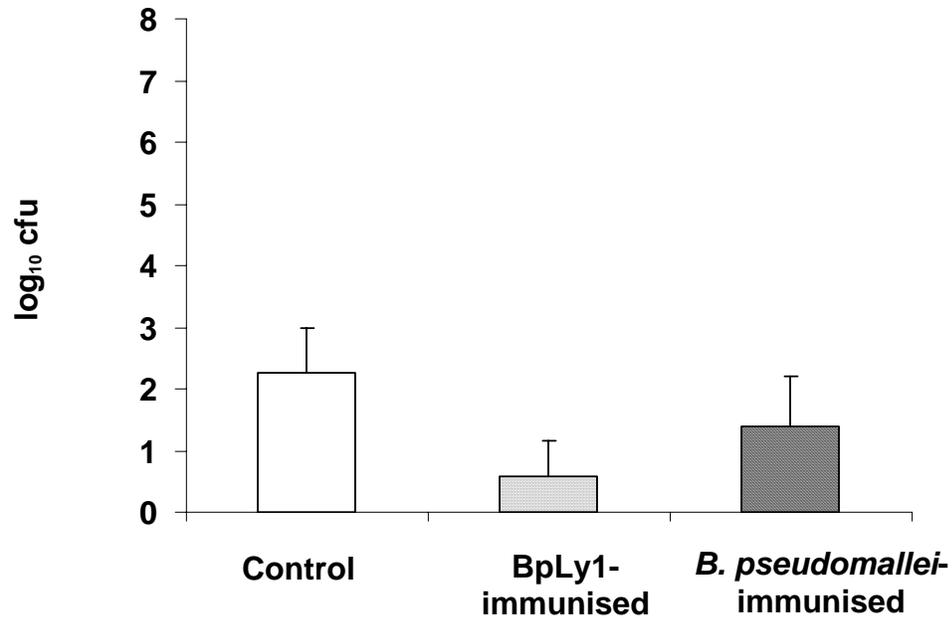


Figure 8.4 Survival rate of low-dose immunised BALB/c mice following challenge with NCTC 13178.

BALB/c mice were immunised *sc* with three low-doses of live *B. pseudomallei* (3 x 290 cfu) or BpLy1 (3 x 5 µg) at day 0, 10 and 30. Control mice received repetitive doses of PBS. At day 45 after initial immunisation, mice were challenged with a lethal dose of NCTC 13178 and monitored for survival over 10 days. Compared to PBS controls, fewer deaths occurred in immunised mice in the first three days of infection. However, by day 10, survival of BpLy1-immunised mice fell to 40%. In contrast, 80% of *B. pseudomallei*-immunised mice survived challenge with NCTC 13178 (P<0.01).

Compared to controls, no deaths were recorded in the immunised groups in the first three days of infection. However, by day 5 post-infection the survival rate of BpLy1-immunised mice had fallen to 40% (Figure 8.4). Mice immunised with live *B. pseudomallei* demonstrated the greatest resistance to challenge with NCTC 13178 with 80% survival during the 10-day experimental period. Bacterial loads in spleen were measured at day 1 and 3 post-challenge. No significant differences were observed between treatment groups at day 1 post-challenge (Figure 8.5a). Compared to controls, significantly fewer bacteria were present at day 3 post-challenge in spleen of *B. pseudomallei*-immunised mice following lethal challenge (Figure 8.5b).

(a) day 1 post-challenge



(b) day 3 post-challenge

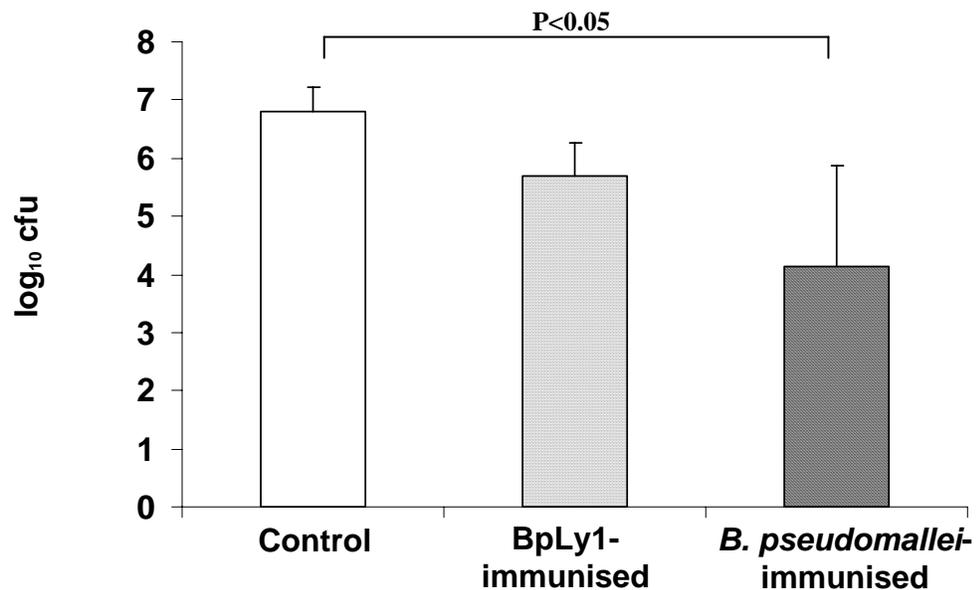
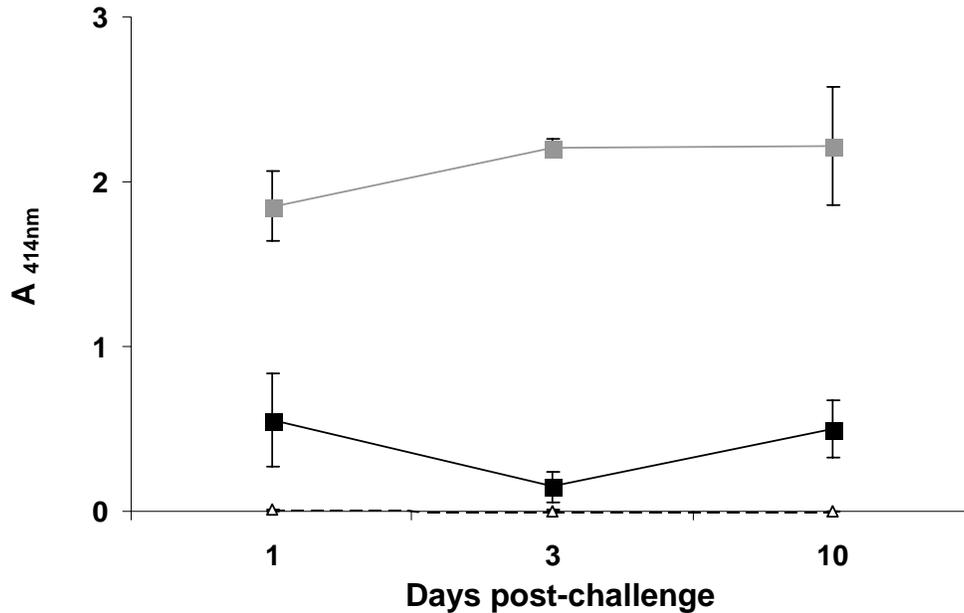


Figure 8.5 Bacterial loads at (a) day 1 and (b) day 3 post-infection in spleen of low-dose immunised BALB/c mice challenged with NCTC 13178.

BALB/c mice were immunised *sc* with three low-doses of live *B. pseudomallei* (3 x 290 cfu) or BpLy1 (3 x 5 µg) at day 0, 10 and 30. Control mice received repetitive doses of PBS. At day 45 after initial immunisation, mice were challenged with a lethal dose of NCTC 13178 and n=5 mice were euthanased at days 1 and 3 to determine bacterial loads in spleen. At day 1 post-challenge bacterial loads were comparable in all treatment groups. In contrast, at day 3 post-challenge bacterial loads in spleen were significantly less in *B. pseudomallei*-immunised mice compared to control mice (P<0.05). Results are expressed as mean bacterial load ± SEM.

△ Control ■ BpLy1-immunised ■ *B. pseudomallei*-immunised

(a) IgG_{2a}



(b) IgG₁

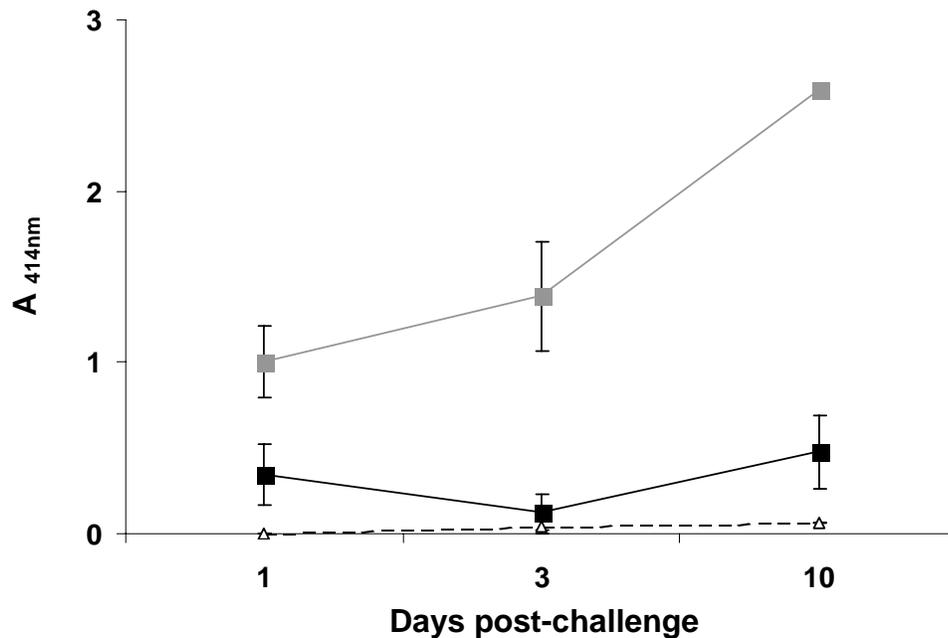


Figure 8.6 (a) IgG_{2a} and (b) IgG₁ levels in serum from low-dose immunised BALB/c mice following challenge with virulent NCTC 13178.

BALB/c mice were immunised *sc* with three low-doses of live *B. pseudomallei* (3 x 290 cfu) or BpLy1 (3 x 5 µg) at day 0, 10 and 30. Control mice received repetitive doses of PBS. At day 45 after initial immunisation, mice were challenged with a lethal dose of NCTC 13178 and serum was collected (n=5) at day 1, 3 and 10 post-challenge. IgG_{2a} and IgG₁ levels were significantly increased in BpLy1-immunised mice compared to *B. pseudomallei*-immunised and control mice (P<0.05 and P<0.05, respectively). Antibody levels in *B. pseudomallei*-immunised mice were not significantly greater than control mice (P>0.05). Results are expressed as mean A_{414nm} ± SEM.

8.3.5 IgG₁ and IgG_{2a} levels

Levels of IgG_{2a} and IgG₁ in serum at day 1, 3 and 10 post-challenge are illustrated in Figure 8.6a and 8.6b, respectively. Significant increases in both IgG_{2a} and IgG₁ were measured in serum from BpLy1-immunised mice compared to *B. pseudomallei*-immunised and control mice ($P < 0.05$ and $P < 0.05$, respectively). Slight increases in both antibody isotypes were also observed for *B. pseudomallei*-immunised mice. However, these levels were not significantly greater than levels in control mice.

8.4 Discussion

Investigations into the generation of resistance to infection with intracellular pathogens suggest that the development of DTH and protection may reflect functionally different immune responses (Tsukuda *et al.* 1991; Goossens, Marchal & Milon 1992; Pais *et al.* 1998; Batoni *et al.* 2000). Bacteria are composed of a multitude of antigenic molecules. The overall immune response to a bacterium that is observed is a combination of specific immune responses targeting each bacterial antigen, and each of these are dependent on a variety of factors, including the type of antigen, its concentration and other characteristics. Therefore, to induce protection against intracellular infection it is necessary to promote a T_H1-type or CMI response against the majority of bacterial immunogenic components. This has been achieved for *L. major* using a repetitive low-dose immunisation strategy (Bretscher *et al.* 1992; Menon & Bretscher 1996). Such a strategy was employed in the current study in an attempt to induce resistance to *B. pseudomallei* in innately susceptible BALB/c mice.

Compared to BpLy1-immunised mice, BALB/c mice immunised with live *B. pseudomallei* had a significantly greater DTH response to BpLy1 and increased survival to challenge with a highly virulent strain of *B. pseudomallei*. Bacterial loads in spleen were also significantly lower in mice immunised with live *B. pseudomallei*.

Therefore, repetitive low-dose immunisation with live *B. pseudomallei* only, induced partial protection from subsequent lethal challenge.

Several studies report that protection against intracellular infection is generated only in the presence of a live pathogen, while non-living preparations induce non-specific resistance and DTH (Nair & Kamat 1982; Goossens, Marchal & Milon 1992). For example, vaccination with live *Salmonella* induces both a DTH response and activation of host macrophages. However, neither response was observed using a formalinised *Salmonella* vaccine (Nair & Kamat 1982). Similarly, a comparison of live and sonicated *Listeria* preparations by Goossens *et al.* (1992) demonstrated that only viable cells are able to generate resistance to the bacterium in mice. This may reflect additional secreted bacterial antigens that would be absent from sonicated preparations, as well as the processing pathway followed by different antigens within APC. Most microbes are phagocytosed and processed by professional APC by two routes called class I and class II pathways, and presented on the cell surface in lymphoid tissues. Presentation of antigen by class I and class II pathways activate CD8⁺ and CD4⁺ T lymphocytes, respectively (Abbas, Lichtman & Pober 2000). Extracellular bacteria and soluble bacterial products are phagocytosed by APC and processed into various peptides via an endosomal/lysosomal pathway. These peptides are loaded onto MHC class II molecules within vesicles and carried to the surface where they are presented to specific CD4⁺ T cells (Abbas, Lichtman & Pober 2000). In contrast, some intracellular bacteria such as *L. monocytogenes* can escape the phagosome and multiply inside the cytosol of the APC (Goebel & Kuhn 2000). Cytosolic proteins are processed and loaded onto MHC class I molecules in the ER. MHC class I molecules are transported directly to the surface of the APC and displays antigen to specific CD8⁺ T cells (Abbas, Lichtman & Pober 2000). Therefore, different components of the immune system are activated depending on the type of antigen and the pathway by which it is processed.

Serum antibody levels were significantly greater in BpLy1-immunised mice. In the proliferation assay, since MNL cultures were stimulated with BpLy1 *in vitro*, the increased proliferation may reflect the presence of a greater number of BpLy1-specific lymphocytes in mice immunised with this preparation. Lymphocytes from

B. pseudomallei-immunised mice would target structural antigens found in BpLy1, as well as secreted antigens produced by viable *B. pseudomallei*.

T cells are pivotal for protection against infection with intracellular pathogens such as *M. tuberculosis*, *L. monocytogenes* and *L. major* and various T cell subsets are involved in this response (Liew, Hale & Howard 1982; Tsukada *et al.* 1991; Batoni *et al.* 2000). Batoni *et al.* (2000) demonstrated that different mycobacterial antigens induce the proliferation of particular T cell types. For example, membrane and secreted mycobacterial antigens predominantly generate CD4⁺ T cells that mediate protection to subsequent infection. In contrast, cytosolic and cell-wall antigens stimulate a NK and $\gamma\delta^+$ T cell response that is non-protective to the host (Batoni *et al.* 2000). However, the authors suggested that for a successful host immune response it is necessary to have the 'correct' combination of activation and regulation of the various T cell types responding to the various antigens (Batoni *et al.* 2000). During an active infection with *L. monocytogenes*, both DTH and protective immunity occur with similar kinetics (Mitsuyama, Nomoto & Takeya 1982). When mice are injected with killed *L. monocytogenes*, only a DTH response is produced and is associated with the generation of CD4⁺ T cells (Koga *et al.* 1987; Goossens, Marchal & Milon 1992). In contrast, both CD4⁺ and CD8⁺ T cells mediate protective immunity to *L. monocytogenes* (Goossens, Marchal & Milon 1992). Also, CD4⁺ T cells that are associated with protection produce high levels of IFN- γ while DTH-mediating CD4⁺ T cells do not (Tsukada *et al.* 1991). The authors proposed that protective T cell clones may be phenotypically different from DTH T cell clones or, alternatively may represent a more mature stage of differentiation of the same cell type that is associated with an IFN- γ secreting ability (Tsukada *et al.* 1991).

Although a slight increase in footpad swelling was observed in BpLy1-immunised mice this did not differ significantly from control mice and may reflect a shift to a T_H2-type response. The high antibody levels detected in serum from BpLy1-immunised mice after challenge support such a shift. However, cytokine studies are necessary to provide evidence of shifts in T_H1/T_H2-type responses following the generation of resistance by low-dose exposure.

Following immunisation, it is possible that the replication of live bacteria within the host will contribute to the generation of a greater number of T cell clones which may perhaps account for the greater efficacy of the live bacteria in producing a protective immune response. It is also feasible that the failure of BpLy1 immunisation to induce resistance to the level that was achieved by immunisation with live *B. pseudomallei* reflects an important role for secreted bacterial antigens in driving a protective immune response toward *B. pseudomallei*. Regardless, it will be necessary to use live bacteria to comprehensively investigate *in vitro* and *in vivo* characteristics of the protective immune response toward *B. pseudomallei* infection. Repetitive exposure to low doses of *B. pseudomallei* will be a useful method for investigating the role of various cellular and molecular components involved in generating protection towards this intracellular bacterium.

CHAPTER 9

A ROLE FOR T CELLS IN SURVIVAL OF PATIENTS WITH *B. pseudomallei* INFECTION

5.3 Introduction

Both innate and acquired immune responses are comprised of a humoral and a cellular arm. The humoral response culminates in the generation of antibodies for a given antigen. In contrast, cellular immunity can only be achieved through the involvement of cells and depends on antigen-specific T lymphocytes. Infectious agents are able to persist in the host if they are capable of resisting or avoiding host immune defenses. There are many mechanisms through which they achieve this including toxin production, antigenic variation and the ability to survive and replicate within host cells. *L. monocytogenes* is an example of an intracellular bacterium that grows within host macrophages allowing the bacterium to avoid the innate responses by these cells (Abbas, Lichtman & Pober 2000). Other examples of facultative intracellular bacteria include *Salmonella* spp., *Francisella tularensis*, *M. tuberculosis* and *L. pneumophila* (Schaible *et al.* 1999).

Intracellular pathogens must either be prevented from invading host cells, or detected within them and eliminated. Control of diseases caused by intracellular bacteria can only be achieved with a CMI response governed by T_H1-type cells. The majority of intracellular bacteria induce the production of IL-12 and IFN- γ from APC's as well as IFN- γ from NK and CD8⁺ T cells, subsequently driving the development of a T_H1-type response (Romagnani 1997). T_H2-type cells are predominantly involved in stimulating B lymphocytes and produce anti-inflammatory cytokines such as IL-4 and IL-10, which exacerbate intracellular infection (Kidd 2003). Based on electron microscopy, *B. pseudomallei* is classified as a facultative intracellular pathogen (Vorachit *et al.* 1995; Jones *et al.* 1996; Hoppe *et al.* 1999). This has important implications for the immune response toward *B. pseudomallei*. One method that has been used to assess the cellular

immune function in melioidosis patients is the measurement of neopterin in urine. Neopterin is a metabolite released by macrophages that become activated by IFN- γ , and its level in urine is thought to reflect the activation of the cellular immune system (Brown *et al.* 1990). While urinary neopterin levels are elevated in all melioidosis patients, significant rises are evident in patients with septicaemic melioidosis. Brown *et al.* (1990) propose that septicemia is preceded by an innate immune response that fails to inhibit bacterial multiplication, therefore allowing the dissemination of *B. pseudomallei* and the initiation of a secondary, non-specific T cell-macrophage activation. It is this secondary response that is reflected in the elevated urinary neopterin levels. However, there is currently no direct evidence in the literature to suggest the development of an adaptive CMI immune response in *B. pseudomallei* infection. In Chapter 6, we demonstrated the development of specific T cell responses in C57BL/6 and BALB/c mice following *B. pseudomallei* infection. Therefore, it was hypothesised that patients surviving melioidosis should develop an adaptive CMI response that would have afforded protection against uncontrolled infection.

For this study, PBML from patients who have recovered from melioidosis were used.

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

- 1 To determine the proliferative response of PBML toward *B. pseudomallei* antigens, *in vitro*
- 2 To determine the production of IFN- γ and IL-10 in response to *B. pseudomallei* antigens
- 3 To determine the activation of CD4⁺ and CD8⁺ T cells following stimulation with *B. pseudomallei* antigens
- 4 To determine correlations between antibody titre, clinical presentation, or time since diagnosis and the strength of a CMI response to *B. pseudomallei* antigens

9.2 Materials and Methods

9.2.1 Subjects

The thirteen patients who volunteered for this study had a clinical presentation consistent with melioidosis and a diagnosis confirmed by isolation of

B. pseudomallei from blood or tissues (Table 9.1). The mean age of the patients was 58 years (range, 31-74 years). Eight of the patients were men, and five were women. The cellular assays were performed with blood samples from these patients, all of who recovered from melioidosis and had no clinical evidence of the disease at the time when the assays were carried out. The mean time after diagnosis of melioidosis in patients was 23 months. Ten control subjects, five men and five women, from the same geographic region were also included in this study. They had no clinical history of melioidosis and were serologically negative for antibodies to *B. pseudomallei*. The mean age of the control subjects was 51 years (range, 38-62 years). Studies were carried out under the approval of the Townsville Health Service District Ethical Committee (68/98).

9.2.2 Antibody titres

A semiquantitative IHA was used to determine serum antibody titres to *B. pseudomallei* antigens according to the method of Ashdown *et al.* (1987). Briefly, antigen was prepared from the supernatants of heat-killed cultures of five *B. pseudomallei* strains. Serum specimens were incubated at 56°C for 30 min and then adsorbed with saline-washed non-sensitized sheep erythrocytes at RT for 15 min before testing. Two-fold dilutions of test serum were performed in isotonic saline (1:5 – 1:5120), and each dilution was incubated with the sensitized ovine erythrocytes for 2 hr at RT. The highest dilution in which hemagglutination occurred was recorded as the end-point titre. Those with a positive reaction (titre \geq 1:40) were confirmed by ELISA for *B. pseudomallei*-specific IgM and IgG (Ashdown *et al.* 1989).

9.2.3 Lymphocyte proliferation assays

Proliferation assays were performed to determine lymphocyte responses to BpLy1 (Chapter 3), a cocktail of *B. pseudomallei* antigens. Each proliferation assay included samples from up to 3 patients and 2 control subjects. PBML separated from heparinised blood were cultured in 96-well plates (10^5 PBML/well) in culture medium (Appendix 1) supplemented with pooled human serum (10% v/v) and antibiotics. Triplicate wells with cells were stimulated with either 1 μ g/mL BpLy1, 15 μ g/mL purified protein derivative (PPD; CSL Biosciences) or left unstimulated. Culture plates were incubated at 37°C in 5% CO₂. Proliferation of cells was determined at 24 h intervals on days 4 to 7 of culture

(4 time points) by measuring [³H]-thymidine incorporation (Amersham-Pharmacia Biotech; 1.25 µCi/mL for 4 hrs). Results were expressed as counts per minute (cpm) or as stimulation index (SI), the proportion of counts per minute in stimulated cultures compared with that in unstimulated culture. The maximum SI or counts per minute out of the 4 time points in individual proliferation assays was compared between patients and control subjects.

9.2.4 Determination of cytokine levels by ELISA

Cell culture supernatant collected on days 2 and 6 of culture from parallel experiments (as described in 9.2.3) were assayed for IFN-γ and IL-10 using capture ELISA techniques. Paired anti-IFN-γ (clones NIB42 and 4S-B3; PharMingen) or anti-IL-10 (clones JES3-19F1 and JES3-12G8; PharMingen) antibodies were used according to the manufacturer's instruction.

9.2.5 Fluorescent-activated cell scanning (FACS) analysis of T cell subsets

Lymphocytes cultured in the presence of BpLy1 for 2 or 6 days were phenotyped, using isotype-matched controls and the following combinations of monoclonal antibodies (1 µg each; Pharmingen): anti-CD3 (Cy-Chrome, HIT3a), anti-CD45 (fluorescein isothiocyanate [FITC], HI30), anti-CD14 (phycoerythrin [PE], M5E2), anti-CD4 (FITC, PRAT4), anti-CD8 (PE, HIT8a), or anti-CD69 (Cy-Chrome, FN50). Cells were analysed using FACScan™ with CellQuest software (BD Immunocytometry Systems, California, USA). The increase in CD4⁺CD69⁺ and CD8⁺CD69⁺ expression between stimulated and unstimulated cells was considered to reflect activation of CD4⁺ and CD8⁺ cells following *in vitro* stimulation.

9.2.6 Statistical analysis

Maximal lymphocyte proliferation between 4 and 7 days of culture (maximum SI and counts per minute from 4 time points) for each individual in the patient and control groups and the production of cytokines were analysed by univariate analysis of variance, using SPSS statistical software (version 8). Dependent variables were tested for normality with a Q-Q plot and were transformed where necessary. Differences in CD4⁺CD69⁺ and CD8⁺CD69⁺ expression in cultured cells from patients and from

control subjects were assessed by Students's *t* Test. Correlation between sets of data was assessed by Pearson's correlation and was considered to be significant if the probability of a type I error was <0.05% ($P<0.05$). Mean values were expressed as mean \pm SEM.

9.3 Results

9.3.1 Serum antibody levels

Antibody titres at the time of the study ranged from 1:5 to 1:5120 (Table 9.1). However, there was no correlation ($P>0.05$) between antibody titre (Table 9.1) and cell proliferation or cytokine production. There was also no correlation between the time since diagnosis and proliferation of lymphocytes in response to BpLy1. No differences in the parameters assessed were observed for patients who initially presented with septicaemic melioidosis versus those patients with non-septicaemic melioidosis.

9.3.2 Lymphocyte proliferation

The maximum SI's and the maximum counts per minute observed in the lymphocyte proliferation assays in response to BpLy1 were compared for patients and control subjects (Table 9.1). There was a significantly higher proliferation of lymphocytes in cell cultures derived from patients (mean cpm, 2120 ± 604 ; mean SI, 75.0 ± 21.4) than in those derived from control subjects (mean cpm, 311 ± 76 ; mean SI, 11.7 ± 2.1), as assessed by both SI ($P<0.001$; Figure 9.1) and counts per minute ($P<0.001$; Table 9.1). Although high levels of proliferation were observed in patients (mean cpm, 4097.7 ± 1108.5 ; mean SI, 152.7 ± 42.6) and in control subjects (mean cpm, 3610.5 ± 653.2 ; mean SI, 155.6 ± 26.3) in response to PPD stimulation, the difference between the groups was not significant ($P>0.05$).

9.3.3 Cytokine levels in supernatants

IFN- γ production in response to BpLy1 was significantly higher on days 2 ($P<0.001$) and 6 ($P<0.001$; Figure 9.2) in cell cultures derived from patients than in those derived from control subjects. There was a positive correlation between IFN- γ production and

Table 9.1 Demographic and clinical details of patients

Patient ID	Sex^a	Age (years)	Time since diagnosis^b (months)	Organ Involvement	Site of Isolation	Antibody Titre (IHA)^c	Maximum cpm, mean	Maximum SI, mean
1JG	F	74	8	lungs	sputum	1:80	6593	254.6
2ES	F	67	10	lungs, spleen, skin (S) ^d	blood, sputum, skin swab ^e	1:640	4741	207.0
3AK	M	31	9	lungs	sputum	>1:5120	5945	146.0
4EM	F	54	9	lungs, skin	skin swab ^e	NS ^f	2467	67.7
5CM	F	54	8	skin	skin swab ^e	1:320	1197	50.9
6GM	M	33	10	lungs	sputum	1:1280	1001	42.7
7TL	M	64	34	lungs	pleural fluid	1:40	1568	41.2
8MH	F	54	54	lungs, bone, skin (S) ^d	blood, skin swab ^e	NS ^f	833	40.7
9WB	M	58	47	testis	scrotal swab	NS ^f	914	30.5
10WS	M	77	23	skin	skin swab ^e	1:1280	654	28.2
11MS	M	54	5	skin	ulcer swab	NS ^f	813	27.3
12JH	M	61	34	prostrate (S) ^d	blood, urine	1:640	555	24.1
13JP	M	68	45	lungs, prostrate	sputum, urine	NS ^f	278	13.6

NOTE ^aSex - F=female; M=male

^bTime since diagnosis - Time in months indicate the period between 1st diagnosis and when the cellular assays were performed. All the patients included in the study had no clinical evidence of melioidosis at the time when the assays were performed.

^cIHA - indirect hemagglutination ^d(S) - patient with septicemia

^eskin swab - swabs were obtained from abscess which were either surgically drained or discharged spontaneously

^fNS - antibody titre of <1:40 was considered non significant

maximal proliferation of lymphocytes obtained from patients after BpLy1 stimulation at days 2 ($r=0.69$; $P<0.001$) and 6 ($r=0.69$; $P<0.001$). Such a positive correlation was not observed in control subjects. There was no significant difference in IFN- γ production between cell cultures derived from patients (2183 ± 958 pg/mL) and control subjects (1436 ± 971 pg/mL) after stimulation with PPD.

Levels of IL-10 in cell culture supernatants of patients and control subjects were not significantly different at day 6 of culture after stimulation with BpLy1 (Figure 9.3). However, high IL-10 levels were detected in cell culture supernatants derived from both patients (704 ± 163 pg/mL) and control subjects (768 ± 134 pg/mL) in response to BpLy1 stimulation. There was no correlation between IL-10 production and cell proliferation, nor was there a correlation between IL-10 and IFN- γ production.

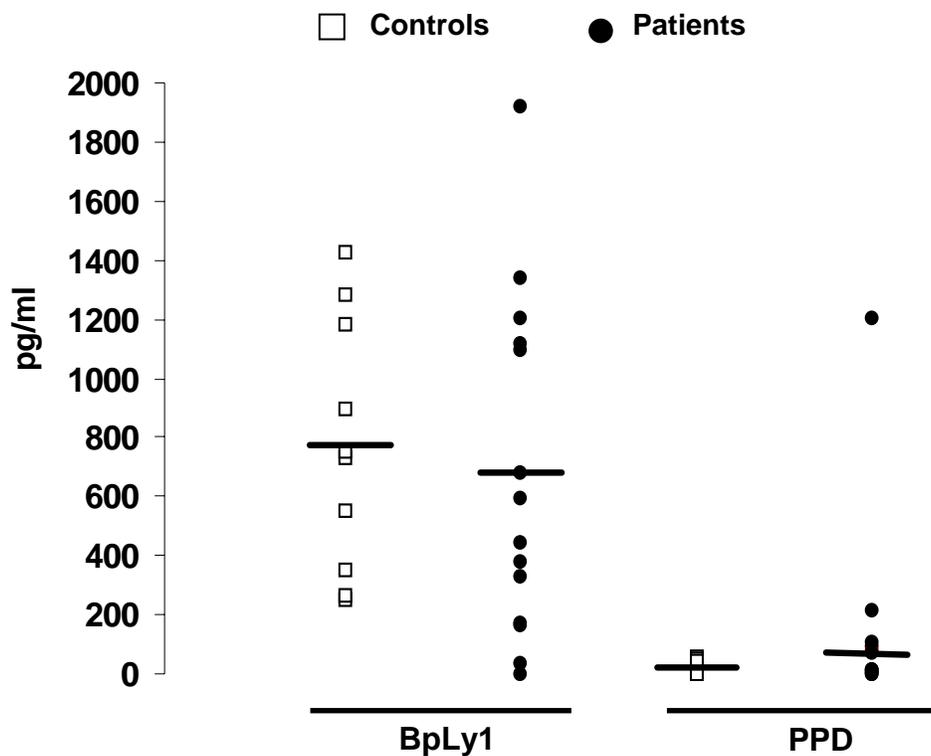


Figure 9.3 IL-10 levels in PBML culture supernatants derived from patients and healthy control subjects in response to BpLy1 and PPD.

Culture supernatants from PBML from healthy control subjects ($n=10$) and melioidosis patients ($n=13$) produced similar amounts of IL-10 at day 6 in response to BpLy1 stimulation ($P>0.05$). Compared to BpLy1 stimulation, decreased IL-10 production was observed for controls and patients following stimulation with PPD ($P>0.05$). Mean values are shown (*horizontal lines*).

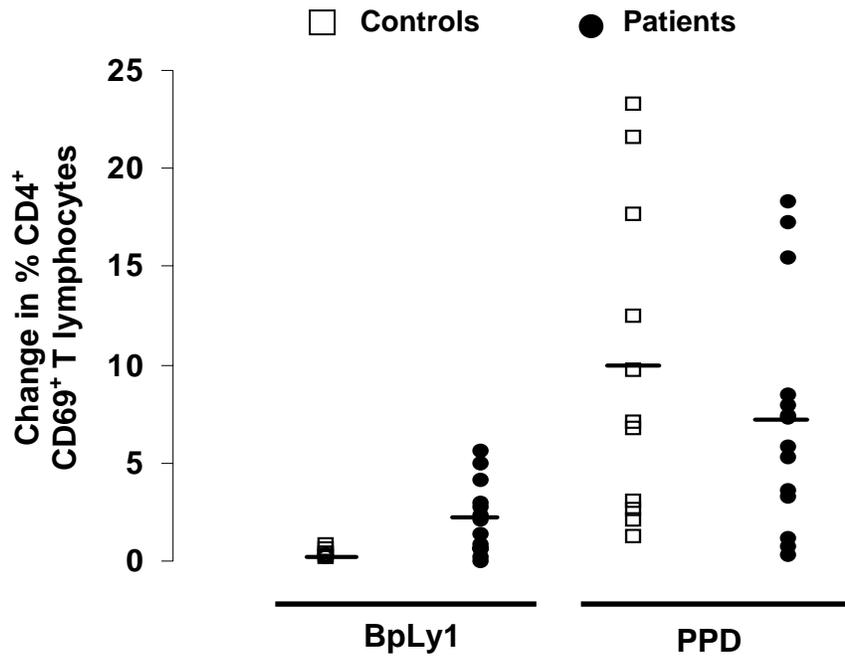


Figure 9.4 Changes in the % of CD4⁺CD69⁺ T lymphocytes at day 6 following stimulation with BpLy1 or PPD.

PBML from healthy controls (n=10) and melioidosis patients (n=13) were cultured in the presence of BpLy1 or PPD for 6 days. The percentage of activated (CD69⁺) CD4⁺ T lymphocytes was higher in cultured cells derived from patients stimulated with BpLy1 than in those derived from controls (P<0.004). Activation of CD4⁺ T lymphocytes from patients and controls was similar following stimulation with PPD (P>0.05). Mean values are shown (*horizontal lines*).

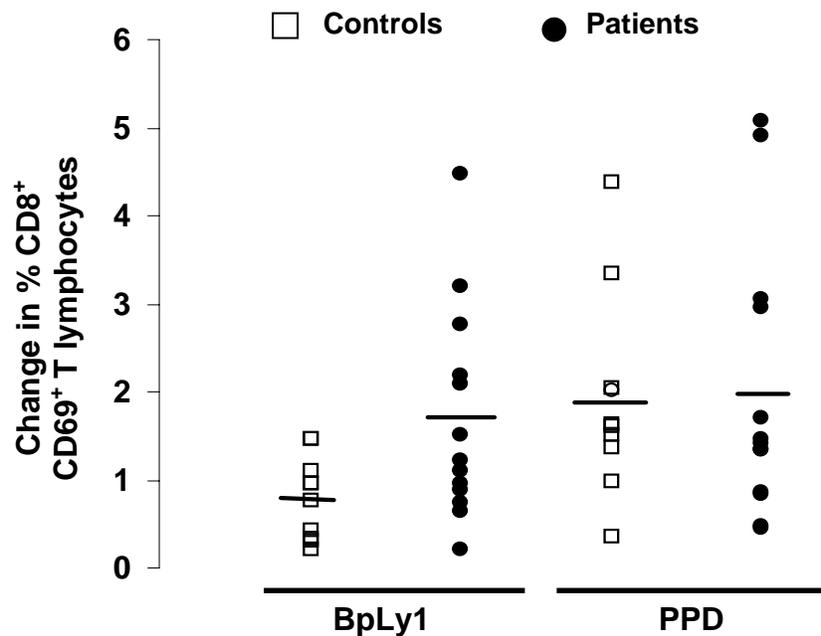


Figure 9.5 Changes in the % of CD8⁺CD69⁺ T lymphocytes at day 6 following stimulation with BpLy1 or PPD.

PBMC from healthy controls (n=10) and melioidosis patients (n=13) were cultured in the presence of BpLy1 or PPD for 6 days. The percentage of activated (CD69⁺) CD8⁺ T lymphocytes was higher in cultured cells derived from patients stimulated with BpLy1 than in those derived from controls (P<0.035). Activation of CD8⁺ T lymphocytes from patients and controls was similar following stimulation with PPD (P>0.05). Mean values are shown (*horizontal lines*).

9.3.4 Activation of T cell subsets

On days 2 and 6, the percentage of activated CD4⁺ (CD4⁺CD69⁺) and CD8⁺ (CD8⁺CD69⁺) T cell populations after BpLy1 stimulation was higher in cultured cells derived from patients than in those from control subjects. At day 6, there was a significant difference (P<0.004) between the percentages of activated CD4⁺ T cells in patient and in control samples (Figure 9.4; 2.09%±0.52% vs. 0.26%±0.12%, respectively). A similar trend was observed between activated CD8⁺ T cells from patient and control samples (Figure 9.5; 1.7%±0.34% vs. 0.80%±0.15%, respectively; P<0.035).

9.4 Discussion

The data presented in this study have demonstrated that compared to controls, lymphocytes from patients who had recovered from melioidosis proliferate *in vitro* in response to *B. pseudomallei* antigens, produce significant amounts of IFN- γ and may involve both activated CD4⁺ T cells and CD8⁺ T cells. To our knowledge this is the first direct demonstration of the possible role of lymphocytes in the development of a cell-mediated adaptive immune response in melioidosis.

A positive IHA test (titre \geq 1:40) is considered as serological evidence of infection with *B. pseudomallei* although it is not indicative of active disease (Ashdown *et al.* 1989). In this study a wide range of antibody titres in the patient group was observed. This is consistent with other studies, which have found the levels of IgG were not predictive of disease outcome suggesting a need for cell-mediated responses in the development of protective immunity (Ho *et al.* 1997).

Assessment of lymphocyte proliferation and production of IFN- γ in PBML cultures following *in vitro* exposure to bacterial antigens provides a measure of antigen-specific lymphocyte responses to recall antigenic challenge. The significantly higher proliferation of lymphocytes and the high levels of IFN- γ in culture supernatants from patients are indicative of the recognition of *B. pseudomallei* antigens by memory T cells.

The patients and controls participating in this study had comparable CMI responses to the antigen PPD as assessed by lymphocyte proliferation and IFN- γ production, indicating they were not immunocompromised.

Resistance to intracellular bacterial infection relies on the rapid production of pro-inflammatory cytokines and an effective CMI response. T_H1-type cells characteristically secrete IL-2 and IFN- γ , whereas T_H2-type cells typically produce IL-4, IL-5 and IL-10. IFN- γ is a potent macrophage activator and a key mediator of CMI responses. If T_H2 – type and not T_H1-type cells respond, CMI responses fail to develop, macrophages are not activated and chronic progression of the disease results (Abbas, Lichtman & Pober 2000). This is best-exemplified in leprosy, the clinical forms of which tend to range between two poles. Patients with tuberculoid leprosy tend to be asymptomatic or have only a mild form of the disease since they have a strong cell-mediated response to the *M. leprae*. In contrast, those patients with lepromatous leprosy who generate high levels of antibody against *M. leprae* but are unable to mount a detectable CMI response, show little resistance to the disease (Romagnani 1997; Schiabile *et al.* 1999; Abbas, Lichtman & Pober 2000). In this form there is a dense infiltration of leprosy bacilli into tissues, which is associated with a poor prognosis. Studies have also demonstrated an important immunoregulatory role for IFN- γ in melioidosis (Brown *et al.* 1991; Santanirand *et al.* 1999). IFN- γ can be beneficial or detrimental, either conferring protection or contributing to the pathology of *B. pseudomallei* infection (Santanirand *et al.* 1999).

IL-10 is a potent inhibitor of IFN- γ and IL-12. It is produced late following endotoxin challenge predominantly by monocyte/macrophages and T cells, supporting the view that it is a natural defense mechanism against excessive inflammatory responses following infection (Opal, Wherry & Grint 1998). High levels of IL-10 in lymphocyte cultures are known to suppress cell proliferation and production of IFN- γ (Plebanski *et al.* 1999). In this study there was no significant difference in the amount of IL-10 in cultures from the two groups. Therefore the inhibitory effects of IL-10 in cell cultures from both groups may have been similar. Although clinical studies have demonstrated an association between elevated levels of IL-10 and other pro-inflammatory mediators

with increased mortality (Suputtamongkol *et al.* 1992; Lauw *et al.* 1999; Lauw *et al.* 2000), the precise role of IL-10 in suppression of immune responses in melioidosis is unclear.

CD69, a protein expressed early on the surface of stimulated T cells, is used as a marker of activation and correlates with the proliferative response of lymphocyte to a specific antigen (Angulo & Fulcher 1998; Marzio, Manuel & Betz-Corradin 1999). In this study, CD69 expression was assessed at both 2 and 6 days post stimulation as it was considered that the detection of a limited number of *B. pseudomallei*-specific T cells in cultures at a very early stage would be technically difficult. Increased CD69 expression can also be partly attributed to the presence of high levels of IFN- γ . In this study we observed an increase in expression of CD69 in both T cell subsets in patients compared to controls indicating a possible role for both CD4⁺ and CD8⁺ T cells in a *B. pseudomallei*-specific immune response. Studies on the host immune response to infections with other intracellular bacteria have also demonstrated a requirement of both T cell subsets. In the host response to *M. tuberculosis*, CD8⁺ T cells undergo phenotypic and functional changes that are similar to CD4⁺ T cells (Feng *et al.* 1999; Serbina & Flynn 2001). Similarly involvement of CD4⁺ and CD8⁺ T cells in response to *L. monocytogenes* infection is supported by large increases in both subpopulations during infection. The results from the current study suggest that future designs of vaccines against *B. pseudomallei* infection should incorporate strategies that activate both CD4⁺ and CD8⁺ T cell subsets.

The significantly higher cell proliferation, IFN- γ production and the activation of T cell subsets observed in the patient group compared to controls upon *in vitro* challenge with *B. pseudomallei* antigens may be explained by antigen-specific memory cell responses. It is tempting to speculate that the patients included in this investigation survived the disease due to the development of an adaptive immune response to *B. pseudomallei*, which was protective. It remains to be determined whether such a response will prevent progression of *B. pseudomallei* infection in individuals exposed to the bacteria.

CHAPTER 10

A ROLE FOR T CELLS IN PREVENTING THE PROGRESSION OF *B. pseudomallei* INFECTION

10.1 Introduction

Melioidosis is regarded as an emerging health problem in certain developing countries, such as Papua New Guinea (PNG; Currie *et al.* 2000c). Newland (1969) and Kingston (1971) reported two cases of latent, reactivated melioidosis that were attributed to service in PNG during World War II more than three decades previously. Since the 1960's several cases of melioidosis have been documented in Port Moresby (Rowlands & Curtis 1965; DeBuse, Henderson & White 1975; Lee & Naraqui 1980; Currie 1993). However, two limited serological studies in PNG failed to detect antibody to *B. pseudomallei*, despite climatic and environmental similarities to neighboring parts of Australia where the seroprevalence is as high as 7.8% in some regions (Rowlands & Curtis 1965; Ashdown & Guard 1984; Barnes *et al.* 1991). Currie *et al.* (2000c) suggest that the low prevalence of melioidosis in Port Moresby may not be representative of other regions of PNG. Recent observations in the Western Province of PNG indicated that melioidosis might be a significant cause of fatal respiratory disease mimicking tuberculosis (Warner *et al.* 1998). The rural community of Balimo in the Western Province of PNG is considered endemic for melioidosis. In 1998, a series of culture confirmed cases of melioidosis that were documented in Balimo, indicated clustering in one particular village within a broad family group (Warner *et al.* 1998). Within this district, seroprevalence, as determined by indirect IgG ELISA ranges from 11-47% (J Warner, *personal communication*).

Intracellular bacterial infections require efficient interaction between infected host cells and antigen-specific T cells for pathogen elimination (Raupach & Kaufmann 2001).

Little is known regarding the role of adaptive CMI responses in melioidosis. However, as *B. pseudomallei* is an intracellular pathogen (Vorachit *et al.* 1995; Jones *et al.* 1996; Harley *et al.* 1998) such responses are believed to be essential, particularly since antibodies against *B. pseudomallei* have little effect in the development of protection (Ho *et al.* 1997). As described in the previous chapter, we have recently demonstrated enhanced lymphocyte proliferation and IFN- γ production in patients who had recovered from melioidosis compared to controls following *in vitro* stimulation of PBML with *B. pseudomallei* antigens (Ketheesan *et al.* 2001).

Latent, or subclinical forms of melioidosis exist due to the ability of *B. pseudomallei* to persist in the host for extensive periods before reactivation of infection many months or years later (Kingston 1971; Thompson & Ashdown 1989; Chaowagul *et al.* 1993). Individuals with latent melioidosis have no apparent clinical signs or symptoms, and are identified only by positive serology. Therefore exposure to, or infection with, *B. pseudomallei* does not necessarily result in development of clinical melioidosis. What characteristics of these seropositive individuals with subclinical infection enable them to control and/or eradicate *B. pseudomallei* compared to individuals who develop clinical melioidosis? An understanding of these characteristics may provide clues for immunotherapy and for the clinical management of patients with melioidosis. In this study, CMI responses to *B. pseudomallei* antigens were compared among a group of individuals from Balimo, Western Province, PNG, with and without a history of culture-confirmed melioidosis. Those included in the study that did not have the disease had antibodies to *B. pseudomallei*, indicating exposure to the bacterium.

Using PBML from individuals with previous exposure to *B. pseudomallei*, the specific aims for the work described in this chapter were:

1. To measure the lymphocyte proliferative response toward *B. pseudomallei* antigens
2. To measure the production of IFN- γ in response to *B. pseudomallei* antigens
3. To compare the proliferation and IFN- γ production in response to antigens derived from two different strains of *B. pseudomallei*

4. To determine correlation between antibody titre, clinical presentation and the magnitude of a CMI response to *B. pseudomallei* antigen

10.2 Materials and Methods

10.2.1 Subjects

The study protocol was approved by the PNG Medical Research Advisory Committee (MRAC 0302). A group of 13 individuals (9 males, 4 females) from Balimo, Western Province, PNG that are part of a prospective study into melioidosis in PNG, were selected to participate in the present investigation. The mean age of the individuals at the time of this study was 22 years (range, 11 to 51 years). The cohort represents two groups, the clinical details of whom are shown (Table 1). The first group was comprised of individuals (n=5) who were symptomatic and culture positive for melioidosis (C⁺). Diagnosis was confirmed by isolation of *B. pseudomallei* from clinical samples. The mean time since diagnosis of melioidosis in the culture positive group was 69 months (range, 53 to 85 months). The second group of individuals (n=8) were related to the melioidosis patients and were sharing accommodation at the time of the outbreak. Subjects in the second group were serology positive, but had never presented with any signs or symptoms of infection (S⁺/C⁻) before, or following the serological tests.

The assays to determine CMI were performed with blood samples from these 13 *B. pseudomallei*-exposed individuals, all of whom had no clinical evidence of melioidosis at the time when the assays were performed. Peripheral blood was collected under field conditions in Balimo, PNG and flown to Townville, Australia where it was processed within 12 hrs of collection. A control group consisted of six control subjects who had no clinical history of melioidosis and were serologically negative for antibody to *B. pseudomallei* (S⁻/C⁻). They included 3 men and 3 women with a mean age of 40 years (range, 25 to 53 years). The study protocol was approved by the PNG Medical Research Advisory Committee (MRAC 0302) and informed consent of the subjects was obtained.

10.2.2 Antibody titres

The IHA was used to determine melioidosis sero-reactivity according to the method of Ashdown *et al.* (1987) and is described in Chapter 9.2.2. An IHA titer of $\geq 1:40$ was considered evidence of recent or remote exposure to *B. pseudomallei*. Positive and negative reference sera were included with each run. Results were expressed as either positive (+) or negative (-) for the presence of *B. pseudomallei*-specific antibodies.

10.2.3 Preparation of *B. pseudomallei* antigens

Proliferation assays were performed to determine lymphocyte responses to lysates prepared from two different strains of *B. pseudomallei* lysate. BpLy1 was prepared from strain NCTC 13179 by sonication using a method described previously (Chapter 3). The second lysate, BpLy2, was prepared from a sonicated, heat-killed isolate derived from a throat swab of subject 3 in this study. A 0.9 McFarland standard suspension of the *B. pseudomallei* isolate was prepared in 30 ml of sterile phosphate-buffered saline (PBS; Appendix 1). This suspension was heated at 60°C for 15 min, then sonicated according to the protocol described previously (Chapter 3). Cell debris and glass beads were pelleted by centrifugation (9000 *g* for 12 min) and the supernatant was filtered using 0.22 μm filters (Millipore, NSW, Australia). After dialysis into PBS, the suspension was again filtered through 0.22 μm filters and protein concentration was estimated using a BCA protein assay kit (Progen, NSW, Australia) as per manufacturers' instructions. Sterility was confirmed by incubating 200 μl aliquots of BpLy2 on Ashdown agar and SBA (bioMérieux, NSW, Australia) at 37°C for 48 hrs. BpLy2 (1 $\mu\text{g}/\mu\text{l}$) was aliquoted into microfuge tubes and stored at -70°C.

10.2.4 Lymphocyte proliferation

PBML separated from heparinized blood were cultured in 96-well plates (10^6 PBML/ml) in culture medium (Appendix 1) supplemented with antibiotics and 10% pooled human serum. Triplicate wells were stimulated with either BpLy1 (1 $\mu\text{g}/\text{ml}$), BpLy2 (1 $\mu\text{g}/\text{ml}$), tetanus toxoid (Ttox; 0.5U/ml) or phytohemagglutinin (PHA; 10 $\mu\text{g}/\text{ml}$). Culture plates were incubated in 5% CO₂ at 37°C. Proliferation of cells was determined at 24 hr intervals between 96 and 168 hrs of culture (4 time points) by measuring [³H]-thymidine

incorporation (Amersham-Pharmacia Biotech; 1.25 μ Ci/ml for 4 h). Results were expressed as stimulation index (SI), the proportion of counts per minute in stimulated cultures compared with that in unstimulated culture. The maximum SI recorded at any one of the 4 time points was compared between seronegative healthy controls (S⁻/C⁻), seropositive/culture negative (S⁺/C⁻) and culture positive (C⁺) individuals.

10.2.5 Measurement of IFN- γ in whole blood

IFN- γ production was determined using the commercially available QuantiFERON-TB assay (Cellestis) with modifications to the manufacturer's instructions. Briefly, heparinized whole blood (1 ml) was added to 24-well culture plates and wells were stimulated with either BpLy1 (1 μ g/ml), BpLy2 (1 μ g/ml), Ttox (0.5U/ml) or PHA (10 μ g/ml). Plates were then incubated for 24 h in 5% CO₂ at 37°C. Following incubation, contents of each well were centrifuged (10 min; 500 g) and plasma removed and stored at -70°C until further analysis. IFN- γ levels were measured in samples from individual wells as per the manufacturer's instructions. Briefly, plasma samples (50 μ l) were added in duplicate to 96-well plates coated with anti-human IFN- γ monoclonal antibody. Conjugate (murine monoclonal anti-human IFN- γ -horseradish peroxidase; 50 μ l) was added simultaneously and plates were incubated for 1 h at RT. After washing, chromogen (3,3',5,5'-tetramethylbenzidine) was added and colour development was stopped (0.5M H₂SO₄) after incubation for 30 min at RT. Absorbance of each well was determined with a microtiter plate reader (Multiskan EX355 v1.0, Labsystems, Finland) at 450 nm, with a reference wavelength of 650 nm. Recombinant human IFN- γ standards were included to construct a standard curve. IFN- γ levels of unstimulated wells were subtracted from stimulated wells and results are expressed as International Units per ml (IU/ml), relative to the standard preparations.

10.2.6 Statistical Analysis

Maximal lymphocyte proliferation between days 4 and 7 of culture (maximum SI from 4 time points) for each individual in the three groups, and the production of IFN- γ was analysed by univariate analysis of variance (ANOVA), using SPSS statistical software

(version 8). Dependent variables were tested for normality with a Q-Q plot and were transformed where necessary. Correlation between sets of data was assessed by Pearson's correlation and was considered significant if the probability of a type I error was <0.05% (P<0.05). Mean values in the text are expressed as mean ± SEM.

10.3 Results

10.3.1 Serum antibody levels

Antibody status at the time of this study was assessed in individuals who had previously been exposed to *B. pseudomallei*. IHA titres of ≥1:40 are considered evidence of past or present infection with *B. pseudomallei*. Results of serology at the time of the current study are shown in brackets in Table 10.1.

Table 10.1 Summary of demographic features and clinical characteristics of individuals exposed to *B. pseudomallei*.

Subject Id.	Culture Status	Disease pattern	Age (yrs)	Gender	Antibody Status*
1	+	Sub-acute respiratory	14	F	+(+)
6	+	Sub-acute respiratory	13	M	+(+)
7	+	Sub-acute respiratory	11	F	+(-)
10	+	Sub-phrenic abscess – long term PUO	12	M	+(+)
13	+	Groin abscess – long term PUO	22	M	-(+)
2	-	NCM	22	M	+(+)
3	-	NCM	20	M	+(+)
4	-	NCM	42	M	+(-)
5	-	NCM	34	M	+(-)
8	-	NCM	18	F	+(+)
9	-	NCM	51	M	+(+)
11	-	NCM	15	F	+(-)
12	-	NCM	11	M	+(+)

*Antibody status is considered positive if >1:40 in IHA assay. The antibody status of individuals at the time of enrolment in this study (1998), and at the time of the CMI assay (2003; within brackets) are provided

PUO – pyrexia of unknown origin

NCM – no clinical melioidosis, asymptomatic

10.3.2 Lymphocyte proliferation

The proliferative responses of lymphocytes from healthy controls and individuals with previous exposure to *B. pseudomallei* were compared (Figure 10.1). There was significantly higher proliferation of lymphocytes in response to BpLy1 ($P < 0.001$; Figure 10.1) and BpLy2 ($P < 0.001$; Figure 10.1) in cell cultures obtained from individuals exposed to *B. pseudomallei* (mean SI, 718.2 ± 153.3 and 1263.5 ± 359.9 , respectively) than in cells obtained from control subjects (mean SI, 42.2 ± 8.4 and 119.9 ± 23.9 , respectively).

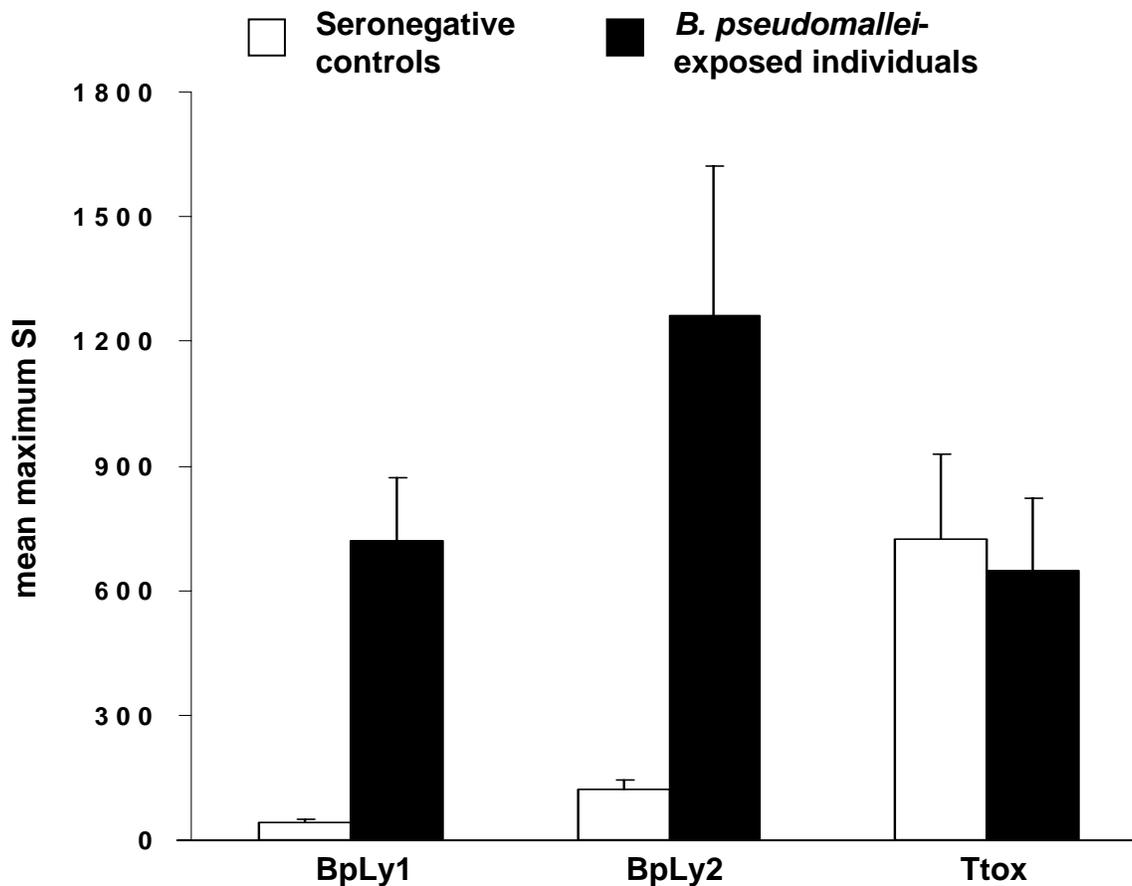


Figure 10.1 Proliferation of lymphocytes from controls and *B. pseudomallei*-exposed individuals in response to BpLy1, BpLy2 and Ttox.

There was a significant increase in lymphocyte proliferation for *B. pseudomallei*-exposed individuals compared to seronegative controls in the presence of BpLy1 ($P < 0.001$) and BpLy2 ($P < 0.001$). In contrast, lymphocyte proliferation following stimulation with Ttox was similar for *B. pseudomallei*-exposed and seronegative individuals ($P > 0.05$) (mean maximum SI \pm SEM).

In contrast, high levels of proliferation in controls (mean SI, 647.3 ± 174.9) and in *B. pseudomallei*-exposed subjects was observed (mean SI, 722.9 ± 205.1) in response to Ttox, with no significant differences between groups ($P > 0.05$; Figure 10.1).

B. pseudomallei-exposed individuals were categorised as culture positive (C^+) and seropositive, culture negative (S^+/C^-) groups, and compared with healthy controls (S^-/C^-) (Figure 10.2). Proliferative responses to BpLy1 for both C^+ ($P < 0.001$; mean SI, 259.3 ± 43.7) and S^+/C^- ($P < 0.001$; mean SI, 1005 ± 230.3) individuals were significantly higher than responses of S^-/C^- (mean SI, 42.2 ± 8.4) (Figure 10.2a). This was also observed in cultures stimulated with BpLy2 (Figure 10.2b). For both BpLy1 and BpLy2, the highest proliferative response was observed in lymphocytes from S^+/C^- versus C^+ individuals ($P < 0.001$; Figure 2a). While the difference in proliferation between C^+ and S^+/C^- was not statistically different for BpLy2 ($P > 0.05$; Figure 10.2b), these responses followed the same trend as BpLy1 stimulated cells ($P < 0.01$; Figure 10.2a). No differences were observed between the three groups following stimulation of lymphocytes with Ttox ($P > 0.05$; Figure 10.2c).

10.3.3 IFN- γ production

Whole blood from S^+/C^- subjects produced the highest levels of IFN- γ (10.1 ± 4.3 IU/ml) when stimulated with BpLy1 (Figure 10.3a), reflecting the proliferation responses observed with this lysate (Figure 10.2a). These levels were significantly higher than for C^+ individuals ($P < 0.05$; 3.9 ± 2 IU/ml) but not compared to S^-/C^- ($P > 0.05$; 2.7 ± 2.7 IU/ml). Production of IFN- γ was significantly elevated for S^-/C^- blood stimulated with BpLy2 (10.2 ± 4.6 IU/ml) when compared to C^+ subjects ($P < 0.05$; 2.0 ± 0.9 IU/ml), but not the S^+/C^- group ($P > 0.05$; 7.3 ± 3.4 IU/ml; Figure 10.3b). While S^+/C^- individuals also produced greater amounts of IFN- γ in response to BpLy2 than C^+ , this difference was not significant. No significant differences in IFN- γ levels were demonstrated between the three groups in whole blood stimulated with Ttox (Figure 10.3c).

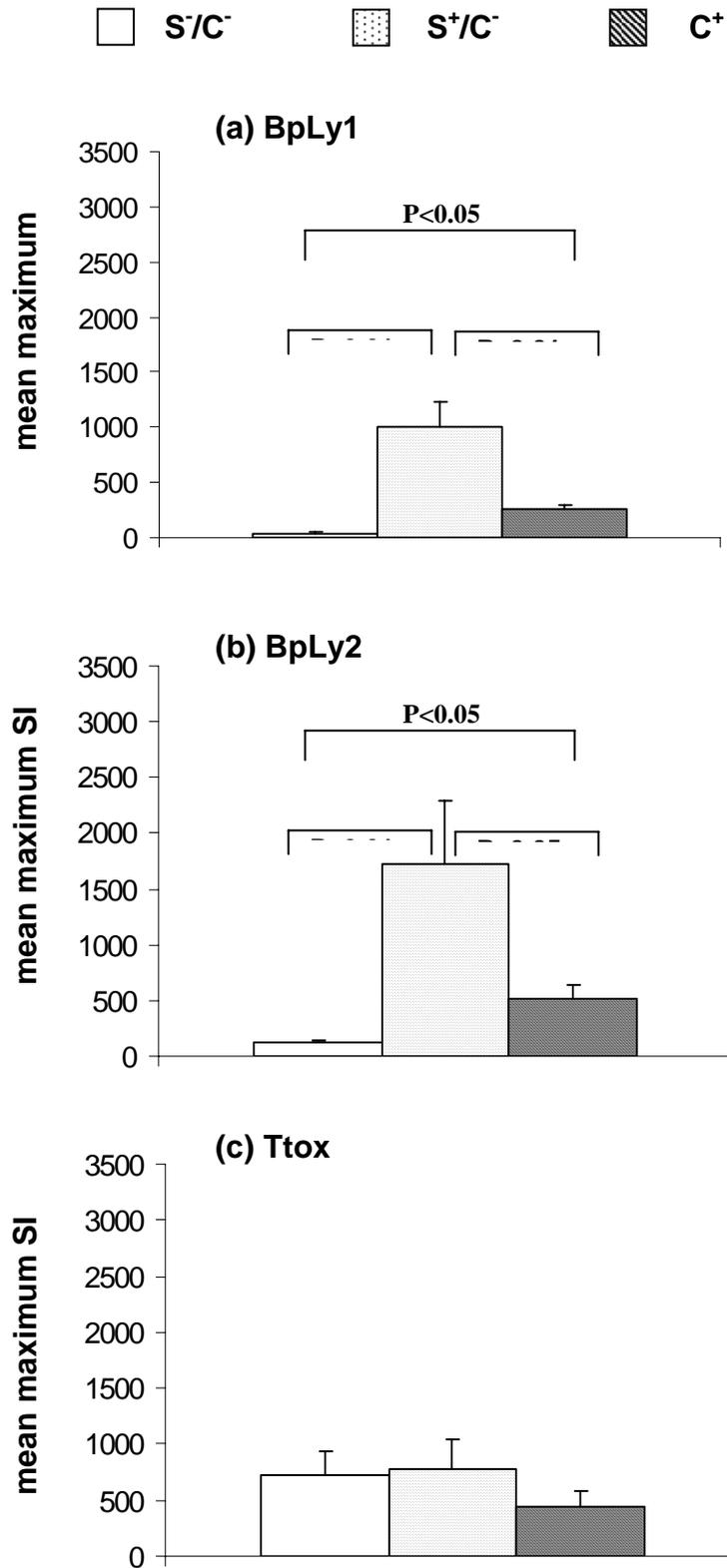


Figure 10.2 Proliferation of controls (S^-/C^-), culture negative (S^+/C^-) and culture positive (C^+) individuals in response to (a) BpLy1, (b) BpLy2 and (c) Ttox. Following stimulation with BpLy1 and BpLy2, significantly greater lymphocyte proliferation was demonstrated in S^+/C^- individuals, compared to both C^+ ($P<0.05$) and seronegative controls (S^-/C^- ; $P<0.01$). There were no significant differences in the proliferation of lymphocytes from S^-/C^- , S^+/C^- and C^+ when cultures were stimulated with Ttox ($P>0.05$; mean maximum SI \pm SEM).

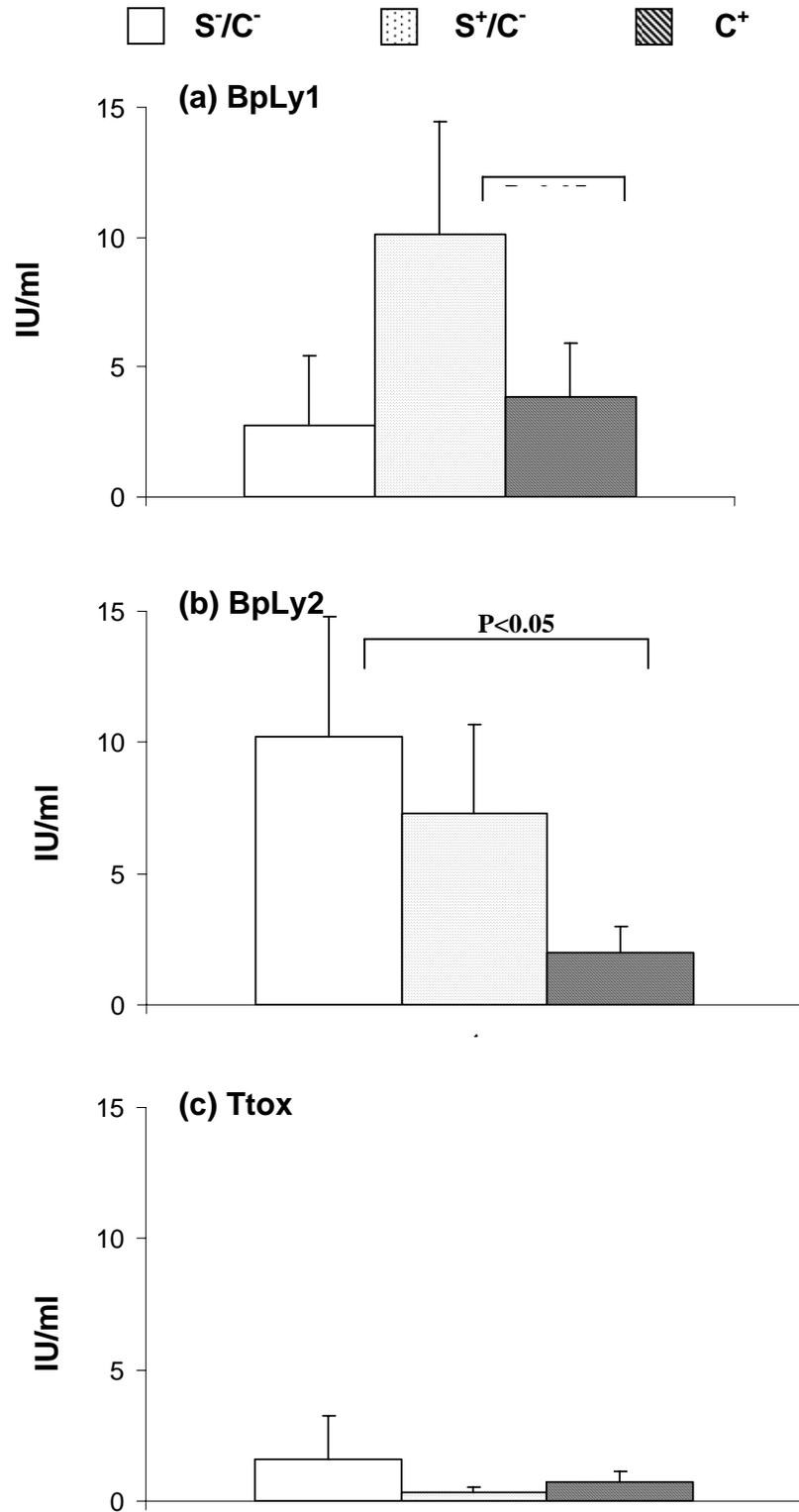


Figure 10.3 IFN- γ production by controls (S^-/C^-), culture negative (S^+/C^-) and culture positive (C^+) individuals in response to (a) BpLy1, (b) BpLy2 and (c) Ttox. IFN- γ levels were significantly higher in S^+/C^- compared to C^+ individuals ($P < 0.05$) following stimulation of whole blood with BpLy1. Following stimulation with BpLy2, IFN- γ was significantly elevated in S^-/C^- blood when compared to C^+ subjects ($P < 0.05$), but not the S^+/C^- group ($P > 0.05$). No significant differences were observed for IFN- γ production between S^-/C^- , S^+/C^- , or C^+ following stimulation with Ttox (IU/ml \pm SEM).

10.4 Discussion

The clinical spectrum of melioidosis is wide, ranging from asymptomatic infection to acute fulminant septicaemia (Leelarasamee & Bovornkitti 1989; Leelarasamee 1998; Currie *et al.* 2000a). *B. pseudomallei* can remain latent in the host for long periods of time and the disease may recrudescence clinically when conditions become favorable for the bacterium to multiply. In endemic areas, a significant proportion of apparently healthy individuals have antibodies to *B. pseudomallei* (Ashdown & Guard 1984; Naigowit *et al.* 1992; Van Phung *et al.* 1993). These antibodies may result from subclinical infection. A serological survey conducted by Thompson and Ashdown (1989) in an Aboriginal community in North Queensland, Australia found 10% of individuals had antibodies to *B. pseudomallei*. Seropositive individuals were assessed annually over a period of 8 years but active infection did not develop in any of the subjects (Thompson & Ashdown 1989). A wide range of antibody titres was observed in the seropositive individuals included in this study although no correlation was found between antibody levels and disease outcome. This is consistent with previous studies (Ho *et al.* 1997; Ketheesan *et al.* 2002).

In the current study, CMI responses to *B. pseudomallei* antigens *in vitro* were compared within *B. pseudomallei*-exposed individuals with disparate outcomes of infection. We focused attention on a group of individuals in whom *B. pseudomallei* infection produced either clinical or subclinical melioidosis. Since neither group had clinical melioidosis at the time of this study, results are unlikely to have been influenced by possible immunoregulatory effects that may be associated with an ongoing systemic infection. It is possible however, that infection persisted, although both groups would have been subject to this risk, since treatment may not eradicate *B. pseudomallei* (Chaowagul *et al.* 1993; Silbermann *et al.* 1997; Inglis *et al.* 2001).

All subjects participating in this study had comparable CMI responses to the Ttox antigen, as assessed by lymphocyte proliferation and IFN- γ production, indicating their

CMI responses were not depressed. Lymphocyte proliferative response to BpLy1 and BpLy2 was significantly higher in seropositive individuals with no clinical history of melioidosis (S⁺/C⁻). Similarly, greatest IFN- γ production following stimulation with BpLy1 was observed in this group. However, when lymphocytes were cultured in the presence of BpLy2 the highest levels of IFN- γ were observed in blood from healthy, seronegative controls S⁻/C⁻ and the lowest in individuals with a history of clinical melioidosis (C⁺). Recently, *in vitro* studies by Lertmemongkolchai *et al.* (2001) demonstrated that *B. pseudomallei* induces IFN- γ production by NK and CD8⁺ T cells from naïve mice within 15 hrs of stimulation. In the current study, PBML were stimulated with *B. pseudomallei* antigens rather than live bacteria. It is not known which antigens are responsible for generating non-specific and specific immune responses toward *B. pseudomallei*. Since BpLy1 and BpLy2 were prepared using different *B. pseudomallei* strains and different antigen extraction methods, it is possible that the high IFN- γ levels observed in cell cultures obtained from controls that were stimulated with BpLy2 are due to non-specific bystander activation of lymphocytes in response to antigens that are only present in the BpLy2 preparation. However, further investigations will be necessary to provide evidence of this.

Proliferation for C⁺ and S⁺/C⁻ individuals was increased in the presence of BpLy2, compared to BpLy1, suggesting greater specificity for this antigen preparation. Since BpLy2 was prepared from a *B. pseudomallei* isolate recovered from subject 3, it is possible that the increased recognition demonstrated by *B. pseudomallei*-exposed individuals to this preparation may be a result of exposure of subjects to a common *B. pseudomallei* strain. Determination of the clonality of the isolates may support such a theory. However, the collection and characterisation of clinical isolates from these individuals comprises the investigations of another researchers' project and as yet have not been determined. Strong CMI responses demonstrated in individuals with subclinical melioidosis may be essential for protection against progression of *B. pseudomallei* infection. Although lower than S⁺/C⁻, C⁺ individuals still demonstrated increased lymphocyte proliferation when compared to S⁻/C⁻.

Protective immunity against pathogens is mediated by antigen-specific memory B and T lymphocytes and triggers a rapid, effective immune response following re-exposure to the microorganism. The extent of *in vivo* T cell expansion in a primary infection influences the size of memory T cell populations (Hou *et al.* 1994). Both antigen and inflammatory factors are believed to jointly promote effector T cell proliferation, although antigen presentation alone is essential for expansion of the memory T cell compartment (Busch *et al.* 2000). The findings of the current study suggest that although C⁺ individuals generate *B. pseudomallei*-specific memory T cells, this lymphocyte subset may be reduced or less responsive compared to the *B. pseudomallei*-specific memory T cells of S⁺/C⁻ individuals. Irrespective of the reasons, individuals who fail to mount an adequate CMI response may succumb to clinical melioidosis.

Disparate CMI responses *in vitro* are documented for subclinical and clinical infections with several intracellular bacteria (Orme, Andersen & Boom 1993; Surcel *et al.* 1994; Bosque *et al.* 2000). In many ways, *B. pseudomallei* infection parallels the characteristics of tuberculosis, caused by the intracellular bacterium *M. tuberculosis*. One-third of the world's population is infected with *M. tuberculosis*, although only 5 to 10% individuals develop active tuberculosis during their lifetimes (Tufariello, Chan & Flynn 2003). Latent tuberculosis is identifiable only by a positive tuberculin skin test. Patients with advanced or disseminated tuberculosis are often anergic on skin testing for DTH and have poor lymphocyte proliferative responses to antigens of *M. tuberculosis in vitro* (Orme, Andersen & Boom 1993; Surcel *et al.* 1994). In contrast, patients who have successfully controlled the primary *M. tuberculosis* infection have a vigorous DTH reaction and their T cells respond well to antigen (Orme, Andersen & Boom 1993). Similarly, in studies of *Leishmania panamensis*, Bosque *et al.* (2000) found a significantly greater *in vitro* expansion of CD4⁺ and CD8⁺ T cells from subclinically infected individuals stimulated with *Leishmania* antigens, than those who had experienced recurrent leishmaniasis. In a mouse model of Leishmaniasis, failure to control disease progression was associated with a population of Lyt-2-T cells that can prevent the induction or expression of protective CMI, thus exacerbating disease development (Liew 1987). Protective T cells produce IFN- γ and macrophage-activating

factor (MAF) when cultured *in vitro* with leishmanial antigens, whereas disease-promoting T cells do not (Liew 1987).

A clinical study by Tanphaichitra and Srimuang (1983) of seven cases of acute melioidosis demonstrated a significant decrease in the total number of lymphocytes and helper T cells, as well as a reduction in the T helper/T suppressor subset ratio. Delayed hypersensitivity responses as assessed by reaction to 2,4-dinitrochlorobenzene (DNCB) were also dramatically reduced to 0% from a normal response of 80% (Tanphaichitra & Srimuang 1983). It was demonstrated that treatment with levamisole, a potent immunostimulant, in combination with conventional antibiotic therapy, improved cellular immunity and recovery from melioidosis (Tanphaichitra & Srimuang 1983). A more recent study by Ramsay *et al.* (2002) similarly describes a reduction in total lymphocyte and T cell numbers in patients with acute melioidosis. In addition, the authors report a decrease in NK cells. Unlike the previous report by Tanphaichitra & Srimuang (1983), Ramsay *et al.* (2002) did not observe any reduction in the ratio of CD4⁺/CD8⁺ T cells. However, the significance of the findings of both groups is clouded by the fact that many of the patients were undergoing chemotherapy or corticosteroid therapy, or had other associated risk factors or diseases which may have influenced the T helper/T suppressor ratio at the time of assessment. For this reason, the true effect of human melioidosis on lymphocyte subsets would be difficult to define from these studies. However, with the use of available murine models, further characterisation and comparison of *B. pseudomallei*-specific T cells in subclinical versus clinical melioidosis is warranted.

In *B. pseudomallei* infection, IFN- γ is essential for host survival and is produced predominantly by NK cells and CD8⁺ T cells (Santanirand *et al.* 1999; Lertmemongkolchai *et al.* 2001). There are several examples of infectious diseases that have an *in vitro* defect in antigen-induced IFN- γ production that is limited to the infecting pathogen alone. Cells from patients with lepromatous leprosy, severe cutaneous and visceral leishmaniasis, and tuberculosis produce low or undetectable levels of IFN- γ in response to their respective antigens but typically generate IFN- γ

normally after stimulation with mitogens or other microbial antigens (Murray 1988). Following treatment, IFN- γ production occurs in response to the specific antigen to which the patients T cells originally failed to react. In contrast, T cells from patients with better-controlled manifestations of the same infections such as tuberculoid leprosy or mucocutaneous leishmaniasis, readily secrete IFN- γ in response to specific microbial antigen (Murray 1988). This may account for the differences in IFN- γ levels observed between C⁺ and S⁺/C⁻ individuals in the current study. Kazi *et al.* (2001) demonstrated that *B. pseudomallei* does induce IFN- γ production in cells from previously healthy individuals, and that the extent of IFN- γ production varies among individual donors. This reflects the findings of the current study, where lymphocytes from S⁻/C⁻ individuals produced levels of IFN- γ that were comparable to C⁺ individuals following stimulation with BpLy1 or to S⁺/C⁻ individuals when stimulated with BpLy2.

Following *in vitro* stimulation with *B. pseudomallei* antigens, the significantly higher lymphocyte proliferation and IFN- γ production observed in asymptomatic, seropositive individuals compared with individuals with a history of clinical melioidosis, may reflect differences in their antigen-specific memory T cell populations. This study provides further evidence of differences in immune responses to *B. pseudomallei* that appear to dictate the outcome of natural infection. The results suggest that individuals who develop strong specific CMI response to *B. pseudomallei* may not develop clinical disease. Alternately, those who fail to mount an adequate CMI response may succumb to infection. Ideally, long-term assessment for evidence of disease reactivation in the S⁺/C⁻ individuals participating in the present study could provide a greater understanding of the host immune responses that are important for maintaining latency of melioidosis.

CHAPTER 11

GENERAL DISCUSSION

Many factors are believed to contribute to the emergence of new disease-causing pathogens and the re-emergence of old ones. Such factors include changes in human behaviour, industrial and economic development, travel and mass movements, civil unrest and wars (Desselberger 2000). Microbial genomic change and adaptation also contribute to the emergence of infectious diseases (Desselberger 2000). Evolutionary mechanisms of pathogens allow them to adapt to new host cells or host species, produce new toxins, bypass or suppress inflammatory and immune responses and develop resistance to drugs and antibodies. The influenza viruses are one example of such pathogens, and can mutate or change in such ways that their virulence is increased. Epidemics and world-wide pandemics of influenza continue to occur (Normile 2003). The increased detection of microbes is likely to contribute to the emergence of some infectious diseases. This is the case of the *L. pneumophila*, the bacterium responsible for the high-mortality pneumonia known as Legionnaire's disease. Following its initial detection during a 1976 outbreak in a convention of American World War II veterans, environmental and retrospective patient cultures subsequently indicated that *Legionellae* had been responsible for 2000 to 6000 deaths previously diagnosed as pneumonias of unknown etiology (Barrett *et al.* 1998). Despite possible increases in detection rates, at least some of these new diseases are contributing to an increase in overall mortality. The most dramatic example of this is the Human Immunodeficiency Virus (HIV). Although retrospective studies have detected cases in Europe and Africa dating back 1959, by 1996 HIV had become the second leading cause of death among adult males aged 25 to 40 years of age in the USA (Barrett *et al.* 1998). Today, about one-quarter of the adult population in South Africa are infected with HIV although it's believed that the full impact of the epidemic is yet to be felt. Deaths due to HIV infection are projected to peak between 2009 and 2012 (www.rand.org).

Other diseases that appeared to be under control are re-emerging. For example, due to the appearance of multi-drug resistant strains of *M. tuberculosis*, tuberculosis is considered a re-emerging infectious disease. It is estimated that 300 million people will be infected with *M. tuberculosis* by the year 2010, and that 30 million of these people will die (www.niaid.nih.gov). Therefore, due to various social and economical impacts, continued research to improve our basic understanding of emerging infectious diseases is warranted.

Melioidosis is also considered an emerging infectious disease and is caused by the potentially deadly bacterium, *B. pseudomallei*. Unlike influenza, tuberculosis and AIDS, many of the basic components involved in the pathogenesis of *B. pseudomallei* infection remain unknown. *B. pseudomallei* can survive for extended periods of time in the environment and has the ability to infect humans by various routes to potentially cause a rapidly fatal disease. For these reasons *B. pseudomallei* is considered a potential biological warfare agent and is listed as a “priority” pathogen by the US National Institutes of Health. Despite its impact and decades of research, relatively little is known regarding the environmental habitat and virulence determinants of the bacterium, as well as the immunopathogenesis of *B. pseudomallei* infection. It is not known what specific factors are responsible for the different clinical presentations that are associated with melioidosis, or why it is that some individuals are extremely susceptible to *B. pseudomallei* infection while others are resistant. Knowledge is also lacking with respect to the predominant immune cell types of the host that are responsible for providing protection against infection with *B. pseudomallei*. Therefore the studies detailed in this thesis sought to provide some basic data relating to the immunopathogenesis of *B. pseudomallei* infection.

In the present studies, a comparison of the disease course in the murine model was made following inoculation of *B. pseudomallei* by *iv*, *ip*, *in*, *po* or *sc* routes of infection. The results of this study emphasize that determination of bacterial virulence is highly dependent on the route of infection. BALB/c mice consistently demonstrated greater susceptibility to *B. pseudomallei*, independent of the route of infection. There is also a

tropism of *B. pseudomallei* for spleen and liver, regardless of the portal of entry. Neurological abnormalities are documented in animals and humans with melioidosis, and a predilection for the brainstem and spinal cord has been suggested (Currie *et al.* 2000b). The current study supports a role for direct brain invasion by *B. pseudomallei* in the pathogenesis of melioidosis following inhalation or ingestion of the bacterium. Interestingly, the colonial morphology of *B. pseudomallei* isolated from brains of C57BL/6 mice infected by the respiratory or oral route differed from brain isolates recovered following intravenous or intraperitoneal infection. Further investigation using intranasal or oral inoculation in the murine model may be useful in studying the pathogenesis of neurological forms of melioidosis and investigation of the significance of morphological changes in isolates recovered from brain tissue.

CMI responses are essential for protection against infection with intracellular pathogens such as *M. tuberculosis* or *L. monocytogenes*. Improved clinical management of patients and the development of preventative measures rely on a more detailed understanding of the CMI responses in melioidosis. The nature of the protective response and the conditions under which it is induced are fundamental for vaccine development. In *B. pseudomallei* infection these basic aspects are not known. Recruitment, migration and activation of leucocyte subsets are crucial events during an immune response that are regulated by pro-inflammatory cytokines and chemokines. However, while these cytokines are indispensable for the effective clearance of a pathogen, an excessive inflammatory response is potentially autodestructive and may contribute to the pathogenesis of an infection (Strieter *et al.* 1996). Studies on patients with melioidosis have previously demonstrated a link between severity of disease and the levels of cytokines and chemokines in serum, including IFN- γ and TNF- α (Brown *et al.* 1991; Friedland *et al.* 1992; Lauw *et al.* 1999). Interferon- γ and TNF- α have also been implicated as important determinants of disease progression and outcome in murine melioidosis (Ulett *et al.* 2000a; Ulett *et al.* 2000b). The kinetics and concentration of various cytokines appear to be critical in determining the generation of resistance or the contribution to immunopathology. Similarly, in the current studies although many of the chemokines and CSF responses detected in C57BL/6 mice were similar to those in

BALB/c mice, the kinetics of mRNA expression and cellular inflammatory response differed considerably. A neutrophil-dominant infiltration was observed in BALB/c compared to C57BL/6 mice during the early stages of *B. pseudomallei* infection. An early neutrophilic influx and an increase in mRNA expression for various chemokines in the present study, as well as increases in pro-inflammatory cytokines in previous studies (Ulett *et al.* 2000b), suggest that BALB/c mice are able to mount an innate immune response toward *B. pseudomallei* infection. However, the significantly greater bacterial loads in BALB/c mice, together with their failure to attract mononuclear cells to the same extent as C57BL/6 mice suggests BALB/c mice may not be able to mount an appropriate cellular response to *B. pseudomallei*. The inability of BALB/c mice to contain the infection at sites of inflammation may underlie the susceptible phenotype of this mouse strain toward *B. pseudomallei* infection.

In intracellular bacterial infections, the successful elimination of the pathogen depends mainly on efficient interaction between infected macrophages and antigen-specific T cells. A previous study in this laboratory demonstrated differences in the microbicidal efficiency of resident monocyte populations from C57BL/6 and BALB/c mice (Ulett, Ketheesan & Hirst 1998). The present study was a preliminary investigation of the contribution of NO production to the resistant and susceptible phenotypes of C57BL/6 and BALB/c mice, respectively. A comparable level of NO, a key molecule involved in microbial killing was produced by IFN- γ -primed peritoneal macrophages from C57BL/6 and BALB/c mice. The findings of the present study, combined with the observation that fewer macrophages are present at sites of *B. pseudomallei* infection in BALB/c mice (Barnes *et al.* 2001) suggest that the differences in macrophage microbial killing reported by Ulett *et al.* (1998) may be a result of differences in priming of BALB/c and C57BL/6 macrophages. A failure of macrophages to become stimulated during the initial stages of *B. pseudomallei* infection may underlie the susceptible phenotype of BALB/c mice since an effective innate, and consequently an adaptive immune response would not be initiated. Future studies in this laboratory will continue to compare the functional abilities of macrophages derived from various tissues of these mouse strains.

T cells are central for protection against infection with other intracellular pathogens. However, until recently few studies had investigated the role of CMI responses in melioidosis. The present studies are the first to demonstrate the role of T cell responses in experimental and human melioidosis. Following exposure to *B. pseudomallei*, both C57BL/6 and BALB/c mice demonstrated significant DTH and lymphocyte proliferation following *in vitro* stimulation with *B. pseudomallei* antigens. These responses were shown to be dependent on the primary challenge dose. Such a response indicates the generation of *B. pseudomallei*-specific lymphocytes after the initial exposure to the bacterium. However, following a subsequent lethal challenge, mortality rates in these mice showed no improvement when compared to controls. Attempts to adoptively transfer either unfractionated MNL or purified T cells from immunised mice to naïve C57BL/6 mice were also unsuccessful in providing complete protection from a subsequent lethal challenge, despite the presence of *B. pseudomallei*-specific lymphocytes. This supports previous intracellular bacterial studies that suggest DTH and a protective, adaptive immune response occur independently (Mitsuyama, Nomoto & Takeya 1982; Orme 1988; Tsukada *et al.* 1991). It appears that exposure to a single small dose of *B. pseudomallei* via the *iv* route is insufficient to generate a protective host immune response. Therefore, development of an effective strategy for induction of protective immunity towards *B. pseudomallei* infection was subsequently investigated, based on the work of Bretscher *et al.* (1992; 1996). Compared to controls, repetitive low-dose immunisation of susceptible BALB/c mice provided a high level of protection against a subsequent *B. pseudomallei* challenge. The failure of BpLy1 immunisation to induce resistance to the level that was achieved by immunisation with live *B. pseudomallei* suggests that secreted bacterial antigens may play an important role in driving a protective immune response toward *B. pseudomallei*. Studies of adaptive immunity to several intracellular pathogens, such as *M. tuberculosis* and *L. monocytogenes*, have also demonstrated that a protective immune response is generated only in the presence of the living organism, since it is mediated by secreted antigens (Mitsuyama, Nomoto & Takeya 1982; Koga *et al.* 1987; Orme 1988). Repetitive exposure to low doses of *B. pseudomallei* will be a useful method for investigating the role of various cellular and molecular components involved in generating protection

towards this intracellular bacterium. Future studies will also be directed at comparing the host immune responses that develop in response to culture filtrate and structural antigens of *B. pseudomallei*.

The present studies have given the first evidence of development of CMI response to *B. pseudomallei* in patients who had recovered from melioidosis. Strong CMI responses were also demonstrated in subclinical *B. pseudomallei* infection in humans. Individuals who had been exposed to *B. pseudomallei* without any clinical manifestations displayed enhanced CMI responses to *B. pseudomallei in vitro*, when compared to individuals who had recovered from clinical melioidosis. The results suggest that individuals who develop strong specific CMI response to *B. pseudomallei* may not develop clinical disease. Alternately, those who fail to mount an adequate CMI response may succumb to infection. Preventative strategies for melioidosis could therefore aim at enhancing specific T cell responses against *B. pseudomallei*.

Collectively, the results contained in this thesis provide basic data regarding the development of protection during *B. pseudomallei* infection. It appears that the susceptibility of BALB/c mice does not affect the development of adaptive immune response following *B. pseudomallei* infection, as indicated by generation of DTH responses and lymphocyte proliferation. However, the susceptible phenotype is associated with a high ratio of neutrophils to mononuclear cells at inflammatory sites, suggesting there may be an innate failure to attract/accumulate the “correct” immune cell types. For the first time, there is evidence supporting an essential role for T cells in the development of a protective adaptive immune response to experimental and human *B. pseudomallei* infection. Differences in the development of T cell responses may influence the outcome of infection. This is of importance for understanding the disease process and development of effective intervention strategies against *B. pseudomallei* infection. Therefore, future studies may focus on further characterisation of cellular infiltrates and the interaction between neutrophils, macrophages and T cells within the susceptible host phenotype. Also, the interaction of individual bacterial products with the host immune system will be an important avenue for future melioidosis research to

identify potential candidate molecules for inclusion into subunit vaccines, or improvements to diagnostic tests. In the future, studies should be carried out to identify key antigens that are able to elicit protective immunity toward *B. pseudomallei* infection.

The similarities between *B. pseudomallei* and other intracellular bacteria, such as *M. tuberculosis* for which there is already a vast knowledge base, provide potential directions for future melioidosis research. Although one-third of the world population is infected with *M. tuberculosis* only 5 to 10% of this population has a lifetime risk of developing active tuberculosis. Similar to melioidosis, when active tuberculosis develops, disease localisation, severity and outcome are highly variable. Tuberculosis can involve any tissue in the body but usually presents as pulmonary infection, ranging from mild infiltration to a severely destructive disease. Miliary tuberculosis, characterised by the hematogenous dissemination of large numbers of mycobacteria throughout the body is the most serious disease manifestation. A recent review by van Crevel *et al.* (2002) on innate immunity to *M. tuberculosis* outlined a series of events that are believed to occur following inhalation of the bacterium. Although research on *M. tuberculosis* is far more advanced than studies on *B. pseudomallei*, the similarities between the diseases they cause suggest the following hypothetical events may potentially apply to infection with *B. pseudomallei*. Following host invasion, *B. pseudomallei* may be immediately destroyed by phagocytes, in which case no observable infection has taken place. Alternatively, *B. pseudomallei* may not be immediately killed and a primary complex consisting of small inflammatory infiltrate develops. A positive DTH response or a *B. pseudomallei*-specific T cell response may identify this 'infection state'. In a minority of cases, active disease may develop either at the site of infection, or in other organs following hematogenous dissemination of *B. pseudomallei* and the infection may stabilize at this point. Months or years afterwards, following conditions of failing immune surveillance, latent infection may reactivate into acute or localised melioidosis. As mentioned previously, this model of disease outcome is hypothetical and based on *M. tuberculosis* research (van Crevel *et al.* 2002). However, it will be interesting to discover the validity of this model as our understanding of the immunopathogenesis of melioidosis continues to advance.

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APPENDIX 1

SOLUTIONS & REAGENTS

A1.1 General Solutions

A1.1.1 1X Phosphate buffered saline (PBS), pH 7.2

NaCl	8 g
(S-9888, Sigma Chemicals, USA)	
Na ₂ HPO ₄	0.64 g
(S-0876, Sigma Chemicals, USA)	
KCl	0.2 g
(AnalaR, BDH Chemicals, Australia)	
KH ₂ PO ₄	0.16 g
(P-5379, Sigma Chemicals, USA)	

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

A1.1.2 Breaking buffer

Leupeptin	0.2 µg/ml
(L2023, Sigma Chemicals, USA)	
Pepstatin	0.2 µg/ml
(P5318, Sigma Chemicals, USA)	
DNase	50 Kunitz Units
(D5025, Sigma Chemicals, USA)	

Combine ingredients in sterile PBS and store aliquots at -20°C until use.

A1.1.3 10% Neutral buffered formalin

40% Formaldehyde	2 L
Distilled water	18 L
Sodium dihydrogen orthophosphate	80 g
Disodium hydrogen orthophosphate	130 g

Combine ingredients and store at RT.

A1.1.4 Haematoxylin and eosin staining technique

Paraffin sections were dewaxed in xylene, treated with graded ethanol (descending concentrations; 100% to 70% v/v), washed in running tap water (1 min) and stained with haematoxylin (8 min). Slides were washed in running tap water for 30 s, immersed in Scott's tap water for 30 s and washed again in running tap water for 2 min. Slides were then stained with eosin (4 min), washed in tap water and dehydrated through graded ethanol (70% to 100%) and xylene. Slides were mounted in DPX

A1.1.4.1 Mayer's haematoxylin

Haematoxylin	2 g
Sodium iodate	4 g
Aluminium ammonium sulphate	100 g
Citric acid	2 g
Chlorol hydrate	100 g

Combine ingredients with 2 L of distilled water.

A1.1.4.2 Eosin

Eosin	15 g
Erythrosin	5 g
Calcium chloride	5 g

Combine ingredients with 2 L of distilled water.

A1.1.4.3 Scott's tap water

Sodium carbonate	8.75 g
Magnesium sulphate	50 g

Combine ingredients with 2.5 L of distilled water.

A1.2 RT-PCR Solutions & Reagents

A1.2.1 10% DECON90

DECON90 (R36/38 S24/25-26, Selby Biolab Scientific)	100 ml
Single distilled water	900 ml

Combine solutions and mix well until DEPC is completely dissolved.

A1.2.2 Diethyl pyrocarbonate (DEPC) treated water

DEPC solution (D-5758, Sigma Chemicals, USA)	1 ml
Single distilled water	1000 ml

Combine solutions and mix well until DEPC is completely dissolved.

A1.2.3 Tris-HCl for RNA work (1 M), pH 7.5

Tris base (T-6791, Sigma Chemicals, USA)	12.10 g
DEPC-treated water	50 ml

Combine ingredients in a nuclease-free bottle and adjust pH to 7.5 with 1N HCl. Bring volume to 100 ml.

A1.2.4 Ethanol for RNA work (75% and 95%)

Ethanol (UNIVAR APS Finechems, 214-2.5L GL)	75 ml (or 95 ml for 95%)
DEPC-treated H ₂ O	Make to 100 ml

Combine solutions in a nuclease-free bottle and store at -20°C until use.

A1.2.5 Reverse transcription mixture

RNA diluted in DEPC-treated water	1 µg in 11 µl
Oligo(dT) ₁₅ primer (500 µg/ml) (C1101Promega)	1 µl

Mix ingredients in thin wall 0.2 ml PCR microfuge tube (1045-20-0, Robbins Scientific Corp), overlay with sterile nuclease-free mineral oil and keep primer mix on ice. To make master mix reaction, combine the following in a separate tube:

5X First Strand Buffer (Y00146, Life Technologies)	4 µl
DTT (0.1M) (C1101, Promega)	2 µl
10 mM dNTP mix diluted in DEPC-treated water (U1240, Promega)	1 µl

Keep master mix on ice. Denature RNA and anneal primer by heating primer mix for 10 min at 70°C on PCR machine. Immediately quench on ice for 5 min. Add master mix reaction and heat for 2 min at 42°C on PCR machine. Add 200 U (1 µl) of Superscript II reverse transcriptase (18064-022, Life Technologies) and incubate for 53 min at 42°C on PCR machine. Inactivate Superscript at 95°C for 4 min. Add 1 µl RNase H (M4281, Promega) and incubate at 37°C for 20 min. Cool to 4°C and store at -20°C.

A1.2.6 PCR mixture

cDNA	2 µl
Forward primer (25 pM/µl)	4 µl
Reverse primer (25 pM/µl)	4 µl
5 mM dNTP mix diluted in DEPC-treated water (U1240, Promega)	4 µl
10X PCR Buffer (Promega)	10 µl
<i>Taq</i> DNA polymerase in Storage Buffer A (M1861, Promega)	0.2 µl
25 mM MgCl ₂	1-4.5 mM
Double distilled sterile water	to 100 µl

Mix ingredients in thin wall 0.2 ml PCR microfuge tube (1045-20-0, Robbins Scientific Corp) in the following order: water, MgCl₂, dNTP mix, primers, 10X buffer and *Taq*.

Overlay with sterile nuclease-free mineral oil and keep on ice. cDNA is added prior to PCR. PCR cycling parameters were: 95°C for 2 min followed by 30 cycles each of denaturation at 94°C for 50 s, annealing of primer and fragment at 60°C for 50 s and primer extension at 72°C for 1 min. A final extension of 72°C for 4 min was included.

A1.2.7 Primers

Custom made primers (Table 4.1; Invitrogen, Melbourne, Australia) were resuspended in DEPC-treated water to a concentration of 100 pM/μl and stored at -20°C. For use, dilute 1 in 4 in DEPC-treated water.

A1.2.8 Tris-acetate (TAE) buffer (50X)

Tris-HCl (T 6791, Sigma Chemicals, USA)	242 g
Glacial acetic acid (100015N, BDH Laboratory Supplies)	57.1 ml
0.5M EDTA pH8.0	100 ml
Double distilled water	Make up to 1000 ml

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

A1.2.9 TE Buffer

Tris-HCl pH8.0	10 mM
EDTA	1 mM

Combine ingredients and store at 4°C until use.

A1.2.10 Agarose gel (2%)

TAE buffer	50 ml
DNA grade Agarose (200-0011, Progen Industries)	1 g

Mix ingredients in glass beaker and microwave for 1 min. Mix by swirling gently and microwave for a further 2 min or until agarose is completely dissolved. Cool at RT for 10 min. Add 2.5 ml of ethidium bromide (10 mg/ml) and swirl to mix. Pour into cast and leave set at RT for at least 30 min.

A1.2.11 Ethidium bromide

Ethidium bromide (200271, Boehringer Mannheim)	100 mg
Distilled water	10 ml

A1.2.12 6X Loading buffer

Glycerol	30%
Bromphenol blue	0.025%

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

A1.2.13 123-base pair DNA ladder (250 ng/μl)

123-bp DNA fragments (1 μg/μl) (15613029, Life Technologies)	100 μl
TE Buffer	233.4 μl
6X DNA loading buffer	66.6 μl

Mix ingredients and store at 4°C.

A1.3 Cell Culture Media & Reagents**A1.3.1 Transport medium**

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

A1.3.2 Culture medium

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

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

A1.3.2.1 L-glutamine stock solution

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

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

A1.3.2.2 Heat-inactivated foetal bovine serum (HIFBS)

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

Combine ingredients and store at -70°C until use. For use add 10 µl of stock to 10 ml of RPMI 1640 (ie. 1 µg/ml) then add 10 µl of this combination to 200 µl of cell culture to give a final concentration of 50 ng/ml.

A1.3.9 [³H]-Thymidine stock solution

³ H-Thymidine (26 Ci/mmol)	1 ml
(B439, Amersham Biosciences)	
1X PBS, pH 7.2	39 ml

Mix ingredients and store at 4°C prior to use. Add 10 µl to 200 µl of culture for a final concentration of 0.25µCi/well.

A1.3.10 Trypan blue solution

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

Combine ingredients and store at RT.

A1.3.11 Lithium heparin

PBS pH 7.2	1 ml
Lithium heparin	
(green top blood collection tube)	10 International Units
(Vacutainer Systems; BD, NSW, Australia)	

Mix PBS and heparin in blood collection tube and use at RT.

A1.4 ELISA Solutions & Reagents

A1.4.1 ELISA wash buffer

Potassium phosphate	0.102 g
Sodium phosphate	1.2 g
Potassium chloride	0.10075 g
Sodium chloride	4.091 g
Tween-20	250 µl
ddH ₂ O	500 mL

A1.4.2 ELISA blocking buffer

0.05% Tween-20	100 µl
1% Fetal bovine serum	2 mL
0.1% skim milk powder	0.2 g

Make up to 200 mL with PBS.

APPENDIX 2

MORTALITY DATA AND BACTERIAL LOADS FOR *B. pseudomallei* strains, NCTC 13178 and NCTC 13179 FOLLOWING INFECTION BY VARIOUS ROUTES

A2.1 Mortality Data

Table A2.1 Mortality data for C57BL/6 mice ten days after subcutaneous (*sc*) infection with NCTC 13178

Challenge (cfu/200µl)	Dilution	Mortality Ratio (#Deaths/#Challenged)	Accumulative Dead	Accumulative Alive	Percentage Morality (%)
3.6×10^7	1:1 (Neat)	5/5	10	0	100
3.6×10^6	1:10 (10^{-1})	2/5	5	3	62.5
3.6×10^5	1:100 (10^{-2})	3/5	3	5	37.5
3.6×10^3	1:10000 (10^{-4})	0/5	0	10	0
0 (Control)	-	0/5	-	-	0

Table A2.2 Mortality data for C57BL/6 mice ten days after intranasal (*in*) infection with NCTC 13178

Challenge (cfu/200µl)	Dilution	Mortality Ratio (#Deaths/#Challenged)	Accumulative Dead	Accumulative Alive	Percentage Morality (%)
2.9×10^4	1:100 (10^{-2})	3/5	9	2	81.81
2.9×10^3	1:1000 (10^{-3})	2/5	6	5	54.54
2.9×10^2	1:10000 (10^{-4})	2/5	4	8	33.33
2.9×10^1	1:100000 (10^{-5})	2/5	2	11	15.38
0 (Control)	-	0/5	-	-	0

Table A2.3 Mortality data for BALB/c mice ten days after subcutaneous (*sc*) infection with NCTC 13178

Challenge (cfu/200µl)	Dilution	Mortality Ratio (#Deaths/#Challenged)	Accumulative Dead	Accumulative Alive	Percentage Morality (%)
6.3 x 10 ³	1:1000 (10 ⁻³)	5/5	8	0	100
6.3 x 10 ²	1:10000 (10 ⁻⁴)	0/5	3	5	37.5
6.3 x 10 ¹	1:100000 (10 ⁻⁵)	1/5	3	9	25
6.3	1:1000000 (10 ⁻⁶)	1/5	2	13	13.33
0 (Control)	-	0/5	-	-	0

Table A2.4 Mortality data for BALB/c mice ten days after intranasal (*in*) infection with NCTC 13178

Challenge (cfu/200µl)	Dilution	Mortality Ratio (#Deaths/#Challenged)	Accumulative Dead	Accumulative Alive	Percentage Morality (%)
2.9 x 10 ³	1:1000 (10 ⁻³)	5/5	9	0	100
2.9 x 10 ²	1:10000 (10 ⁻⁴)	3/5	4	2	66.67
2.9 x 10 ¹	1:100000 (10 ⁻⁵)	1/5	1	6	14.28
2.9	1:1000000 (10 ⁻⁶)	0/5	0	11	0
0 (Control)	-	0/5	-	-	0

Table A2.5 Mortality data for C57BL/6 mice ten days after subcutaneous (*sc*) infection with NCTC 13179

Challenge (cfu/200µl)	Dilution	Mortality Ratio (#Deaths/#Challenged)	Accumulative Dead	Accumulative Alive	Percentage Morality (%)
5.9 x 10 ⁸	1:1 (Neat)	1/5	1	4	20
5.9 x 10 ⁷	1:10 (10 ⁻¹)	0/5	0	9	0
5.9 x 10 ⁶	1:100 (10 ⁻²)	0/5	0	14	0
5.9 x 10 ⁵	1:1000 (10 ⁻³)	0/5	0	19	0
0 (Control)	-	0/5	-	-	0

Table A2.6 Mortality data for C57BL/6 mice ten days after intranasal (*in*) infection with NCTC 13179

Challenge (cfu/200µl)	Dilution	Mortality Ratio (#Deaths/#Challenged)	Accumulative Dead	Accumulative Alive	Percentage Morality (%)
2.9 x 10 ⁸	1:1 (Neat)	2/5	5	3	62.5
2.9 x 10 ⁷	1:10 (10 ⁻¹)	2/5	3	6	33.33
2.9 x 10 ⁶	1:100 (10 ⁻²)	1/5	1	10	9.1
2.9 x 10 ⁵	1:1000 (10 ⁻³)	0/5	0	15	0
0 (Control)	-	0/5	-	-	0

Table A2.7 Mortality data for BALB/c mice ten days after subcutaneous (*sc*) infection with NCTC 13179

Challenge (cfu/200µl)	Dilution	Mortality Ratio (#Deaths/#Challenged)	Accumulative Dead	Accumulative Alive	Percentage Morality (%)
5.9 x 10 ⁴	1:1000 (10 ⁻³)	30/34	70	4	94.6
5.9 x 10 ³	1:10000 (10 ⁻⁴)	26/30	40	8	83.33
5.9 x 10 ²	1:100000 (10 ⁻⁵)	12/23	14	19	42.42
5.9 x 10 ¹	1:1000000 (10 ⁻⁶)	2/21	2	38	5
0 (Control)	-	0/5	-	-	0

Table A2.8 Mortality data for BALB/c mice ten days after intranasal (*in*) infection with NCTC 13179

Challenge (cfu/200µl)	Dilution	Mortality Ratio (#Deaths/#Challenged)	Accumulative Dead	Accumulative Alive	Percentage Morality (%)
3 x 10 ⁶	1:10 (10 ⁻¹)	2/5	4	3	57.14
3 x 10 ⁵	1:100 (10 ⁻²)	1/5	2	7	22.22
3 x 10 ⁴	1:1000 (10 ⁻³)	1/5	1	11	8.33
3 x 10 ³	1:10000 (10 ⁻⁴)	0/5	0	16	0
0 (Control)	-	0/5	-	-	0

A2.2 LD₅₀ Calculations (Reed & Muench 1938)

Formula: $LD_{50} = [\text{Neat}] \text{ cfu}/40\mu\text{l} / 10^{\log_{10} \text{ endpoint}}$ (for subcutaneous inoculation), or
 $LD_{50} = [\text{Neat}] \text{ cfu}/20\mu\text{l} / 10^{\log_{10} \text{ endpoint}}$ (for intranasal inoculation)

Where: $\log_{10} \text{ endpoint} = \log_{10} x - (\text{PD} \times \log_{10} \text{ dilution factor between } x \text{ \& } y)$

And: $x = \text{dilution of Neat resulting in \% Mortality immediately below } 50\%$, and
 $y = \text{dilution of Neat resulting in \% Mortality immediately above } 50\%$
Proportional Distance (PD) = $(50\% - X\%) / (Y\% - X\%)$

Where: $X = \text{mortality immediately below } 50\%$, $Y = \text{mortality immediately above } 50\%$

A2.2.1 Subcutaneous inoculation of C57BL/6 mice with NCTC 13178

Proportional Distance = $50 - 37.5 / 62.5 - 37.5$
= 0.5

$\log_{10} \text{ endpoint} = \log_{10} 100 - (0.5 \times \log_{10} 10)$
= 1.5

$LD_{50} \text{ (cfu)} = 3.6 \times 10^7 / 10^{1.5}$
= $1.14 \times 10^6 \text{ cfu}$

A2.2.2 Intranasal inoculation of C57BL/6 mice with NCTC 13178

Proportional Distance = $50 - 33.33 / 54.54 - 33.33$
= 0.786

$\log_{10} \text{ endpoint} = \log_{10} 10000 - (0.786 \times \log_{10} 10)$
= 3.214

$LD_{50} \text{ (cfu)} = 2.9 \times 10^6 / 10^{3.214}$
= $1.8 \times 10^3 \text{ cfu}$

A2.2.3 Subcutaneous inoculation of BALB/c mice with NCTC 13178

Proportional Distance = $50 - 37.5 / 100 - 37.5$
= 0.2

$\log_{10} \text{ endpoint} = \log_{10} 10000 - (0.2 \times \log_{10} 10)$
= 3.8

$LD_{50} \text{ (cfu)} = 6.3 \times 10^6 / 10^{3.8}$
= $0.998 \times 10^3 \text{ cfu}$

A2.2.4 Intranasal inoculation of BALB/c mice with NCTC 13178

Proportional Distance = $50 - 14.28 / 66.67 - 14.28$
= 0.682

$\log_{10} \text{ endpoint} = \log_{10} 100000 - (0.682 \times \log_{10} 10)$
= 4.318

$LD_{50} \text{ (cfu)} = 2.9 \times 10^6 / 10^{4.318}$
= $1.4 \times 10^2 \text{ cfu}$

A2.2.5 Subcutaneous inoculation of C57BL/6 mice with NCTC 13179

The LD₅₀ value was not able to be calculated since only one death was recorded following challenge with the neat preparation of 5.9×10^7 cfu.

A2.2.6 Intranasal inoculation of C57BL/6 mice with NCTC 13179

$$\begin{aligned}\text{Proportional Distance} &= 50-33.33 / 62.5 - 33.33 \\ &= 0.571 \\ \log_{10} \text{ endpoint} &= \log_{10} 10 - (0.571 \times \log_{10} 10) \\ &= 0.429 \\ \text{LD}_{50} \text{ (cfu)} &= 2.9 \times 10^8 / 10^{0.429} \\ &= 1.08 \times 10^8 \text{ cfu}\end{aligned}$$

A2.2.7 Subcutaneous inoculation of BALB/c mice with NCTC 13179

$$\begin{aligned}\text{Proportional Distance} &= 50-42.42 / 83.33 - 42.42 \\ &= 0.185 \\ \log_{10} \text{ endpoint} &= \log_{10} 100000 - (0.185 \times \log_{10} 10) \\ &= 4.815 \\ \text{LD}_{50} \text{ (cfu)} &= 5.9 \times 10^7 / 10^{4.815} \\ &= 9.03 \times 10^2 \text{ cfu}\end{aligned}$$

A2.2.8 Intranasal inoculation of BALB/c mice with NCTC 13179

$$\begin{aligned}\text{Proportional Distance} &= 50-22.22 / 57.14 - 22.22 \\ &= 0.796 \\ \log_{10} \text{ endpoint} &= \log_{10} 100 - (0.796 \times \log_{10} 10) \\ &= 1.204 \\ \text{LD}_{50} \text{ (cfu)} &= 3 \times 10^7 / 10^{1.204} \\ &= 1.88 \times 10^6 \text{ cfu}\end{aligned}$$

A2.3 Bacterial Loads

C57BL/6 and BALB/c mice were inoculated with 5.7×10^5 or 570 cfu of NCTC 13178.

Mice were challenged by either an intravenous (*iv*), intraperitoneal (*ip*), intranasal (*in*) or oral/ingestion (*ing*) route. At days 1 to 3 post infection, mice were euthanased and the bacterial loads in various tissues were determined (Chapter 3). Results for individual mice, given as log₁₀ cfu per organ, are shown in Tables A2.9 and A2.10 below.

Table A2.9 Growth kinetics data for C57BL/6 mice

B L O O D											
Mouse No.	Day 1 pi				Day 2 pi				Day 3 pi		
	IV	IP	IN	ING	IV	IP	IN	ING	IP	IN	ING
1	3.15	3.40	2.37	0	-	-	0	-	4.56	0	0
2	3.79	3.73	0	1.92	-	-	2.26	0	2.07	0	0
3	3.74	1.70	0	0	-	2.62	2.22	0	0	0	0
4	3.75				-						
5	3.82				-						
Mean	3.65	2.94	0.79	0.64	-	2.62	1.50	0	2.21	0	0
SD	0.28	1.09	1.37	1.11	-	-	1.29	0	2.28	0	0
SEM	0.13	0.63	0.79	0.64	-	-	0.75	0	1.32	0	0
S P L E E N											
Mouse No.	Day 1 pi				Day 2 pi				Day 3 pi		
	IV	IP	IN	ING	IV	IP	IN	ING	IP	IN	ING
1	5.23	5.22	4.29	5.15	7.40	5.75	4.03	2.52	7.15	2.77	3.06
2	5.17	5.06	3.90	3.92	6.60	6.11	3.86	3.40	6.57	3.74	3.40
3	5.02	4.78	3.02	3.08	4.79	5.78	3.15	3.45	5.10	3.16	3.49
4	4.97				6.54						
5	5.28				6.58						
Mean	5.13	5.02	3.74	4.05	6.38	5.88	3.68	3.12	6.27	3.22	3.32
SD	0.13	0.22	0.65	1.04	0.96	0.20	0.47	0.52	1.06	0.49	0.23
SEM	0.06	0.13	0.38	0.60	0.43	0.12	0.27	0.30	0.61	0.28	0.13
L I V E R											
Mouse No.	Day 1 pi				Day 2 pi				Day 3 pi		
	IV	IP	IN	ING	IV	IP	IN	ING	IP	IN	ING
1	5.53	5.38	3.52	5.12	7.40	7.08	2.52	3.75	7.84	2.52	2.40
2	5.49	5.48	3.27	2.82	7.09	7.00	3.54	3.70	5.53	3.24	3.35
3	5.78	4.85	2.57	2.57	5.29	6.28	3.47	2.82	6.07	1.92	2.85
4	5.59				7.02						
5	5.79				7.19						
Mean	5.64	5.24	3.12	3.51	6.80	6.79	3.18	3.42	6.48	2.56	2.87
SD	0.14	0.34	0.49	1.40	0.85	0.44	0.57	0.52	1.21	0.66	0.48
SEM	0.06	0.20	0.28	0.81	0.38	0.25	0.33	0.30	0.70	0.38	0.28
L U N G											
Mouse No.	Day 1 pi				Day 2 pi				Day 3 pi		
	IV	IP	IN	ING	IV	IP	IN	ING	IP	IN	ING
1	2.62	2.70	1.62	5.88	5.32	4.09	1.92	2.22	4.78	2.62	0
2	3.71	2.57	0	2.73	3.40	3.30	2.62	3.35	2.82	0	2.22
3	3.98	0	0	0	3.59	3.71	1.92	2.22	3.04	0	3.04
4	3.18				3.18						
5	3.79				3.50						
Mean	3.46	1.76	0.54	2.87	3.80	3.70	2.15	2.60	3.55	0.87	1.75
SD	0.55	1.52	0.94	2.94	0.87	0.39	0.40	0.65	1.08	1.51	1.57
SEM	0.25	0.88	0.54	1.70	0.39	0.23	0.23	0.38	0.62	0.87	0.91
L Y M P H N O D E											
Mouse No.	Day 1 pi				Day 2 pi				Day 3 pi		
	IV	IP	IN	ING	IV	IP	IN	ING	IP	IN	ING
1	3.75	3.45	0	3.34	4.28	4.71	0	2.40	5.26	0	0
2	3.33	3.83	0	0	5.10	4.70	0	2.88	3.04	0	0
3	4.11	2.85	0	0	5.24	4.19	0	0	3.35	0	0
4	3.38				4.74						
5	3.86				5.16						

Mean	3.68	3.38	0	1.11	4.90	4.53	0	1.76	3.88	0	0
SD	0.33	0.49	0	1.93	0.40	0.30	0	1.54	1.20	0	0
SEM	0.15	0.28	0	1.11	0.18	0.17	0	0.89	0.69	0	0
B R A I N											
Mouse No.	Day 1 pi				Day 2 pi				Day 3 pi		
	IV	IP	IN	ING	IV	IP	IN	ING	IP	IN	ING
1	1.92	1.92	2.98	1.92	3.04	2.62	3.37	0	4.09	0	2.22
2	2.32	1.92	3.25	0	2.96	3.74	0	4.33	2.62	2.52	3.80
3	2.57	0	0	0	3.68	2.22	3.51	0	0	2.40	0
4	2.22				3.44						
5	2.10				4.19						
Mean	2.23	1.28	2.08	0.64	3.46	2.86	2.29	1.44	2.24	1.64	2.01
SD	0.24	1.11	1.80	1.11	0.50	0.79	1.99	2.50	2.07	1.42	1.91
SEM	0.11	0.64	1.04	0.64	0.22	0.45	1.15	1.44	1.20	0.82	1.10

Table A2.10 Growth kinetics data for BALB/c mice

B L O O D											
Mouse No.	Day 1 pi				Day 2 pi				Day 3 pi		
	IV	IP	IN	ING	IV	IP	IN	ING	IP	IN	ING
	1.22	1.22	0	0	3.57	3.89	2.74	3.13	4.63	-	0
	1.22	2.34	0	0	3.47	3.33	1.22	0	3.94	0	0
	1.82	1.92	1.52	0	3.23	3.58	3.16	0	3	3.48	0
	1.52				2.07						
	1.52				2.54						
Mean	1.46	1.83	0.51	0	2.98	3.60	2.37	1.04	3.86	1.74	0
SD	0.25	0.56	0.88	0	0.65	0.28	1.02	1.81	0.82	2.46	0
SEM	0.11	0.33	0.51	0	0.29	0.16	0.59	1.04	0.47	1.42	0
S P L E E N											
Mouse No.	Day 1 pi				Day 2 pi				Day 3 pi		
	IV	IP	IN	ING	IV	IP	IN	ING	IP	IN	ING
1	3.98	1.62	2.47	0	6.77	7.07	4.28	4.25	7.79	7.43	6.07
2	4.29	4.65	0	0	7.00	7.08	4.11	0	7.39	4.77	0
3	4.48	4.73	2.85	0	7.09	7.03	4.21	0	6.26	5.74	0
4	4.25				6.16						
5	4.16				6.24						
Mean	4.23	3.67	1.77	0	6.65	7.06	4.20	1.42	7.15	5.98	2.02
SD	0.18	1.77	1.55	0	0.43	0.03	0.09	2.45	0.79	1.35	3.51
SEM	0.08	1.02	0.89	0	0.19	0.02	0.05	1.42	0.46	0.78	2.02
L I V E R											
Mouse No.	Day 1 pi				Day 2 pi				Day 3 pi		
	IV	IP	IN	ING	IV	IP	IN	ING	IP	IN	ING
1	4.18	1.62	0	0	7.40	7.02	4.11	4.07	7.68	6.85	4.77
2	3.94	4.26	0	0	6.59	6.23	3.99	0	6.10	4.52	0
3	3.98	4.36	3.41	0	6.45	7.00	4.09	0	6.04	5.45	0
4	4.57				3.94						
5	3.45										
Mean	4.02	3.41	1.14	0	5.99	6.75	4.06	1.36	6.61	5.61	1.59
SD	0.41	1.55	1.97	0	1.31	0.45	0.07	2.35	0.93	1.17	2.75
SEM	0.18	0.90	1.14	0	0.59	0.26	0.04	1.36	0.54	0.67	1.59
L U N G											
Mouse No.	Day 1 pi				Day 2 pi				Day 3 pi		
	IV	IP	IN	ING	IV	IP	IN	ING	IP	IN	ING
1	0	0	0	0	4.13	3.59	2.70	3.10	4.60	3.88	2.22

2	0	0	0	0	3.82	3.77	3.13	0	3.52	0	0
3	0	3.48	1.88	0	3.43	4.03	3.30	0	2.82	3.24	0
4	0				3.07						
5	0				2.40						
Mean	0	1.16	0.63	0	3.37	3.80	3.04	1.03	3.65	2.37	0.74
SD	0	2.01	1.08	0	0.67	0.22	0.31	1.79	0.90	2.08	1.28
SEM	0	1.16	0.63	0	0.30	0.13	0.18	1.03	0.52	1.20	0.74
L Y M P H N O D E											
Mouse No.	Day 1 pi				Day 2 pi				Day 3 pi		
	IV	IP	IN	ING	IV	IP	IN	ING	IP	IN	ING
1	0	2.10	0	0	3.64	4.21	3.85	3.54	4.40	4.69	3.15
2	0	2.32	0	0	4.18	2.40	0	0	3.61	0	0
3	0	0	0	0	3.68	4.39	0	0	2.40	0	0
4	0				1.92						
5	0				3.18						
Mean	0	1.47	0	0	3.32	3.67	1.28	1.18	3.47	1.56	1.05
SD	0	1.28	0	0	0.86	1.10	2.22	2.05	1.01	2.71	1.82
SEM	0	0.74	0	0	0.38	0.64	1.28	1.18	0.58	1.56	1.05
B R A I N											
Mouse No.	Day 1 pi				Day 2 pi				Day 3 pi		
	IV	IP	IN	ING	IV	IP	IN	ING	IP	IN	ING
1	0	0	0	0	3.32	3.26	4.18	0	3.57	3.44	0
2	0	0	0	0	3.07	2.22	0	0	3.26	0	0
3	0	0	0	0	2.62	3.28	0	0	0	0	0
4	0				0						
5	0				2.22						
Mean	0	0	0	0	2.25	2.92	1.39	0	2.28	1.15	0
SD	0	0	0	0	1.32	0.61	2.42	0	1.98	1.99	0
SEM	0	0	0	0	0.59	0.35	1.39	0	1.14	1.15	0

(-) indicates mice in which blood collection was not possible due to death of the mouse

APPENDIX 3

GENERATION OF RESISTANCE IN SUSCEPTIBLE BALB/c MICE

A3.1 Determination of suitable immunising dose

Figure A3.1 Footpad swelling in BALB/c mice following *sc* inoculation with various doses of *B.pseudomallei* NCTC 13179 (mm±SEM).

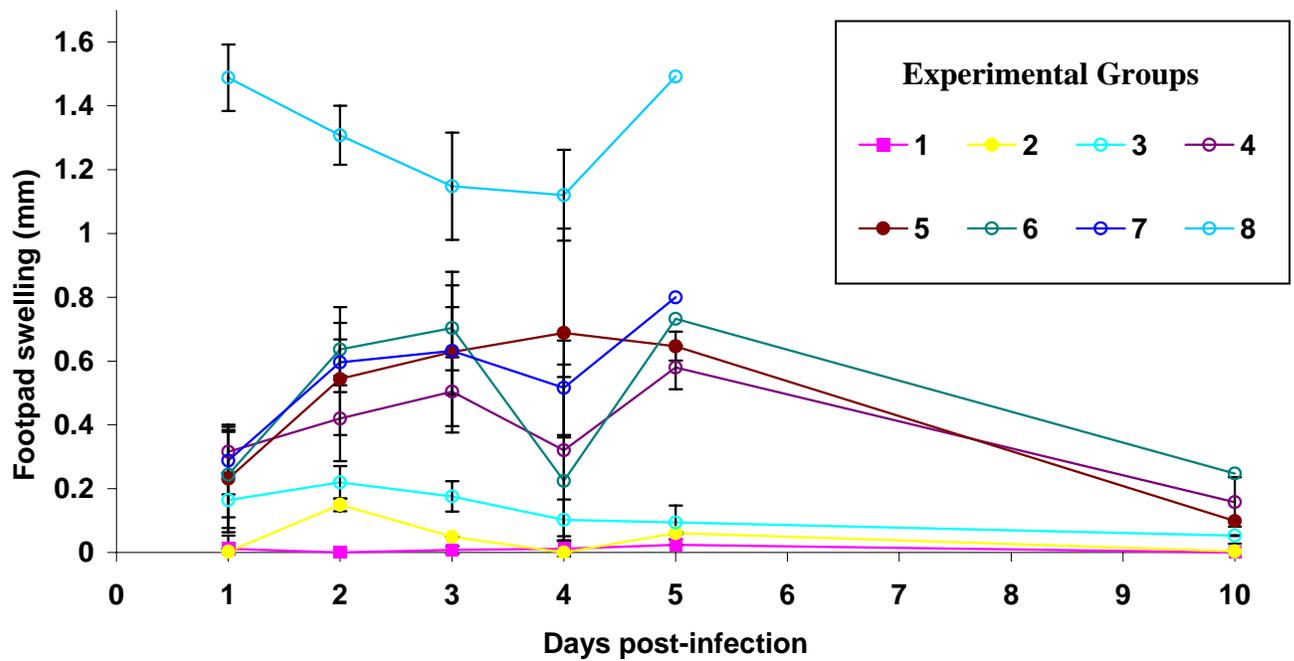


Figure A3.2 Change in spleen index following *sc* infection with various doses of *B. pseudomallei* NCTC 13179 (spleen index \pm SEM).

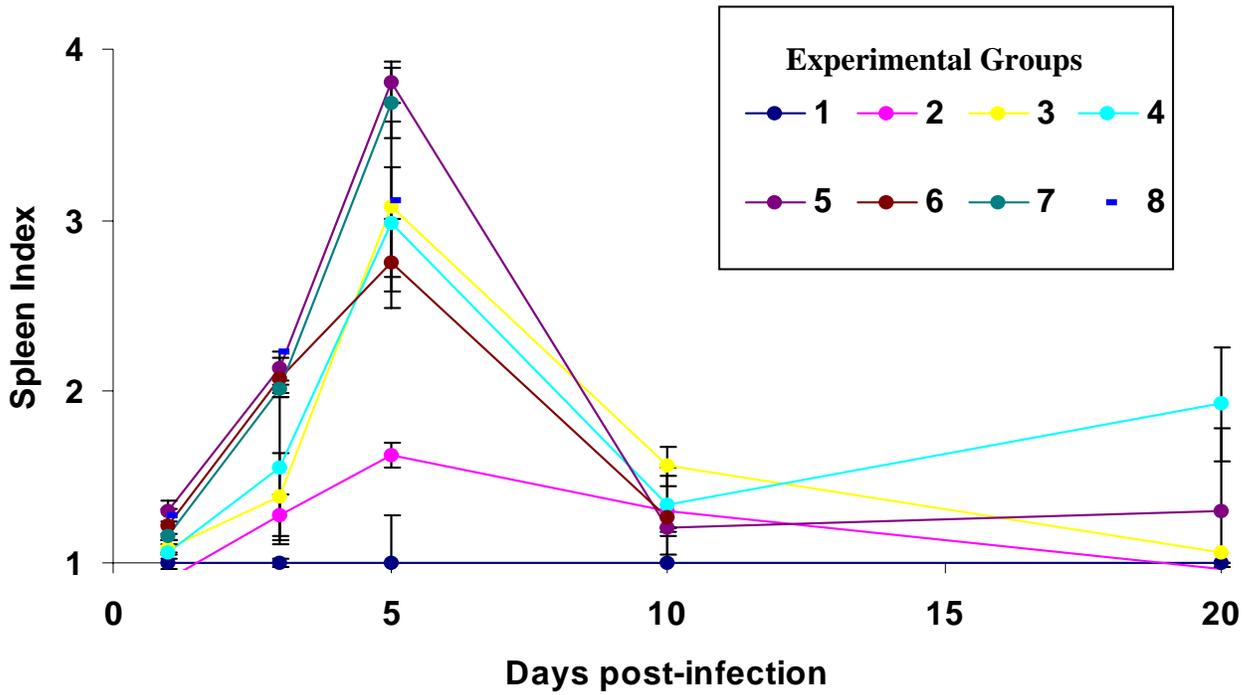


Figure A3.3 Bacterial load in spleen of BALB/c mice following *sc* infection with various doses of *B. pseudomallei* NCTC 13179 (\log_{10} cfu \pm SEM).

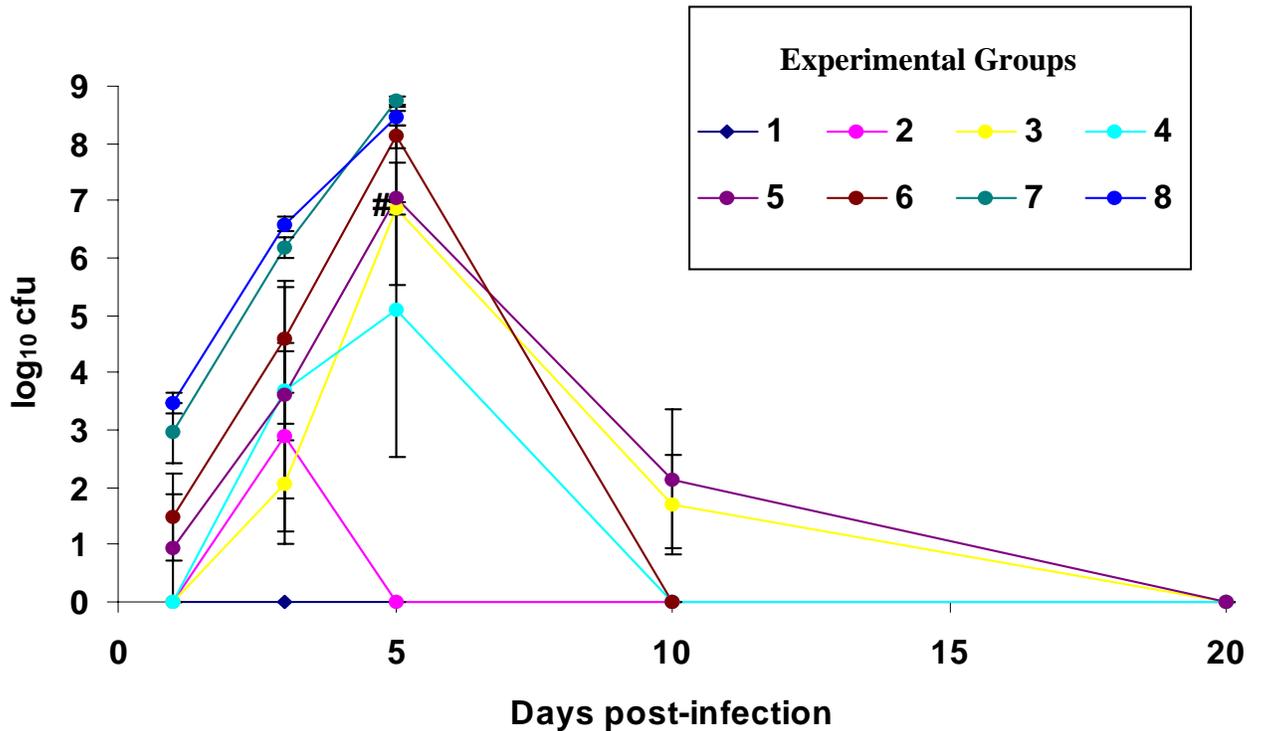


Figure A3.4 Percentage mortality in BALB/c mice following *sc* infection with various doses of *B. pseudomallei* NCTC 13179.

