The role of dendritic cells in the dissemination of *Burkholderia pseudomallei* infection

Thesis submitted by
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DECLARATION OF ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human (*1999*),* the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics*. *Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review (Approval #A1601 and #H4470) and from The Queensland Health Townsville Hospital and Health Services Ethic Committee (Approval #71/04).

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STATEMENT OF CONTRIBUTION OF OTHERS

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ABSTRACT

Burkholderia pseudomallei infection, known as melioidosis in humans and animals, is an important cause of community-acquired bacterial sepsis that is associated with high mortality rates in endemic regions including Northern Australia and Southeast Asia. In Thailand, melioidosis causes a significant number of fatalities, placing it as the third leading cause of deaths from an infectious disease behind AIDS and tuberculosis. In individuals exposed to *B. pseudomallei*-contaminated soil or water, the bacteria can rapidly disseminate to a variety of organs resulting in diverse manifestations that range in severity from asymptomatic to acute, chronic and latent infections. Irrespective of the initial presentation, bacteraemia and sepsis are common complications associated with melioidosis. Early diagnosis and provision of appropriate antibiotic therapy are crucial for preventing mortality but is hampered by the lack of an accurate, reliable and cost-effective diagnostic assay. Improved diagnosis, treatment and future vaccine development is dependent on understanding host-pathogen interactions and the mechanisms involved in the progression of melioidosis.

The work presented in this thesis endeavoured to further our understanding of host-pathogen interactions between dendritic cells (DC) and *B. pseudomallei* and the potential use of *B. pseudomallei*-specific T cell assays for monitoring the development of protective immune responses in patients. The research presented is the first to investigate the capacity of specialised type I interferon (IFN)-producing plasmacytoid DC (pDC) to respond to *B. pseudomallei*. The current research also describes a novel mechanism for *B. pseudomallei* dissemination, whereby migrating DC traffic the bacterium to sites distal from the infection site. In addition, the work presented demonstrates the benefit of *B. pseudomallei*-specific T cell assays as an alternative method to current serological assays, such as the indirect haemagglutination assay (IHA), for detecting the development of adaptive immune responses. These assays could become useful tools for detecting exposure to *B. pseudomallei* and improve monitoring of patients with melioidosis.

Plasmacytoid DC are a subset of DC that provide innate immune responses by rapidly producing large quantities of type I IFN (IFN-α and IFN-β) to modulate

immune cell activation, such as NK cell cytolytic activity and cytokine production. Although the role of pDC during viral infections is well described, their functional responses to bacterial infections are underappreciated. The limited evidence available suggests that pDC and type I IFN may drive beneficial anti-bacterial responses or alternatively detrimental immune suppressive functions. Recently, leukocytes from patients with acute melioidosis were found to activate type I IFN-mediated signalling pathways. However, the dominant cell type producing type I IFN and the significance of inflammatory signalling via type I IFN during *B. pseudomallei* infection is yet to be defined. Because type I IFN modulate a range of immune cell functions, excessive signalling via type I IFN during *B. pseudomallei* infection could potentially contribute to the mechanisms driving the development of septic shock in melioidosis. Therefore, the functional responses of pDC following *in vitro* exposure to *B. pseudomallei* were investigated. Human and murine pDC internalised and killed *B. pseudomallei* as efficiently as conventional DC. Interestingly, pDC generated from *B. pseudomallei*-susceptible BALB/c mice demonstrated significantly increased IFN-α production and were unable to kill intracellular *B. pseudomallei* in comparison to pDC generated from *B. pseudomallei*-resistant C57BL/6 mice. The findings indicate that pDC are an additional innate immune cell capable of responding to *B. pseudomallei* that potentially contribute to the excessive cytokine response and increased bacterial burden causing rapid mortality in BALB/c mice. The outcomes of this work provide the first evidence of pDC bactericidal activity against *B. pseudomallei.*

Conventional dendritic cells (DC) are considered professional antigen presenting cells that link the innate and adaptive immune responses. *In vitro* studies have demonstrated that highly phagocytic, immature DC internalise and kill *B. pseudomallei*. These *in vitro* DC-*B. pseudomallei* interactions triggered DC maturation, a process whereby DC up-regulated expression of antigen presenting molecules (MHC class II) and T cell co-stimulatory molecules (CD80, CD86). Due to the rapid dissemination of disease in acute melioidosis, it was hypothesised that migrating DC may serve as a vehicle for *B. pseudomallei* dissemination. Using an *in vitro* migration assay, it was demonstrated that *B. pseudomallei* stimulated significantly increased migration of bone marrow derived DC (BMDC) compared to uninfected BMDC. Importantly, migrated BMDC were found to harbour live

B. pseudomallei. Subsequent studies provided evidence that *B. pseudomallei* stimulated *in vivo* migration of fluorescently labelled BMDC from the footpad to the draining popliteal lymph node (pLN). Furthermore, *in vivo* migration of *B. pseudomallei*-infected BMDC facilitated dissemination of the bacterium to distal secondary lymphoid tissue and lungs of mice. DC-associated dissemination of *B. pseudomallei* corresponded with significantly increased bacterial burden in the spleen and lungs of mice in comparison to mice infected with *B. pseudomallei* alone. These novel findings demonstrate that *B. pseudomallei* is capable of persisting within migrating DC, which enhances the ability of the bacteria to rapidly disseminate and colonise lymphoid tissue.

The DC migration assays used *in vitro* cultured BMDC exposed to *B. pseudomallei* to demonstrate that *B. pseudomallei* hijacks DC migration. To determine whether DC at the infection site also traffic *B. pseudomallei,* C57BL/6 mice with infected with *B. pseudomallei* via the footpad then the intracellular bacteria within tissue-resident DC in the footpad injection site and also in the draining pLN, spleen and lung were enumerated. The findings presented are the first to provide evidence of *B. pseudomallei* internalisation by skin DC at the site of infection. In addition, the findings demonstrate that dissemination of *B. pseudomallei* was higher in tissue-resident DC compared to non-DC in the pLN and spleen of infected mice. Of interest, spleen DC appear to facilitate intracellular persistence of *B. pseudomallei.* Collectively, the findings of the current investigation are the first to prove that the migratory response of DC to secondary lymphoid tissues for antigen presentation inadvertently facilitates the systemic spread of *B. pseudomallei*.

Activation of adaptive cell-mediated immune (CMI) responses for elimination of intracellular pathogens including *B. pseudomallei* are important for protection against disease progression. *B. pseudomallei*-specific T cell responses are evident in patients who have recovered from melioidosis. Furthermore, studies suggest that a strong CMI may protect an individual from the development of clinical melioidosis. Although CMI-based assays are used to diagnose other diseases, such as the QuantiFERON assay for diagnosing tuberculosis, assessment of *B. pseudomallei*-specific T cell responses in clinical practice is underutilised. Instead, the IHA is typically used as an affordable, serological diagnostic aid despite its poor sensitivity

and specificity. Furthermore, approximately 10 % of patients with culture-confirmed melioidosis remain persistently IHA negative (IHA-negative patients). Given the unreliability of the IHA, it was proposed that assessment of *B. pseudomallei*-specific T cell recall responses would be useful to demonstrate the development of adaptive immune responses in IHA-negative patients. *B. pseudomallei*-specific T cell responses were detected in peripheral blood mononuclear cells isolated from IHA-negative patients confirming both exposure and the development of an adaptive immune response to *B. pseudomallei*, despite negative IHA serology. This response was predominantly driven by $CD4^+$ T cells, with a strong IFN-γ response. The findings of this study are the first to demonstrate *B. pseudomallei*-specific T cell responses in patients with culture-confirmed melioidosis who are persistently IHA negative and support the development of a CMI-based assay, possibly an IFN-γ detection assay akin to the tuberculosis QuantiFERON assay, which would be beneficial for monitoring the immune status of patients with melioidosis.

In summary, the current study is the first to describe the innate response of pDC exposed to *B. pseudomallei* and demonstrate that increased IFN-α production and impaired bacterial killing by pDC may contribute to host susceptibility. Importantly, the current study provides novel data on the migratory capacity of DC exposed to *B. pseudomallei*, which demonstrates that DC migration to secondary lymphoid tissues is a mechanism that facilitates the rapid systemic dissemination of the bacterium. In addition, the current study confirmed the development of *B. pseudomallei*-specific T cell responses in patients who have recovered from melioidosis but have no detectable *B. pseudomallei*-specific antibody response as determined by the IHA. Collectively, the work described has contributed to significantly furthering our understanding of early host-pathogen interactions following infection with *B. pseudomallei* and have demonstrated that assessment of *B. pseudomallei*-specific T cell responses would be beneficial to determine exposure to this pathogen and for monitoring patients with melioidosis.

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LIST OF ABBREVIATIONS

GENERAL

AIDS	Acquired immunodeficiency syndrome
AF	Alexa fluor
ANOVA	Analysis of variance
APC	Allophycocyanin
BCA	Bicinchoninic acid
BM	Bone marrow
BMDC	Bone marrow derived dendritic cells
CBA	Cytometric bead array
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD	Cluster of differentiation
cDC	Conventional dendritic cells
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CFU	Colony forming unit
CO_2	Carbon dioxide
CMI	Cell-mediated immune
CMTMR	Chloromethyl benzoylamino-tetramethylrhodamine
CNS	Central nervous system
CPM	Counts per minute
CXCL	CXC-chemokine ligand
CXCR	CXC-chemokine receptor
Cy	Cyanine
DC	Dendritic cells
DNA	Deoxyribonucleic acid
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FLT-3L	Fms-like tyrosine kinase 3 ligand
GM-CSF	Granulocyte/macrophage colony stimulating factor
HBSS	Hanks buffered salt solution
HI-FBS	Heat-inactivated foetal bovine serum

HIV	Human immunodeficiency virus
HLA	Human leukocyte antigens
HRP	Horseradish peroxidase
IFN	Interferon
IFNAR	Interferon alpha receptor
IHA	Indirect haemagglutination assay
IL	Interleukin
iLN	Inguinal lymph node
IRF	Interferon regulatory factor
JCU	James Cook University
LD_{50}	Lethal dose (50%)
LPS	Lipopolysaccharide
MDC	Monocyte-derived dendritic cell
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
NET	Neutrophil extracellular traps
NK	Natural killer
OD	Optical density
ODN	Oligodeoxynucleotide
OmpA	Outer membrane protein A
PAMP	Pathogen-associated molecular patterns
PALS	Periarteriolar lymphoid sheaths
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PerCP	Peridinin-chlorophyll proteins
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
pLN	Popliteal lymph node
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptors
RBC	Red blood cells
RNA	Ribonucleic acid

SD	Standard deviation
SEM	Standard error of the mean
SI	Stimulation index
T2D	Type 2 diabetes
T3SS	Type three secretion system
T_{fh}	T follicular helper
T_h	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T_{reg}	T regulatory
TSB	Tryptic soy broth

UNITS OF TIME

sec	Seconds
min	Minutes
hr	Hours

UNITS OF MEASURMENTS

µm	Micrometer
nm	Nanometer
ml	Milliliter
µl	Microliter
g	Gram
mg	Milligram
µg	Microgram
pg	Picogram
M	Molar
mM	Millimolar
µM	Micromolar
˚C	Degrees Celsius
U	Units
G	Gravitational force

SYMBOLS

α	Alpha
β	Beta
γ	Gamma
μ	Micro
%	Percentage
$\pm$	Plus/minus
n	Number/sample size

CHAPTER 1
INTRODUCTION

Melioidosis is a bacterial infection caused by *Burkholderia pseudomallei*, a soil saprophyte that is typically found in tropical locations between the latitudes 20 °N and 20 °S (Cheng and Currie, 2005). In endemic regions, such as Northern Australia and Southeast Asia, high rates of incidence and mortality from melioidosis are reported. In Northern Australia, the annual incidence is 16.5 per 100,000 population, of which up to 25 % of cases are fatal (Currie *et al.*, 2004; Malczewski *et al.*, 2005). This is higher again in Northeast Thailand where the annual incidence is 21.3 per 100,000 population, with a mortality rate of up to 40.9 % (Limmathurotsakul *et al.*, 2010). Transmission of melioidosis occurs via inhalation or cutaneous inoculation of *B. pseudomallei*-contaminated water or soil. Following exposure, *B. pseudomallei* is a versatile intracellular pathogen capable of invading and persisting within a variety of host cells in various organs causing asymptomatic, acute, chronic or latent infections (Cheng and Currie, 2005). Although *B. pseudomallei* can cause disease in healthy individuals, a strong association is observed between melioidosis and type 2 diabetes (T2D; Currie *et al.*, 2004). In addition, individuals with melioidosis and T2D comorbidity demonstrate significantly increased disease severity and mortality rates from melioidosis (Currie *et al.*, 2010).

Clinical presentations of melioidosis vary widely from skin ulcers, pneumonia, parotid infections, urinary tract infections or abscesses in organs such as the spleen and prostate. Typically, patients develop acute melioidosis and the most common presentation is pneumonia (Currie *et al.*, 2010). Bacteraemia and systemic dissemination to multiple organs is a common complication observed in 60 % of cases in Australia and 100 % of cases in Thailand (Suputtamongkol *et al.*, 1994; Malczewski *et al.*, 2005; Currie *et al.*, 2010). Consequently, patients can rapidly develop sepsis and septic shock, significantly increasing the patient's risk of death (Currie *et al.*, 2000b; Wiersinga *et al.*, 2007a).

Mortality rates for melioidosis are influenced by early diagnosis and treatment, underlying patient risk factors and disease severity which is influenced by the virulence of the infecting *B. pseudomallei* strain and the route of transmission

(Barnes and Ketheesan, 2005; Cheng and Currie, 2005). Melioidosis is a challenge to diagnose and treat as there is a wide spectrum of clinical manifestations, which often mimic symptoms of other bacterial infections such as tuberculosis (Wiersinga *et al.*, 2012). Misdiagnosis of melioidosis has a significant impact on patient outcome as *B. pseudomallei* is notoriously resistant to the common antibiotics used to treat other Gram-negative infections (Ashdown, 1988; Dance *et al.*, 1988). Because *B. pseudomallei* is an intracellular pathogen capable of establishing latent infection, an extended period of antimicrobial therapy (up to 20 weeks) is required to eradicate the bacteria and reduce the potential for relapse (Rode and Webling, 1981; Currie *et al.*, 2000a; Limmathurotsakul *et al.*, 2012).

The early control of intracellular pathogens requires robust innate cell-mediated immune (CMI) responses to internalise and kill extracellular bacteria and also to destroy infected host cells. Investigations of immune cell interactions with *B. pseudomallei* have found that during the early phases of infection, macrophages and neutrophils provide important innate defence by killing *B. pseudomallei* and producing pro-inflammatory cytokines (Leakey *et al.*, 1998; Barnes and Ketheesan, 2007; Barnes *et al.*, 2008; Morris *et al.*, 2012; Rinchai *et al.*, 2012; Riyapa *et al.*, 2012; Woodman *et al.*, 2012). However, *B. pseudomallei* can also evade intracellular killing mechanisms and replicate unchecked in the host cell cytoplasm (Harley *et al.*, 1998b; Kespichayawattana *et al.*, 2000; Stevens *et al.*, 2002; Wiersinga *et al.*, 2006). Professional antigen presenting cells, such as dendritic cells (DC), play an important role in controlling intracellular infections by activating pathogen-specific cell-mediated effector function and antibody production leading to the development of immunity and immunological memory. The subsequent activation of appropriate *B. pseudomallei*-specific T cells that produce interferon (IFN)-γ upon reactivation, provides protection against disease progression in mice (Barnes *et al.*, 2004; Haque *et al.*, 2006b; Barnes and Ketheesan, 2007). It appears that dysregulated immune responses allow localised *B. pseudomallei* infection to rapidly progress to a systemic disease, characterised by significantly increased pro-inflammatory cytokine production and the development of multiple organ failure (Wiersinga *et al.*, 2007a). However, studies to date have been unable to define the signalling pathways driving the dysregulated immune cell responses and excessive cytokine production associated with melioidosis sepsis.

During infection, inflammatory responses are regulated by pro-inflammatory and anti-inflammatory cytokine networks. Pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-8, IL-12, IFN-γ and tumour necrosis factor (TNF)-α, initiate an inflammatory state including the recruitment and activation of inflammatory cells, such as macrophages, neutrophils and NK cells, which is essential for controlling the infection (Dinarello, 2000; Beutler, 2004). However, uncontrolled pro-inflammatory cytokine signalling can cause excessive immune cell infiltration and activation that can lead to pathological damage to tissues and organs, and fatal complications such as septic shock. Anti-inflammatory cytokines such as IL-4, IL-10 and IL-13, promote tissue repair and act to prevent excessive and perpetuating feedback signalling by pro-inflammatory cytokines. Excessive anti-inflammatory cytokine signalling can also be detrimental and cause immune suppression (Dinarello, 2000; Opal and DePalo, 2000). Investigations using *B. pseudomallei*-resistant (C57BL/6) and *B. pseudomallei*-susceptible (BALB/c) mice have demonstrated that resistant hosts (C57BL/6 mice) appear to produce an appropriate cytokine response against *B. pseudomallei* involving elevated, but not hyper-production of pro-inflammatory cytokines IFN-γ, TNF-α, IL-1β and IL-6. In contrast, *B. pseudomallei*-susceptible hosts (BALB/c mice) exhibit excessive production of pro-inflammatory cytokines following infection which is associated with pathological damage and mortality (Ulett *et al.*, 2000a; Koo and Gan, 2006). Similarly, patients with melioidosis demonstrate elevated plasma concentrations for IFN-γ, IL-12, IL-15 and IL-18 and this is significantly higher in patients with bacteraemic melioidosis (Brown *et al.*, 1991; Lauw *et al.*, 1999). Recently, profiling of gene expression signatures from whole blood of patients with melioidosis demonstrated that type I IFN responses were prominent (Koh *et al.*, 2013a). Although type I IFN signalling can be beneficial, excessive type I IFN signalling during infection can drive inflammatory syndromes that lead to pathological damage in the host (Trinchieri, 2010). Therefore, future studies to determine the cell types responsible for producing type I IFN in response to *B. pseudomallei* infection, along with quantifying the cytokine levels and the implications of type I IFN on disease progression are required.

Specialised IFN-producing cells, also known as plasmacytoid DC (pDC), are a subset of DC that provide innate immune responses against pathogens, primarily by rapidly

producing large quantities of type I IFN which has a range of immunomodulatory effects such as activating the cytolytic activity of NK cells and $CD8^+$ T cells (Reizis *et al.*, 2011a). The importance of pDC and type I IFNs is well described for viral infection. However, evidence that pDC are also capable of eliciting anti-bacterial activity that can be beneficial or detrimental to the host at an early stage of infection is accumulating (Schiavoni *et al.*, 2004; Decker *et al.*, 2005; Meyer-Wentrup *et al.*, 2008; Parcina *et al.*, 2013). Investigations on the type I IFN response to bacterial infections, such as *Legionella pneumophila, Chlamydia pneumoniae, Staphylococcus aureus* and *Mycobacterium tuberculosis*, have described both protective and detrimental immune suppressive functions (Ang *et al.*, 2010; Simmons *et al.*, 2010; Crother *et al.*, 2012; Parcina *et al.*, 2013). While pDC and type I IFN activate immune responses against intracellular pathogens, excessive type I IFN secretion during infection can also be detrimental by over activating anti-cellular effects, such as type I IFN-mediated apoptosis of pathogen infected cells, causing pathological damage and inflammatory syndromes in the host (Trinchieri, 2010). Although pDC are specialised IFN producing cells that are likely to be the main leukocyte responsible for type I IFN-production in response to *B. pseudomallei*, their contribution to the clearance or persistence of *B. pseudomallei* infection is unknown.

The main effector function of conventional dendritic cells (DC) is antigen presentation, which provides potent activation of naïve T cells (Steinman, 2008; Villadangos and Young, 2008). Sentinel DC at the site of infection quickly recognise and phagocytose pathogens. In response to the pathogen, DC mature into professional antigen presenting cells by up-regulating the expression of chemokine receptors driving migration to secondary lymphoid tissues, along with antigen presenting molecules and T cell co-stimulatory molecules (Banchereau *et al.*, 2000). Consequently, mature DC are equipped with the machinery to efficiently navigate through tissue and migrate to lymphoid tissues where they activate naïve T cells and polarise the activation of pathogen-specific effector T cells. In response to intracellular bacteria such as *Salmonella typhimurium* and *Listeria monocytogenes*, mature DC play an important role in the activation of adaptive immune responses, including the activation of $CD4^+$ and $CD8^+$ T cells (Yrlid *et al.*, 2001a; Brzoza *et al.*, 2004). *In vitro* investigations have demonstrated internalisation and killing of intracellular *B. pseudomallei* by DC (Charoensap *et al.*, 2008; Williams *et al.*, 2008;

Horton *et al.*, 2012). In response to infection with *B. pseudomallei*, DC developed a mature phenotype with increased expression of molecules involved in antigen presentation (MHC class II) and T cell co-stimulation (CD80 and CD86), along with increased cytokine production (IL-12 and IL-6; Williams *et al.*, 2008; Horton *et al.*, 2012). While these studies demonstrate the capacity for DC to respond to *B. pseudomallei in vitro*, the role of DC during *B. pseudomallei* infection is ill-defined and critical aspects of DC effector functions are yet to be investigated. To date there have been no *in vivo* investigations to support the findings of published *in vitro* studies on the interactions of DC and *B. pseudomallei*. Furthermore, research on the role of DC at the site of *B. pseudomallei* infection, the antigen presenting capacity and the type of adaptive immune responses activated by *B. pseudomallei*-infected DC is limited. An important aspect of DC and *B. pseudomallei* interactions that remains undetermined is the capacity of *B. pseudomallei*-infected DC to migrate from the site of infection to secondary lymphoid tissues.

Migration of mature, antigen-loaded DC from the site of infection to secondary lymphoid tissues is essential for antigen presentation to T cells. The migration of DC to and within secondary lymphoid tissues is co-ordinated by the CC-chemokine receptor 7 (CCR7) expressed on DC and its ligands, CC-chemokine ligand 19 and CCL21 (Comerford *et al.*, 2013). However, modulation or interference of DC migration could potentially affect the ability of the host to mount a protective immune response. Some intracellular bacteria, such as *Brucella suis* and *Yersinia pestis*, have been shown to evade immune detection by impairing DC migration (Velan *et al.*, 2006; Billard *et al.*, 2007). In contrast, DC migration has been reported to facilitate the dissemination of intracellular bacteria, such as *Listeria monocytogenes, Francisella tularensis* and *Streptococcus pneumoniae* (Pron *et al.*, 2001; Bar-Haim *et al.*, 2008; Rosendahl *et al.*, 2013). Similarly, *B. pseudomallei* is an intracellular pathogen capable of rapid systemic spread to multiple organs within hours of exposure and persistence within diverse host cells (Leakey *et al.*, 1998; Hoppe *et al.*, 1999; Barnes and Ketheesan, 2005). Recently, colonisation of the brain in a murine model of neurological melioidosis was found to occur via the transmigration of *B. pseudomallei*-infected monocytes/neutrophils across endothelial cells (Liu *et al.*, 2013). These studies support the hypothesis that the migration of

DC to secondary lymphoid tissue, a crucial step for initiating adaptive immune responses, may in fact facilitate the systemic dissemination of *B. pseudomallei*.

Activation of the adaptive immune system provides pathogen-specific immune responses and the development of immunological memory. Activated B cells produce pathogen-specific antibodies which facilitate neutralisation of extracellular pathogens via the classical complement cascade and promote phagocytosis by innate immune cells (Dempsey *et al.*, 2003). Although *B. pseudomallei*-specific antibody responses are detected following infection, the bacteria are able to evade humoral immune responses by actively invading host cells (Ashdown, 1981; Jones *et al.*, 1996; Ho *et al.*, 1997; Harley *et al.*, 1998b). Thus, *B. pseudomallei*-specific T cells play an important role during infection and are essential for protection against disease progression (Barnes *et al.*, 2004; Haque *et al.*, 2006b; Barnes and Ketheesan, 2007). *B. pseudomallei*-specific T cell recall responses have been demonstrated in patients who had recovered from culture confirmed melioidosis (Ketheesan *et al.*, 2002). Protective *B. pseudomallei*-specific T cell responses are also evident in healthy individuals with a history of exposure to *B. pseudomallei* but no clinical symptoms of melioidosis. Together, these studies suggest that strong *B. pseudomallei*-specific CMI responses may protect an individual from the development of clinical melioidosis (Barnes *et al.*, 2004). The assessment of *B. pseudomallei*-specific T cell responses has also proven useful for testing the efficacy of potential immunisation strategies against *B. pseudomallei* (Barnes and Ketheesan, 2007; Tippayawat *et al.*, 2009). However, assessment of *B. pseudomallei*-specific T cell responses to demonstrate exposure and to monitor the recovery of patients with melioidosis is underutilised. Rather, serological assays are often used as a diagnostic aid to detect antibody responses in presumptive cases, as confirmation of *B. pseudomallei* infection requires incubation of cultures from clinical specimens for up to 48 hr (Peacock *et al.*, 2005; Cheng *et al.*, 2006a). The indirect haemagglutination assay (IHA) is used internationally as a diagnostic aid for melioidosis. However, this assay is not standardised between laboratories and notoriously provides results with variable sensitivity (56-73%) and specificity (64-99%; Ashdown, 1987; Cheng *et al*., 2006a; Chantratita *et al.,* 2007c). In addition, despite repeat testing for antibodies on admission, during and post recovery, a subset of patients with culture-confirmed melioidosis (10%) remain persistently IHA

negative (IHA-negative patients), presumably due to the limitations associated with the IHA. Furthermore, the IHA is a poor method for monitoring patient recovery as quantification of *B. pseudomallei*-specific antibodies does not correlate with disease severity (localised infection compared to septicaemic infection) or with patient outcome (death as opposed to recovery; Ho *et al.*, 1997). Consequently, it is imperative that alternative methods to the IHA are developed that enable not only rapid diagnosis but are also useful for monitoring patient recovery.

The development of future diagnostic and vaccine strategies is reliant on understanding the immune responses to *B. pseudomallei*. Dendritic cells are likely to be central to the development of protective *B. pseudomallei*-specific CMI responses, but in doing so may facilitate disease progression. Therefore, as part the current study, the functional responses of human and murine pDC following *in vitro* exposure to *B. pseudomallei* were investigated. In addition, the effect of *B. pseudomallei* infection on DC migration *in vitro* and whether *B. pseudomallei* use the *in vivo* migration of DC as a mechanism for dissemination from the site of infection was investigated. While defining the protective CMI responses against *B. pseudomallei* is important, development of appropriate methods for monitoring patient *B. pseudomallei*-specific CMI responses is essential for patient care and for testing the efficacy of future vaccines. The current study also aimed to demonstrate the development of *B. pseudomallei*-specific T cell responses in IHA-negative patients and in doing so assess the usefulness of this assay as an informative tool for monitoring the recovery of patients with melioidosis.

Therefore, the broad aims of the work presented in this thesis are to investigate:

i) The functional responses of human and murine plasmacytoid DC to *in vitro* infection with *B. pseudomallei* (Chapter 4)
ii) The effect of *B. pseudomallei* infection on the migratory capacity of DC (Chapter 5)
iii) The potential for migrating DC to facilitate the dissemination of *B. pseudomallei* (Chapter 6)
iv) The effect of persistent *B. pseudomallei* on the ability of DC to stimulate T cell responses (Chapter 6)

v) The suitability of T cell recall assays for confirming the development of *B. pseudomallei*-specific immune responses in IHA-negative patients (Chapter 7)

CHAPTER 2
LITERATURE REVIEW

2.1 History of Melioidosis

The first description of melioidosis was by Alfred Whitmore and C.S. Krishnaswami in Rangoon, Burma 1911, who identified a fatal septicaemic disease, primarily afflicting morphine addicts (Whitmore and Krishnaswami, 1912). Whitmore and Krishnaswami demonstrated that the causative bacterium, a Gram-negative bacillus had similar biochemical and morphological characteristics to *Bacillus mallei*, now known as *Burkholderia mallei*. Initially, Whitmore and Krishnaswami reported the disease as the first human case of glanders, a then common pulmonary disease in donkeys and horses caused by *B. mallei* (Whitmore and Krishnaswami, 1912). However, upon further investigation, Whitmore and Krishnaswami discovered that the isolated bacterium could in fact be differentiated from *B. mallei.* Unlike *B. mallei*, the novel bacterium was motile and caused rapid fatality in male guinea pigs with no evidence of the Strauss reaction, exudative swelling characterised by necrotising inflammation of the scrotum (Whitmore and Krishnaswami, 1912; Whitmore, 1913).

The first documented reports of melioidosis outside of Burma were by Ambrose Thomas Stanton and William Fletcher who described the occurrence of melioidosis in humans and animals in Malaysia 1917. In 1921, the disease was officially named melioidosis by Stanton and Fletcher, based on the Greek derivatives, "melis" (distemper of asses) and "eidos" (resemblance; Stanton and Fletcher, 1921). Reports of melioidosis between 1925 and 1950 were largely confined to Southeast Asia. In 1949, melioidosis was reported for the first time in Australia. In this particular case, an outbreak of melioidosis was identified in sheep in Winton (Cottew *et al.*, 1952). The first case of human melioidosis in Australia was reported the following year when a diabetic patient died of septicaemic melioidosis in Townsville, Australia (Rimington, 1962). Appreciation of *B. pseudomallei* as a pathogen, capable of causing severe disease via inhalation of infected aerosols, increased following its classification as a category B pathogen that has the potential to be used in biological warfare by the US Centers for Disease Control and Prevention (CDC) in 2002 (Rotz

et al., 2002). Melioidosis is now recognised as a disease of significance in tropical regions, with Northern Australia and Northeast Thailand considered highly endemic with an annual incidence of 19.6 and 21.3 cases per 100,000 population, respectively (Currie *et al.*, 2004; Limmathurotsakul *et al.*, 2010).

2.2 Burkholderia pseudomallei

The *Burkholderia* genus contains over 30 species, most of which are plant pathogens or soil saprophytes (Coenye and Vandamme, 2003). Important exceptions include *B. pseudomallei* (causes melioidosis in humans and animals; Cheng and Currie, 2005), *B. mallei* (host-restricted pathogen that causes glanders in horses and donkeys, rare cases of zoonotic transmission to humans have also been documented; Khan *et al.*, 2013; Van Zandt *et al.*, 2013) and *B. cepacia* (important opportunistic pathogen that commonly afflicts patients with cystic fibrosis; Ramsay *et al.*, 2013). *B. thailandensis* is not considered a human pathogen, but has important relevance to *B. pseudomallei* as it was originally identified as an avirulent form of *B. pseudomallei* that can assimilate L-arabinose (Wuthiekanun *et al.*, 1996; Coenye and Vandamme, 2003). Our understanding of *B. pseudomallei* pathogenesis has been improved through comparative studies using avirulent *B. thailandensis* and *B. pseudomallei* (Scott *et al.*, 2013).

Burkholderia pseudomallei is a Gram-negative bacillus that is a facultative anaerobe, non-spore forming, oxidase positive and motile (Whitmore and Krishnaswami, 1912; Cheng and Currie, 2005). Wide variation in biofilm formation, colonial morphology and growth rates are observed across *B. pseudomallei* isolates (Koh *et al.*, 2013b). In addition, *B. pseudomallei* is a robust bacterium that is well documented for its ability to persist in soil and water in adverse environmental conditions such as low nutrient levels, low pH and high temperatures (Dejsirilert *et al.*, 1991; Wuthiekanun *et al.*, 1995; Lee *et al.*, 2007; Moore *et al.*, 2008; Robertson *et al.*, 2010). Our understanding of how *B. pseudomallei* isolates from different regions are related has improved since the development of molecular typing techniques. Cheng *et al.* (2004) used Multilocus Sequence Typing (MLST) to compare isolates from Australia and Thailand. This study confirmed that Australian isolates are unique to those from Thailand (Cheng *et al.*, 2004). Furthermore, no association was observed between

strain type and virulence or disease presentation. Interestingly, the discovery of a dominant ancestral gene cluster in an Australian *B. pseudomallei* isolate indicates that *B. pseudomallei* may have originated in Australia and subsequently spread to Southeast Asia (Tuanyok *et al.*, 2007).

In 2004, the entire genome of a clinical *B. pseudomallei* isolate (K96243) was sequenced for the first time (Holden *et al.*, 2004). Analysis of the sequence revealed an exceptionally large genome (7.25 megabase pairs) that is distributed across two chromosomes. Of these chromosomes, Chr1 largely controls essential functions such macromolecule biosynthesis, amino acid metabolism, cofactor and carrier synthesis, nucleotide and protein biosynthesis, chemotaxis, and mobility. Chr2 appears to be an acquired chromosome that encodes accessory functions such as adaptation to atypical conditions, osmotic protection and iron acquisition, secondary metabolism, regulation, and laterally acquired DNA (Holden *et al.*, 2004). Furthermore, comparison of *B. pseudomallei* genome with that of *B. mallei* and *B. thailandensis* has enabled understanding of the evolution of *B. pseudomallei* virulence and niche specialisation (Kim *et al.*, 2005; Yu *et al.*, 2006).

2.3 Geographical Distribution of *B. pseudomallei*

Burkholderia pseudomallei is a saprophyte that typically inhabits soil and surface water of tropical environments within the latitudes 20 ºN and 20 ºS (Figure 2.1). Since the first description of melioidosis in Burma, the world wide incidence of melioidosis has increased such that Northern Australia, along with Southeast Asia are now recognised as endemic for this disease (Cheng and Currie, 2005; Currie *et al.*, 2008). In other tropical locations, the prevalence of *B. pseudomallei* is thought to be under reported due to poor vigilance in diagnosing and reporting melioidosis cases and environmental surveying for the bacteria (Cheng and Currie, 2005). Although tropical climates are conducive for environmental persistence of *B. pseudomallei*, high prevalence of melioidosis is observed in non-tropical regions such as Taiwan (Su *et al.*, 2007). Similarly, the first Australian melioidosis outbreak in sheep in Winton is another example of this tropical disease occurring outside the typical 20 ˚N- 20 ˚S latitudes (Cottew *et al.*, 1952). The increasing reports of

melioidosis outside the typical tropical latitudes suggest that the distribution of this bacterium may be worldwide (Dance, 1991; Dance, 2000; Currie *et al.*, 2008).

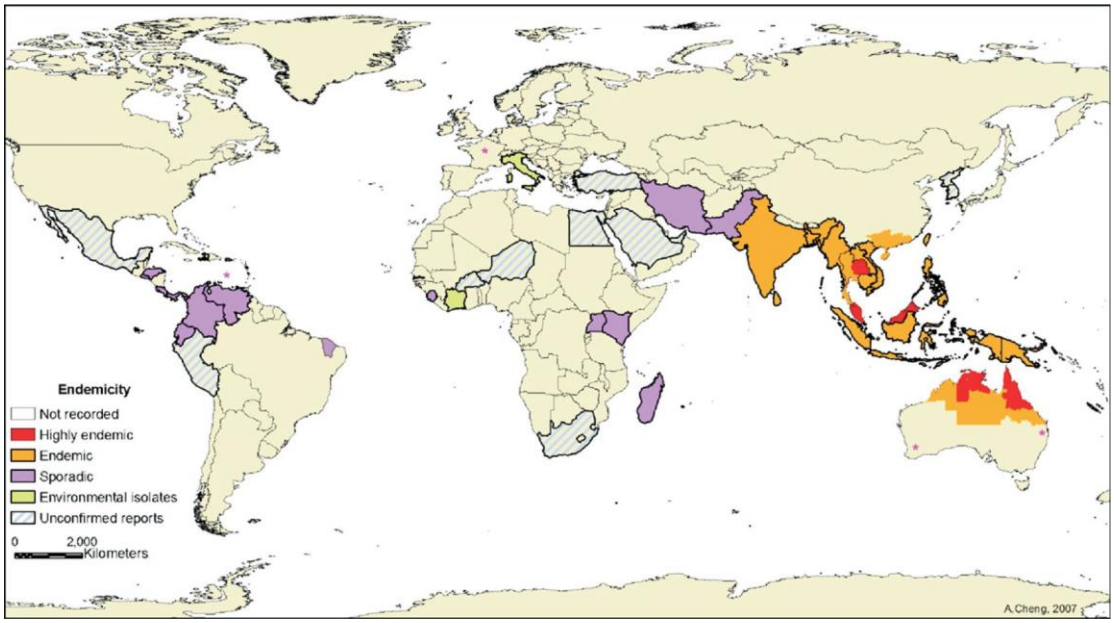

Figure 2.1 Global distributions of melioidosis
Climate and soil conditions in the tropics are conducive for *B. pseudomallei* persistence within the environment. Melioidosis is endemic in Northern Australia and Southeast Asia. In these regions, vigilant diagnosis, reporting and surveying for melioidosis occurs. In other regions, the prevalence of melioidosis is likely to be under reported due to poor clinician awareness and inability to diagnose melioidosis (Adapted from Currie *et al.*, 2008).

2.4 Incidence and Mortality Resulting from Melioidosis

Ubon Ratchatani in Northeast Thailand is reported to have the highest incidence of melioidosis. Since 1994, reports of melioidosis in Thailand has risen dramatically from approximately 4.4/100,000 population per year to 21.3/100,000 population per year in 2006 (Suputtamongkol *et al.*, 1994; Limmathurotsakul *et al.*, 2010). In Northern Australia, melioidosis is frequently reported in Darwin, where the average incidence of melioidosis is 16.5/100,000 population per year. Interestingly, the first reported case in each of these countries was not recorded until more than 30 years after the discovery of *B. pseudomallei* (Cheng and Currie, 2005). In Australia, a strong association between incidence and monsoonal rainfall is observed with 85 % of new cases occurring during the monsoonal wet season (Figure 2.2; Currie *et al.*, 2000a; Currie and Jacups, 2003). Similarly, association between incidence of melioidosis and high annual rainfall occurs in Thailand. Approximately 75 % of new

patients with melioidosis presented during the monsoonal season in Thailand between 1987 and 1991 (Suputtamongkol *et al.*, 1994). Furthermore, extreme weather events such as tropical cyclones with intense rainfall and tsunamis correlate with increased incidence of melioidosis in endemic regions. During 1998 in Darwin, Australia, the incidence of melioidosis temporarily spiked to 41.7/100,000 (Currie *et al.*, 2004; Cheng and Currie, 2005; Currie *et al.*, 2010). This increase in patients with melioidosis correlated with two tropical cyclones, Les (January 1998) and Thelma (December 1998), indicating that the incidence of melioidosis is associated with high annual rainfall and also severe weather events. Similarly, extreme weather conditions created by a category 5 Typhoon in Taiwan, correlated with a dramatic increase in incidence to 70/100,000 population (Su *et al.*, 2007). Notably, these associations are not observed in Malaysia and Singapore where distinct dry and wet seasons are not as pronounced (Heng *et al.*, 1998; Sam and Puthucheary, 2007).

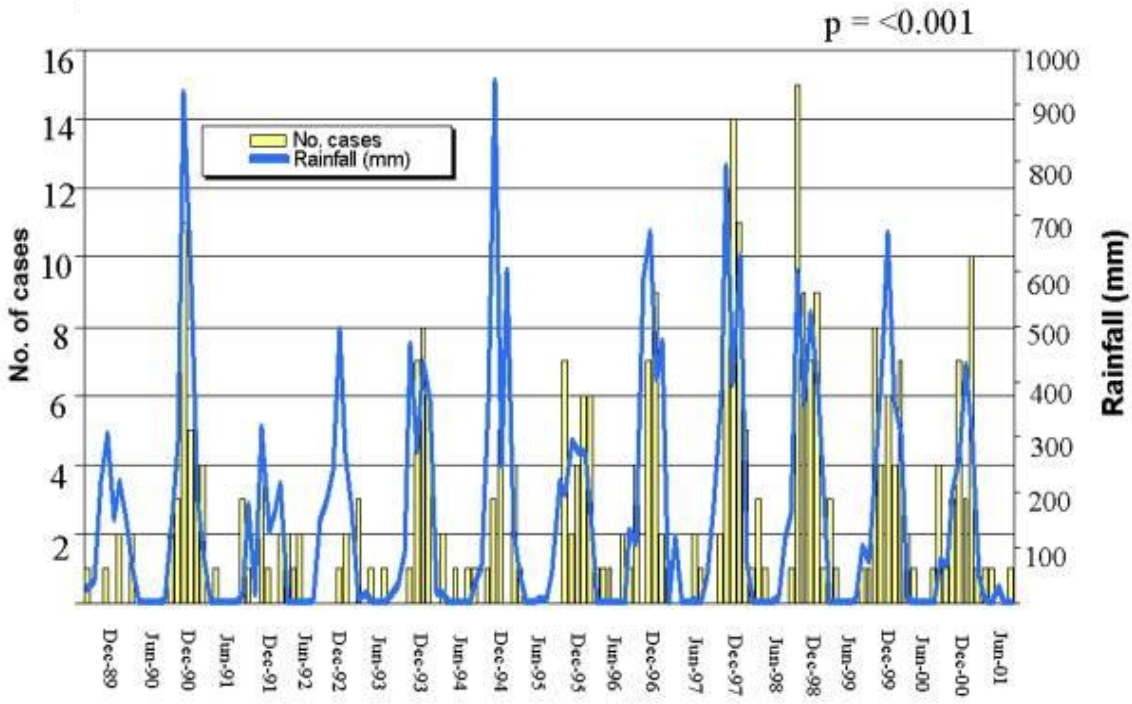

Figure 2.2 Correlation between melioidosis and rainfall in Northern Australia
In Darwin, Australia, a significant correlation (P=<0.001) is observed between the incidence of melioidosis and the intensity of rainfall, such that 85 % of patients with melioidosis present during the December to February monsoonal period (Adapted from Currie and Jacups, 2003; Currie *et al.*, 2000a;).

In addition to high incidence, significantly high mortality rates of patients with melioidosis are observed; up to 25 % for Northern Australia and up to 68 % for Northeast Thailand (White, 2003; Malczewski *et al.*, 2005; Currie *et al.*, 2010;

Limmathurotsakul *et al.*, 2010). Due to improved diagnosis and treatment of melioidosis, the mortality rate of melioidosis has declined in Australia. However, the average mortality rate remains 14 % in Darwin and 25 % in Townsville, Australia (Malczewski *et al.*, 2005; Currie *et al.*, 2010). In Ubon Ratchatani, Thailand, the mortality rate from melioidosis is 40.9 % (Limmathurotsakul *et al.*, 2010). The incidence and mortality rates associated with melioidosis highlight its importance as an emerging infectious disease that is now the third most common cause of death from an infectious disease in Thailand (Figure 2.3; Dance, 2000; Limmathurotsakul *et al.*, 2010).

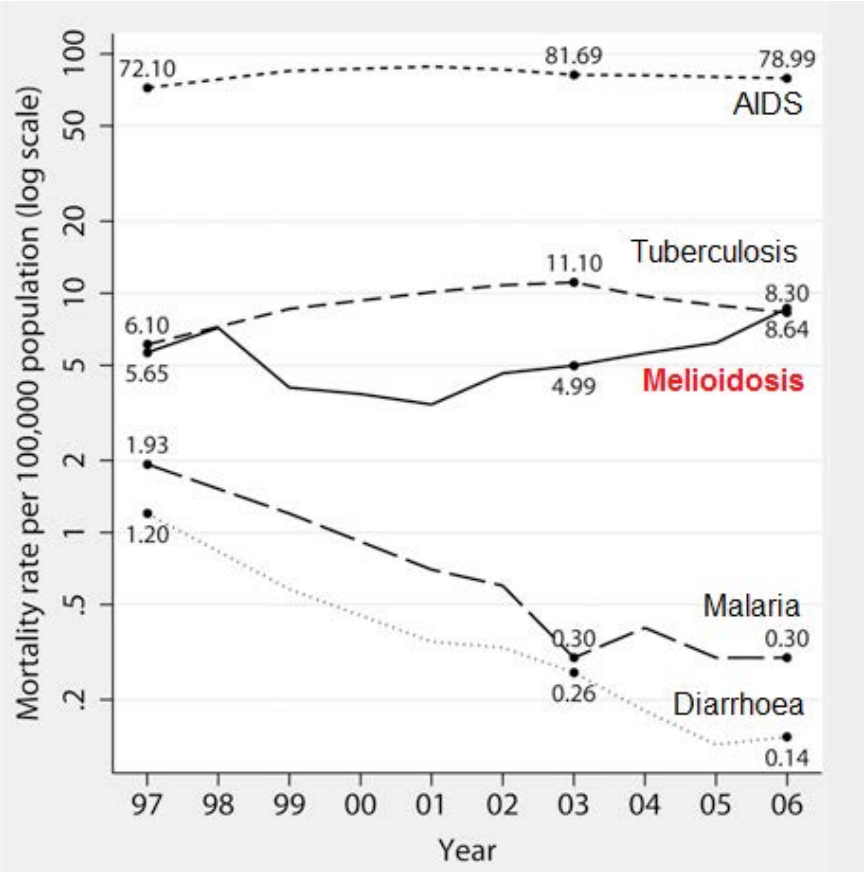

Figure 2.3 Mortality rates for the top 5 infectious diseases in Thailand between 1997 - 2006
Melioidosis causes a significant number of deaths each year in Thailand where it is the 3rd leading cause of death from an infectious disease (Adapted from Limmathurotsakul *et al.*, 2010).

2.5 Transmission of Melioidosis

The two main routes of infection for *B. pseudomallei* are inhalation of contaminated aerosols or cutaneous inoculation via exposed wounds (Cheng and Currie, 2005). The association of intense annual rainfall with increased number of new cases and disease severity, including pneumonia as the initial presentation, suggest inhalation is the primary route for infection (Suputtamongkol *et al.*, 1994; Currie *et al.*, 2000a; Currie *et al.*, 2000b; Currie, 2003; Currie and Jacups, 2003; Currie *et al.*, 2004). Further support for inhalation as the major route of infection has come from observations in the aftermath of tsunamis. After the December 2004 tsunami in Southern Thailand, an increase in the incidence of melioidosis was observed, typically aspiration pneumonia termed 'tsunami lung' following inhalation of *B. pseudomallei*-contaminated water (Potera, 2005). Wuthiekanun *et al*. (2006) also reported serological conversion of Thai residents, who were not directly affected but were bystanders to the tsunami, presumably due to exposure to aerosolised *B. pseudomallei*.

Inhalation was also suggested as a route of *B. pseudomallei* transmission during World War II and the Vietnam War when melioidosis was identified as an important disease among military personnel (Howe *et al.*, 1971). It was proposed that helicopter crew members who developed melioidosis were infected following the inhalation of contaminated aerosols generated by the updrafts of helicopter propellers (reviewed by Currie, 2003). However, the living conditions of soldiers and prisoners of war in Southeast Asia were also conducive for transmission to occur via cutaneous inoculation of wounds exposed to *B. pseudomallei*-contaminated soil and water (Howe *et al.*, 1971).

In Australia, cutaneous inoculation is a commonly reported route of transmission and epidemiological studies report that involvement of the lung following *B. pseudomallei* infection does not necessarily indicate inhalation as the route of transmission (Currie *et al.*, 2000b; Currie *et al.*, 2000c). Rather, patients with melioidosis presenting with pneumonia often reported presumptive cutaneous inoculation, suggesting that pneumonia developed as a consequence of haematogenous spread (Currie *et al.*, 2000b; Currie *et al.*, 2000c). Currie *et al*.

(2000c) also observed that following percutaneous inoculation, active *B. pseudomallei* infection often did not develop at the inoculation site rather the bacteria disseminated and colonised distant sites. Consequently, the true contribution of inhalation or cutaneous inoculation as the route of transmission has remained difficult to determine.

Non-typical routes of *B. pseudomallei* infection have also been reported and include ingestion of contaminated food or water (McCormick *et al.*, 1975; Ketterer *et al.*, 1986; Currie *et al.*, 2000a; Draper *et al.*, 2010; Mayo *et al.*, 2011). Evidence of *B. pseudomallei* infection of the stomach wall causing gastric ulcers with microabscesses, which can lead to peritonitis, is thought to occur following ingestion *B. pseudomallei*-contaminated food or water (Currie *et al.*, 2000c). Studies have also correlated *B. pseudomallei*-contaminated water supply with cases of melioidosis in Northern Australia and Northeast Thailand (Inglis *et al.*, 2000; Currie *et al.*, 2001; Mayo *et al.*, 2011; Limmathurotsakul *et al.*, 2013; Limmathurotsakul *et al.*, 2014). In experimental animal studies, ingestion of *B. pseudomallei* was found to cause disseminated infection with various bacterial colonisation sites including the brain, lung, spleen, liver and/or mesenteric lymph node (West *et al.*, 2010). The ability of *B. pseudomallei* to disseminate following presumptive inhalation, cutaneous inoculation or ingestion and colonise distal sites without causing active infection at the inoculation site makes it difficult to conclusively confirm the mode of transmission (West *et al.*, 2010).

2.6 Risk Factors for Melioidosis

Although *B. pseudomallei* can cause disease in immune-competent individuals, a number of pre-existing conditions are considered significant risk factors for the development of melioidosis. Currie *et al.* (2004) identified that susceptibility to *B. pseudomallei* infection within the Northern Territory, Australia can be attributed to but not limited to a number of risk factors including type 2 diabetes (T2D), excessive alcohol consumption and chronic lung disease (Table 2.1). Additionally, individuals that are over 45 years of age, are male or Indigenous, have a higher risk of contracting melioidosis than other members of the Australian population. It was suggested that this was likely to correlate with increased occupational or recreational

exposure (Currie *et al.*, 2004). Of the risk factors for melioidosis that were highlighted by Currie *et al.* (2004), only chronic lung disease was reported to be significantly associated with high mortality following *B. pseudomallei* infection.

A comparative study of culture confirmed cases at the Townsville Hospital within Queensland, Australia by Malczewski *et al.* (2005) reported similar findings to the Northern Territory study (Currie *et al.*, 2004; Malczewski *et al.*, 2005). Key risk factors identified for Queensland included T2D, excessive alcohol consumption, chronic lung disease, immunosuppressive drug use and renal disease (Table 2.1). Similarly, in Thailand, T2D is the most commonly associated risk factor with 46.6-60 % of patients diagnosed with melioidosis and T2D comorbidity (Suputtamongkol *et al.*, 1999; Limmathurotsakul *et al.*, 2010). However, there appear to be regional differences in other identified risk factors between Australia and Thailand. Whilst excessive alcohol consumption, chronic lung disease and the use of immunosuppressive drugs are significant risk factors for melioidosis in Australia, this association is not observed in Thailand (Suputtamongkol *et al.*, 1999; Malczewski *et al.*, 2005).

Overall T2D is the most commonly associated and well documented risk factor for melioidosis within endemic regions. In Northern Australia and Northeast Thailand, 42 % and 60 % of patients with melioidosis also have pre-existing diabetes respectively (Suputtamongkol *et al.*, 1999; Currie *et al.*, 2000b; Malczewski *et al.*, 2005; Currie *et al.*, 2010; Limmathurotsakul *et al.*, 2010). The incidence rate of melioidosis in the diabetic population is over 100 times higher at 260 and 146 cases per 100,000 population in Northern Australia and Northeast Thailand, respectively (Currie *et al.*, 2010; Limmathurotsakul *et al.*, 2010). Furthermore, significantly increased disease severity and mortality rates are observed in patients with melioidosis and comorbid T2D (Currie *et al.*, 2010). Interestingly, murine infection studies indicate that the clinical isolates responsible for causing severe forms of melioidosis in T2D individuals are significantly less virulent in mice compared to clinical isolates from non-diabetic patients (Ulett *et al.*, 2001). As the global prevalence of T2D increases, the incidence of severe life threatening forms co-morbid T2D-melioidosis infections is also predicated to increase (Whiting *et al.*, 2011; Koh *et al.*, 2012; Hodgson *et al.*, 2013).

Table 2.1 Regional differences in risk factors for melioidosis

Risk Factor	Australia		Thailand[4,5,6] (%)	Malaysia[7] (%)	Singapore[8,9] (%)	India[10] (%)
	Townsville[1] (%)	Darwin[2,3] (%)				
Male	63	69	58.5 - 62	76	83	80
ATSI origin	30	52	-	-	-	-
T2D	42	39	46.6 - 60	38	64	68
Excess Alcohol	42	39	12	-	-	28
Renal Disease	11	12	20	10	4	-
No Risk	18	20	36	24	19	25
Mortality	25	14	26 - 68	65	17 - 46	8

[1] n=57, Malczewski *et al.* (2005)
[2] n=252, Currie *et al.* (2000b)
[3] n=504, Currie *et al.* (2010)
[4] n=63, Chaowagul *et al.* (1989)
[5] n=204, Suputtamongkol *et al.* (1999)
[6] n=2217, Limmathurotsakul *et al.* (2010)
[7] n=50, Puthucheary *et al.* (1992)
[8] n=185, Singapore Committee on Epidemic Diseases (1995)
[9] n=23, Lim *et al.* (1997)
[10] n=25, Saravu *et al.* (2010)

2.7 Clinical Presentations of Melioidosis

Exposure to *B. pseudomallei* can lead to the onset of infection and symptoms within 24 hr to a number of weeks. In the Northern Territory, Australia the documented incubation period for *B. pseudomallei* infection ranges between 1 to 21 days with a mean of 9 days (Currie *et al.*, 2000a). However, in some instances *B. pseudomallei* infection can remain latent for many years (Ngauy *et al.*, 2005). Following exposure, patients with melioidosis can develop a variety of clinical presentations, such as pneumonia, skin ulcers or abscesses in organs, with varying degrees of severity from acute to chronic or even asymptomatic/latent infections (Figure 2.4; Wiersinga *et al.*, 2012).

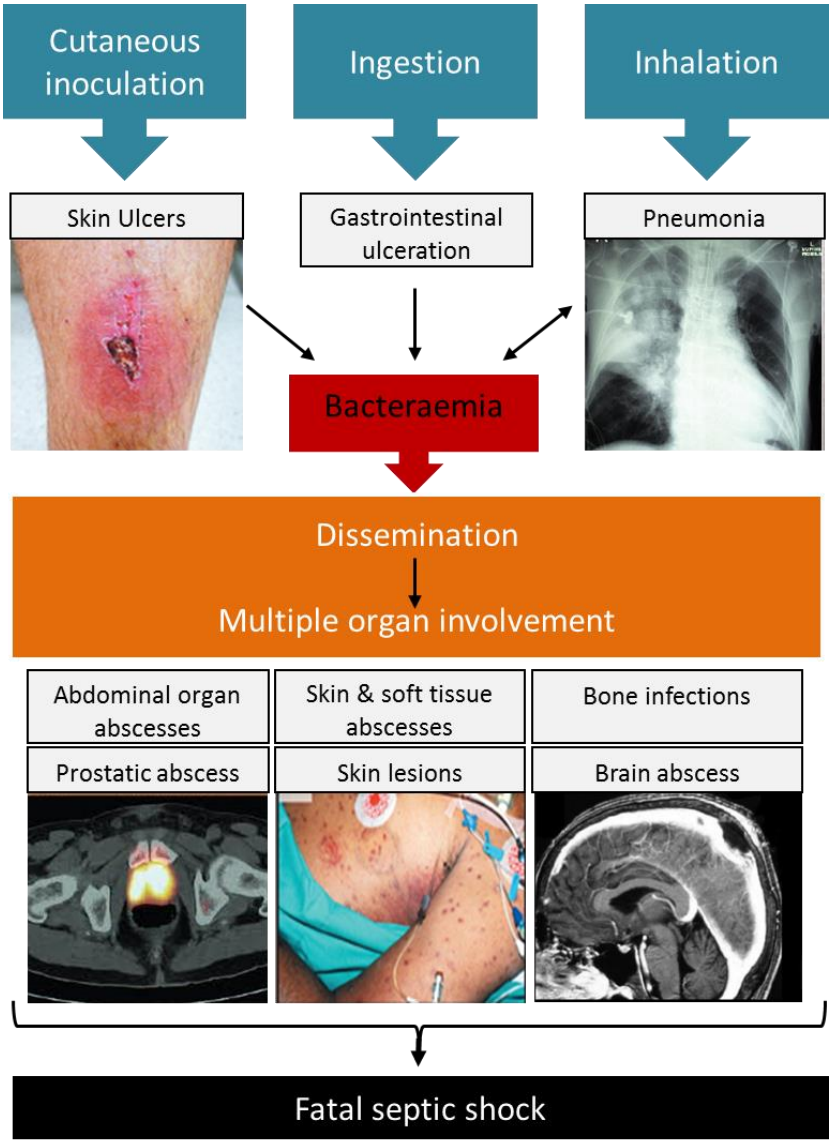

Figure 2.4 Presentations of melioidosis
Patients with melioidosis can develop a wide range of clinical presentations that are commonly complicated by bacteraemia, which can rapidly lead to fatal septic shock (Arzola *et al.*, 2007; Bommakanti *et al.*, 2010; Wiersinga *et al.*, 2012).

Host risk factors, along with infectious dose, differences in *B. pseudomallei* strain virulence and route of infection all influence the clinical presentation, disease severity and patient outcome (Currie, 2008). Early diagnosis and provision of appropriate antibiotic therapy are crucial for preventing mortality (Cheng and Currie, 2005). The wide variation in symptomatic presentation makes diagnosis of melioidosis difficult. Typically, pneumonia is the most common presentation (Table 2.2). Irrespective of the initial presentation, bacteraemia leading to systemic spread of *B. pseudomallei* is a common complication (Cheng and Currie, 2005; Malczewski *et al.*, 2005).

Table 2.2 Comparison of organ involvement during initial presentation with melioidosis

Organ Involved	Australia		Thailand[4,5,6] (%)	Malaysia[7] (%)	Singapore[8,9] (%)
	Townsville[1] (%)	Darwin[2,3] (%)			
Lung	58	50	25	22	52
Genitourinary	11	15	0	-	0
Skin/Soft tissue	9	17	22	10	48
Bone/Joint	4	4	-	0	0
CNS	4	4	0	-	0
No source	12	-	51	24	0
Other	4	11	2	44	-
Bacteraemia	60	46 - 55	100	100	-
Mortality	25	14 - 19	26 - 68	65	17 - 46

Abbreviations: CNS – central nervous system
[1] n=57, Malczewski *et al.* (2005)
[2] n=252, Currie *et al.* (2004)
[3] n=504, Currie *et al.* (2010)
[4] n=63, Chaowagul *et al.* (1989)
[5] n=204, Suputtamongkol *et al.* (1999)
[6] n=2217, Limmathurotsakul *et al.* (2010)
[7] n=50, Puthucheary *et al.* (1992)
[8] n=85, Singapore Committee on Epidemic Diseases (1995)
[9] n=23, Lim *et al.* (1997)

Acute melioidosis is defined as an illness with a duration of less than two months (Currie *et al.*, 2000a; Malczewski *et al.*, 2005). Subacute melioidosis refers to acute cases of short duration but where symptoms were present for longer than 2 weeks (up to 2 months) prior to presentation. In Northern Territory, Australia 85 % of patients

with melioidosis presented with acute melioidosis between 1989 and 2009 (Table 2.3; Currie *et al.*, 2000b; Currie *et al.*, 2010). A similar study within North Queensland, Australia, reported acute melioidosis in 92 % of patients that presented between 1996 to 1994 (Malczewski *et al.*, 2005). Common presentations of acute melioidosis are pneumonia or an acute septicaemic illness in conjunction with a high fever and multi-organ dissemination, which is difficult to distinguish from other acute bacterial infections (Ip *et al.*, 1995; Currie *et al.*, 2010). Other clinical presentations of acute melioidosis include localised suppurative infection or a disseminated disease that may develop as a milder form of acute pneumonia. The increased association of mortality with acute melioidosis and the development of septic shock are highlighted in Table 2.3 (Currie *et al.*, 2010).

Table 2.3 Presentation and outcomes of melioidosis in Darwin, Australia between 1989 - 2009

	Total		Bacteraemic	
	Number	**Deaths (Mortality %)**	**Number**	**Deaths (Mortality %)**
Septic Shock	**116**	**58 (50%)**	**103**	**48 (47%)**
Pneumonia	88	43 (49%)	78	35 (45%)
No focus	13	8 (62%)	12	7 (58%)
Genitourinary	10	5 (50%)	9	4 (44%)
Osteomyelitis/ Septic Arthritis	4	2 (50%)	4	2 (50%)
Soft Tissue Abscess	1	0	0	0
Non-septic shock	**424**	**19 (4%)**	**195**	**13 (7%)**
Pneumonia	190	12 (16%)	89	9 (10%)
Skin Infections	68	0	1	0
Genitourinary	66	2 (3%)	41	2 (5%)
No focus	52	2 (4%)	47	2 (4%)
Soft Tissue Abscess(es)	18	0	4	0
Osteomyelitis/ Septic Arthritis	16	0	10	0
Neurological	14	3 (21%)	3	0
Total	**540**	**77 (14%)**	**298**	**61 (20%)**

(Adapted from Currie *et al.*, 2010)

Chronic melioidosis is defined as disease of duration for greater than two months, often a long standing localised suppurative abscess, typically a skin infection but can involve any organ (Cheng and Currie, 2005). Symptoms associated with chronic pulmonary melioidosis can mimic tuberculosis (Everett and Nelson, 1975; Ip *et al.*, 1995). In Australia, chronic melioidosis has been documented in 8 % and 11 % of melioidosis cases in North Queensland and Northern Territory, respectively (Currie *et al.*, 2000b; Malczewski *et al.*, 2005; Currie *et al.*, 2010). As patients diagnosed with chronic melioidosis respond well to appropriate antibiotic therapy, fatalities from chronic melioidosis are uncommon (Currie *et al.*, 2000a).

In some instances, no apparent clinical disease develops immediately following infection with *B. pseudomallei*. In these asymptomatic or latent forms of melioidosis, the host immune response is sufficient to control but not eliminate the bacterium. Thus, disease may become clinically apparent at a later time if the immune system subsequently becomes compromised (Ip *et al.*, 1995). Cases of latent melioidosis have spanned decades with the longest documented latency period being 62 years (Ngauy *et al.*, 2005). In this particular case, the only known exposure to *B. pseudomallei* occurred while the patient was held as a prisoner of war in Thailand during WWII by Japanese forces. The patient was diagnosed with melioidosis 62 years later after presenting with a non-healing ulcer on his right hand following a dog bite. In this case, the activation of symptomatic melioidosis from a latent *B. pseudomallei* infection after a long period of latency was associated with the development of diabetes (Ngauy *et al*., 2005). Between 1989 and 1998 in the Northern Territory, Australia, 4 % of patients with melioidosis had a history that suggested reactivation from a latent focus (Currie *et al.*, 2000b; Currie *et al.*, 2010).

2.8 Diagnosing Melioidosis

Confirmation of presumptive melioidosis is by culture of *B. pseudomallei* from clinical specimens. As this bacterium is not considered to be part of the normal microbial flora of humans, the detection of *B. pseudomallei* in any clinical specimen collected from a patient is deemed a definitive diagnosis of melioidosis (Cheng *et al.*, 2013). Isolation of *B. pseudomallei* from clinical specimens can be difficult as the bacterium is slow growing and variations in colonial morphology occur. Therefore,

all clinical specimens from patients with suspected melioidosis are cultured on blood agar and on a *B. pseudomallei* selective culture media, such as Ashdown agar (Ashdown, 1979), which promotes growth and are selective and differential for this bacterium. It was suggested by Howard and Inglis (2003) that their new *B. pseudomallei* selective agar (BPSA) was a more powerful diagnostic tool than Ashdown agar, facilitating faster growth of the bacteria and increased the ability to isolate persistently mucoid *B. pseudomallei* strains that are normally inhibited by crystal violet in Ashdown agar. However, when Peacock *et al*. (2005) independently compared Ashdown agar, *B. cepacia* medium and BPSA for clinical isolation of *B. pseudomallei*, it was found that BPSA was less selective for *B. pseudomallei* than Ashdown agar and supporting the continued use of Ashdown agar as the standard medium for isolation.

Burkholderia pseudomallei can be cultured from a variety of patient samples. Typically, blood culture is the most common although urine, sputum and specimens from infected lesions can also be used. Positive detection and quantification of *B. pseudomallei* in blood or urine can also be used as a predictor of mortality. Blood specimens with more than 100 colony forming units (CFU) per ml of blood are associated with 96 % mortality (Wuthiekanun and Peacock, 2006). Similarly, urine specimens with more than 10^5 CFU/ml are associated with 71 % mortality (Limmathurotsakul *et al.*, 2005)

The indirect haemagglutination assay (IHA) is internationally used as a diagnostic aid for quantifying the antibody response to *B. pseudomallei* but provides results with variable sensitivity (73 %) and specificity (64 %; Chantratita *et al.*, 2007c). Furthermore, the assay is poorly standardised between laboratories, which use different *B. pseudomallei* strains and protocols for antigen preparation (Ashdown, 1987; Wuthiekanun *et al.*, 2006a; Harris *et al.*, 2009). Antigens derived from *B. pseudomallei* are used to sensitise sheep red blood cells (RBC). Inactivated human serum, pre-absorbed for non-specific RBC agglutinins, is serially diluted then incubated with sensitised or non-sensitised RBC. The presence of *B. pseudomallei*-specific antibodies in the human serum is detected when agglutination of the sensitised RBC occurs. The highest serum dilution is recorded as the antibody titre. In Australia, a titre of ≥ 1:40 is indicative of positive serology for melioidosis where

2.4 % of the healthy population in Northern Australia are sero-positive for *B. pseudomallei* (Lazzaroni *et al.*, 2008). In Thailand, where 20-30 % of the healthy population are sero-positive for melioidosis, the cut-off titre is set at ≥ 1:160 (Naigowit *et al.*, 1992; Wuthiekanun *et al.*, 2006b). Although the IHA is useful for quickly confirming whether a patient has been exposed to *B. pseudomallei*, as a rapid diagnostic assay for melioidosis the IHA performs poorly and is unable to confirm active *B. pseudomallei* infection. Interpretation is also complicated by the sero-positivity rates in the population and increasing the positive titre cut-off decreases sensitivity, for example using a diagnostic cut-off titre of ≥ 1:160 reduces the sensitivity to 40 % in Australia (Cheng *et al.*, 2006a). Furthermore, only 51 % of patients with culture confirmed melioidosis have positive IHA serology (IHA-positive patients) on admission (Harris *et al.*, 2009). On subsequent testing, the majority of patients with initial negative IHA serology demonstrate sero-conversion and yield a positive IHA result. However, approximately 10 % of patients with culture-confirmed melioidosis remain persistently negative (IHA-negative patients). While the presence of bacteraemia predicted an initially negative IHA serology, no specific patient factors correlate with persistently negative IHA serology (Harris *et al.*, 2009). Therefore, the adaptive cell-mediated immune (CMI) response of IHA-negative patients has been investigated in Chapter 7.

Due to the poor sensitivity and specificity of the IHA, a number of attempts have been made to develop an improved, rapid, sensitive and specific diagnostic assay for melioidosis (Table 2.4 and 2.5). Although new diagnostic assays are often reported to have improved sensitivity and specificity compared to the IHA, unlike the IHA, these new assays are often not suitably validated and are not generally accepted as a reliable alternative for diagnosing melioidosis (Peacock *et al.*, 2011). A limitation of all antibody detection assays is the inability of these assay formats to confirm active *B. pseudomallei* infection. *B. pseudomallei* antigen detection assays using latex agglutination, lateral flow, polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP) formats have the potential to rapidly detect active *B. pseudomallei* infection (Table 2.5). Currently, a new point-of-care Active Melioidosis Detect Lateral Flow Immunoassay (AMD LFI) is undergoing testing in Australia and Thailand (Houghton *et al.*, 2014). When tested using bacterial

suspensions or 'spiked' patient serum, the AMD LFI was reported to have high sensitivity (98.7) and specificity (97.2; Table 2.5) when compared to near neighbor isolates such as *B. thailandensis* and *B. cepacia*. However, the assay has 90.9% cross-reactivity to *B. mallei.* Although the assay is designed to test a range of clinical samples including serum and urine, the limit of detection for this assay is estimated to be greater than 1.2 x 10^4 CFU/ml. This high limit of detection poses a significant limitation for the assay (Houghton *et al*., 2014).

Table 2.4 Antibody detection assays for melioidosis

Assay	Clinical sample	Detects	Sensitivity (%)	Specificity (%)	Benefits and Limitations	Reference
IHA	Serum	Antibodies against *B. pseudomallei*	56-73	64-99	Inexpensive, validated, protocols not standardised	Ashdown (1987); Cheng *et al.* (2006a); Chantratita *et al.* (2007c)
ELISA	Serum	IgG / IgM antibodies against *B. pseudomallei*	64-95	72-99	Sensitivity and specificity is affected by antigen preparation	Ashdown *et al.* (1989); Chantratita *et al.* (2007c)
IFAT	Serum	IgG and/or IgM antibodies against *B. pseudomallei*	91-95	95-100	Impractical for resource poor countries	Ashdown *et al.* (1989); Vadivelu and Puthucheary (2000)
ICT	Serum	IgG / IgM antibodies against *B. pseudomallei*	50.6-100	69-95	No specialised expertise or equipment required, discrepancy in sensitivity and specificity	Cuzzubbo *et al.* (2000); Chuah *et al.* (2005); Cheng *et al.* (2006b)
2DIA	Serum	Antibodies against *B. pseudomallei*	100	87.1	Tested in a small study	Sorenson *et al.* (2013)

Abbreviations: 2DIA – 2-dimensional immunoarray, ELISA – enzyme linked immunosorbent assay, ICT – immunochromatographic card test, IHA – indirect haemagglutination assay, IFAT – immunofluorescent antibody test, LPS – lipopolysaccharide.

Table 2.5 Antigen detection assays for melioidosis

Assay	Clinical sample	Detects	Sensitivity (%)	Specificity (%)	Benefits and Limitations	Reference
Latex agglutination	Blood cultures	Exopolysaccharide of *B. pseudomallei*	99.1 - 100	99.7	Requires 1-3 days for blood culture incubation step, 'in-house' assays yet to be standardised between laboratories	Amornchai *et al.* (2007); Ekpo *et al.* (2007); Hodgson *et al.* (2009); Duval *et al.* (2014)
PCR	Any clinical specimen	*B. pseudomallei* DNA	34-41$^{(c)}$ 86.7-100$^{(RT)}$	47.4-100$^{(c)}$ 88.2-100$^{(RT)}$	Expensive, sensitivity and specificity has been improved by using RT PCR	Chantratita *et al.* (2007b)
LAMP	Any clinical specimen	*B. pseudomallei* DNA	44	98.4	Inexpensive but lacks sensitivity especially when applied to blood samples	Chantratita *et al.* (2008)
Mab-IFA	Blood cultures	Exopolysaccharide of *B. pseudomallei*	97.4	100	Requires 1-3 days for blood culture incubation step, expensive	Chantratita *et al.* (2013)
AMD LFI	Serum and urine	Capsular polysaccharide of *B. pseudomallei*	98.7	97.2	Currently being tested for commercialisation	Houghton *et al.* (2014)

Abbreviations: AMD LFI - active melioidosis detect lateral flow immunoassay, c – conventional, LAMP – loop-mediated isothermal amplification, Mab-IFA – monoclonal antibody-based immunofluorescent assay, PCR – polymerase chain reactions, RT – real-time.

2.9 Pathogenesis of *B. pseudomallei* Infection

2.9.1 Murine models of melioidosis

Experimental infection of laboratory animals, including mice, has facilitated improved understanding of the pathogenesis of melioidosis. Studies using murine models have enabled comparison of the virulence of different *B. pseudomallei* strains, investigation of how this bacterium evades host defences, the importance of particular components of the immune system in providing protection and the testing of strategies for providing protection or developing immunity against melioidosis. The genetically defined inbred BALB/c mouse strain (Table 2.6) has been identified as a murine model for acute human melioidosis. This particular strain demonstrates high susceptibility to *B. pseudomallei* and appears to mimic acute melioidosis as seen in humans (Leakey *et al.*, 1998; Barnes and Ketheesan, 2005). Following infection, BALB/c mice demonstrate poor ability to control *B. pseudomallei* replication, which rapidly leads to dissemination, colonisation of multiple organs and hyper production of pro-inflammatory cytokines causing excessive inflammation that damages host tissues resulting in mortality within 72 hr (Ulett *et al.*, 2000a; Liu *et al.*, 2002; Massey *et al.*, 2014).

In contrast, the genetically defined black inbred C57BL/6 mouse strain (Table 2.6) is partially resistant to *B. pseudomallei* infection and provides an animal model for chronic human melioidosis (Leakey *et al.*, 1998; Conejero *et al.*, 2011). The course of infection and persistence of *B. pseudomallei* in C57BL/6 mice varies with solate virulence, dose and route of infection (Barnes and Ketheesan, 2005). In C57BL/6 mice infected with a low dose of *B. pseudomallei*, bacterial persistence in the lungs, spleen and blood can be observed 50 days post-infection. Similar to the heterogeneous clinical spectrum seen in patients with chronic melioidosis, the organ load and presence of visible abscesses varied from animal to animal (Conejero *et al.*, 2011). Partial resistance of the C57BL/6 strain has been attributed to early control of bacterial replication by innate immune responses in these mice, including enhanced microbial efficiency of peritoneal macrophages compared to susceptible BALB/c mice. (Leakey *et al.*, 1998; Hoppe *et al.*, 1999; Ulett *et al.*, 2000a). The C57BL/6 mice also develop a protective T_h1 response and appear to produce an appropriate

cytokine response against *B. pseudomallei* involving elevated, but not hyper-production of pro-inflammatory cytokines (Ulett *et al.*, 2000b; Ulett *et al.*, 2000a; Tan *et al.*, 2008). In contrast, *B. pseudomallei*-infected BALB/c mice exhibit excessive production of pro-inflammatory cytokines that is detrimental to the host (Ulett *et al.*, 2000a; Koo and Gan, 2006; Tan *et al.*, 2008). These studies indicate that susceptibility to *B. pseudomallei* is due to uncontrolled bacterial replication initiating an overwhelming cytokine response that drives excessive inflammation and death (Gan, 2005).

Together, BALB/c and C57BL/6 mice are used as contrasting models of acute and chronic melioidosis to improve our understanding of host-pathogen interactions during *B. pseudomallei* infection. Infection studies have shown that although host factors play a role in the acute susceptibility of BALB/c mice compared to C57BL/6 mice, the route of infection and differences in the virulence of *B. pseudomallei* isolates also influences disease severity. Barnes and Ketheesan (2005) used the Reed and Muench (1938) method to calculate the 50% lethal dose (LD_{50}) for two *B. pseudomallei* isolates of high and low virulence in BALB/c and C57BL/6 mice using different routes of infection (intranasal, oral, intravenous, intraperitoneal and subcutaneous). Lower doses of *B. pseudomallei* consistently caused lethality in BALB/c mice compared to C57BL/6 mice however; the route of infection also dramatically affected the virulence of the bacterium in these murine models of melioidosis (Barnes and Ketheesan, 2005). The findings of Barnes and Ketheesan (2005) has been supported by a number of studies using different *B. pseudomallei* isolates (Liu *et al.*, 2002; Conejero *et al.*, 2011; Laws *et al.*, 2011; Massey *et al.*, 2014).

Table 2.6 Differences identified between C57BL/6 and BALB/c mice following infection with *B. pseudomallei*

BALB/c	C57BL/6	Reference
		The Jackson Laboratory (2014)
Highly susceptible, model for acute human melioidosis	Partially resistant, model for chronic human melioidosis	Leakey *et al.* (1998); Hoppe *et al.* (1999); Liu *et al.* (2002); Barnes and Ketheesan (2005); Conejero *et al.* (2011); Massey *et al.* (2014)
LD_{50} values when infected with NCTC 13178 s.c. = $1x10^3$ CFU i.v. = <10 CFU i.n. = $1.4x10^2$ CFU	LD_{50} values when infected with NCTC 13178 s.c. = $9x10^5$CFU i.v. = $5x10^3$ CFU i.n. = $1.8x10^3$CFU	Barnes and Ketheesan (2005)
LD_{50} values when infected with NCTC 13179 s.c. = $9x10^2$ CFU i.v. = $9x10^3$ CFU i.n. = $1.9x10^6$ CFU	LD_{50} values when infected with NCTC 13179 s.c. = $>10^8$ CFU i.v. = $6x10^6$ CFU i.n. = $>10^8$ CFU	Barnes and Ketheesan (2005)
Hyper production of pro-inflammatory cytokines	Moderate production of pro-inflammatory cytokines	Hoppe *et al.* (1999); Ulett *et al.* (2000b); Ulett *et al.* (2000a); Barnes *et al.* (2001); Liu *et al.* (2002); Tan *et al.* (2008)
Cytokines peak at 24-48 hr post-infection	Cytokines peak at 48-72 hr post-infection	Ulett *et al.* (2000b); Ulett *et al.* (2000a)
Excessive neutrophil infiltration, reduced recruitment of macrophages and lymphocytes	Influx of neutrophils, macrophages and lymphocytes	Santanirand *et al.* (1999); Conejero *et al.* (2011); Massey *et al.* (2014)
Poor killing by macrophages	Efficient killing by macrophages	Leakey *et al.* (1998); Breitbach *et al.* (2006); Barnes and Ketheesan (2007)
Increased bacterial loads and tissue necrosis	Lower bacterial loads with focal containment	Hoppe *et al.* (1999); Conejero *et al.* (2011); Massey *et al.* (2014)

Abbreviations: i.n. – intranasal, i.v. – intravenous, LD_{50} – 50 % lethal dose; CFU – colony forming units, s.c. – subcutaneous.

In addition to providing a model for investigating the pathogenesis of *B. pseudomallei*, murine models of inhalation melioidosis have been further

characterised in order to test therapeutic and preventative strategies (Liu *et al.*, 2002; Conejero *et al.*, 2011; Gelhaus *et al.*, 2013; Massey *et al.*, 2014). To date, a range of potential vaccine strategies have been tested using live attenuated, whole killed and a variety of antigens prepared from *B. pseudomallei* which have been administered using a number of routes including intranasal, intraperitoneal, subcutaneous and intramuscular injection (Peacock *et al.*, 2012). Studies suggest that the protective efficacy of tested vaccines is affected by the strain of *B. pseudomallei*, the antigen preparation used and also the route of challenge post immunisation (Barnes and Ketheesan, 2007; Sarkar-Tyson *et al.*, 2009). To date, vaccine strategies have been unsuccessful and despite vaccine candidates providing up to 80% protection against intraperitoneal challenge most fail to provide protection against intranasal challenge (Sarkar-Tyson *et al.*, 2009; Peacock *et al.*, 2012).

2.9.2 *B. pseudomallei* virulence factors

Immunity to an infectious agent requires co-ordination of the innate and adaptive immune responses. Following infection, the innate immune responses provide early defence, recognising and controlling foreign pathogens as they enter the body (Murphy *et al.*, 2011). The subsequent activation of the adaptive immune system is central for providing pathogen-specific effector function and the development of immunity and immunological memory (Ketheesan *et al.*, 2002; Haque *et al.*, 2006a; Barnes and Ketheesan, 2007). *B. pseudomallei* is a competent intracellular bacterium that has a broad host cell range infecting both non-phagocytic cells (such as epithelial cells of the skin and respiratory tract) and phagocytic cells (such as macrophages and neutrophils). Following invasion of host cells, the bacteria evade intracellular killing mechanisms then persist within the cytoplasm of host cells (Allwood *et al.*, 2011). Interest in understanding the virulence factors that enable *B. pseudomallei* to establish infection and evade the immune responses of the host has been the key focus of numerous studies over the past two decades (Table 2.7). Resistance to antimicrobial agents and attachment of *B. pseudomallei* to epithelial cells is aided by pili, capsule and lipopolysaccharide (LPS; Ahmed *et al.*, 1999; Gori *et al.*, 1999; Reckseidler *et al.*, 2001; Kespichayawattana *et al.*, 2004). Following attachment, *B. pseudomallei* invades non-phagocytic cells and actively promotes its

internalisation by phagocytic cells (Jones *et al.*, 1996; Kespichayawattana *et al.*, 2000).

Table 2.7 Virulence factors described for *B. pseudomallei*

Virulence Factor	Putative Role	Reference
Capsule	Epithelial attachment, resistance to complement	Ahmed *et al.* (1999); Reckseidler-Zenteno *et al.* (2005); Reckseidler-Zenteno *et al.* (2010)
Pili	Epithelial attachment, microcolony/biofilm formation	Brown *et al.* (2002); Essex-Lopresti *et al.* (2005); Boddey *et al.* (2006)
Adhesins	Epithelial attachment	Balder *et al.* (2010)
LPS	Resistance to complement and defensins, evade innate immune recognition	DeShazer *et al.* (1998); Burtnick and Woods (1999); Novem *et al.* (2009)
Flagella	Motility	DeShazer *et al.* (1997); Chantratita *et al.* (2014)
Quorum sensing	Stationary phase gene regulation, including secreted enzymes and oxidative stress protein	Valade *et al.* (2004); Lumjiaktase *et al.* (2006)
Type III secretion system 1 (T3SS1)	Intracellular survival and replication	Rainbow *et al.* (2002); D'Cruze *et al.* (2011)
Type III secretion system 3 (T3SS3)	Invasion and vacuolar escape. Secretion of proteins, BsaZ and BipD - lyse endosomal membrane, BopE - rearrangement of actin cytoskeleton, BopA - lyse cell membrane.	Stevens *et al.* (2002); Stevens *et al.* (2003); Burtnick *et al.* (2008)
Type VI secretion system 1 (T6SS1)	Intracellular survival	Burtnick *et al.* (2011)
Morphotype switching	Alteration of surface determinants for *in vivo* phenotypic changes	Chantratita *et al.* (2007a); Chantratita *et al.* (2012)
Siderophore	Iron acquisition	Yang *et al.* (1993)
Global regulator RpoS	Regulate multinuclear cell formation, interfere with macrophage iNOS expression and induces apoptotic cell death in macrophages	Utaisincharoen *et al.* (2006); Lengwehasatit *et al.* (2008)
TssM	Deubiquitinase, suppresses host immune response by inhibiting NF-κB signalling and type I IFN production in macrophages	Tan *et al.* (2010)

(Adapted from Lazar *et al*., 2009)

2.9.3 Invasion of host cells by *B. pseudomallei*

The type III secretion system of *B. pseudomallei* is responsible for its ability to invade host cells. Three loci within the *B. pseudomallei* genome are reported to encode a T3SS designated T3SS1, T3SS2 and T3SS3 (Rainbow *et al.*, 2002; Stevens *et al.*, 2002). T3SS1 and T3SS2 share homology with T3SS of the plant pathogens *Ralstonia solanacearum* and *Xanthomonas* spp (Winstanley *et al.*, 1998). Warawa and Woods (2005) demonstrated that the T3SS1 and T3SS2 of *B. pseudomallei* facilitates infection of tomato plants but are not required to establish an infection in hamsters. The contribution of T3SS1 and T3SS2 to the virulence of *B. pseudomallei* is poorly defined, although D'Cruze *et al*. (2011) suggest that the T3SS1 is involved the intracellular survival of *B. pseudomallei* (D'Cruze *et al.*, 2011).

Investigations of T3SS3 illustrate the importance of this secretion apparatus for invasion and intracellular survival of *B. pseudomallei* within eukaryotic cells (Sun and Gan, 2010). The *B. pseudomallei* T3SS3 is predicted to share homology with the *inv/spa/prg* T3SS from *Salmonella enterica* subspecies *enterica* serotype typhimurium (hence forth referred to as *S. typhimurium*) and *ipa/mxi/spa* T3SS from *Shigella flexneri* (Stevens *et al.*, 2002). These particular T3SS play an important role in facilitating invasion and intracellular survival of *S. typhimurium* and *S. flexneri* (Wiersinga *et al.*, 2006). The mechanism of these T3SS is described as a molecular syringe; the apparatus secretes translocator proteins, which interact with the host cell membrane injecting secreted effector proteins directly into the cytoplasm of a target cell (Figure 2.5). These effector proteins mimic eukaryotic enzyme structure and function, hijacking host cell processes to benefit the bacteria, such as promoting bacterial internalisation, which has been shown for *S. typhimurium* T3SS (Stevens and Galyov, 2004).

A type VI secretion system encoded by 6 gene clusters (T6SS1, T6SS2, T6SS3, T6SS4, T6SS5 and T6SS6) has also been described for Gram-negative bacteria, including *B. pseudomallei* (Schell et al., 2007). Structurally, the T6SS is thought to resemble the tail of a bacteriophage and functions to inject effector proteins into the cytosol of host cells (Hood *et al.*, 2010). The T6SS1 appears to be involved in actin-based motility, multinucleated giant cell formation and intracellular survival of

Burkholderia mallei, although this is yet to be proven for *B. pseudomallei* (Burtnick et al., 2011).

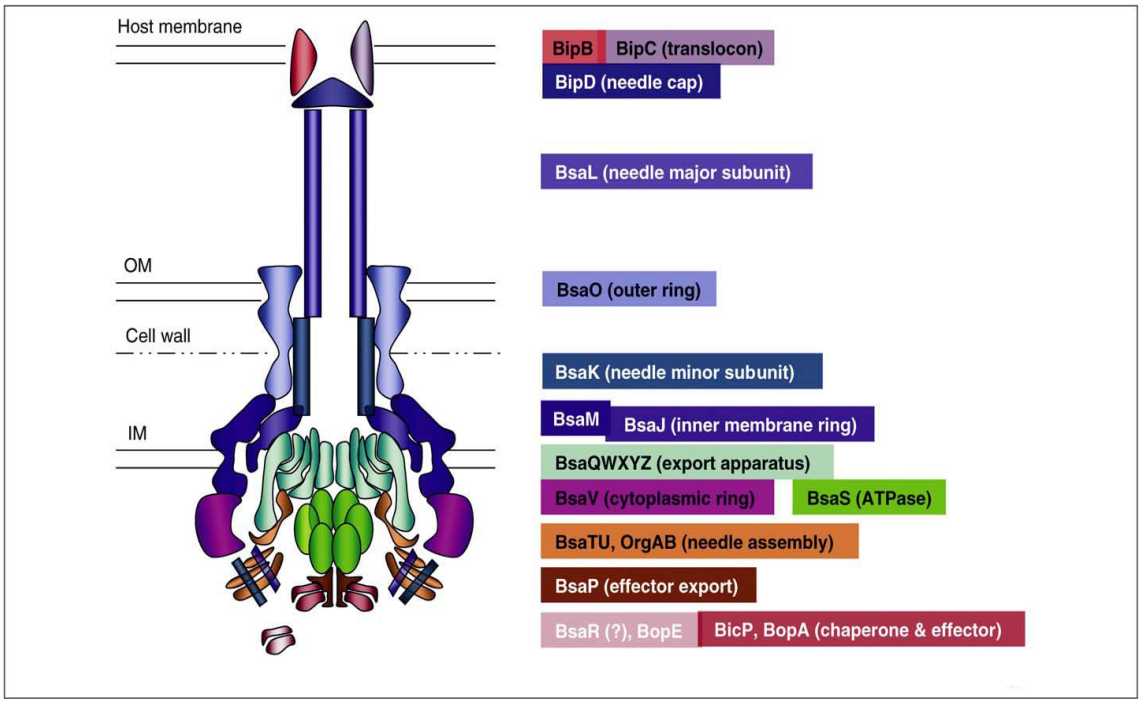

Figure 2.5 T3SS3 syringe-like model
Putative structure and gene functions described for the molecular syringe-like T3SS3 (Adapted from Sun and Gan, 2010)

2.9.4 Host recognition of *B. pseudomallei*

Burkholderia pseudomallei actively invades non-phagocytic host cells, such as epithelial cells, and promotes internalisation by phagocytic cells including macrophages, neutrophils and DC (Jones *et al.*, 1996; Harley *et al.*, 1998a; Harley *et al.*, 1998b; Ahmed *et al.*, 1999). Phagocytic cells express or secrete a variety of pattern recognition receptors (PRR), which enable the recognition of the pathogens as they enter the host (Medzhitov, 2001; Takeuchi and Akira, 2010; Miyaji *et al.*, 2011; Suresh and Mosser, 2013). These PRR recognise conserved pathogen molecules that perform essential physiological functions termed pathogen-associated molecular patterns (PAMP; Medzhitov, 2001).

Table 2.8 Pattern recognition receptors shown to recognise *B. pseudomallei*

PRR	Location	Ligands	Function	References
TLR1	Cell surface	Peptidoglycan, lipoprotein	Activate inflammatory cells and pro-inflammatory cytokine production	Wiersinga *et al.* (2007b)
TLR2	Cell surface	Peptidoglycan, lipoprotein	LPS of *B. pseudomallei* can activate TLR2 but may be detrimental to the host as TLR2 deficiency was protective in *B. pseudomallei*-infected mice	Wiersinga *et al.* (2007b); Feterl *et al.* (2008); Hii *et al.* (2008); West *et al.* (2008)
TLR4	Cell surface	Gram-negative bacterial LPS	Recognition of *B. pseudomallei* LPS requires co-receptors MD2 and CD14 and activates macrophage cytokine responses	Wiersinga *et al.* (2007b); Feterl *et al.* (2008); Hii *et al.* (2008); West *et al.* (2008)
TLR5	Cell surface	Flagellin	Impaired TLR5 signalling correlates with decreased IL-6 and IL-10 production and reduced patient fatality	Feterl *et al.* (2008); Hii *et al.* (2008); West *et al.* (2013); (Chantratita *et al.*, 2014)
TLR9	Endosomal membranes	Unmethylated CpG DNA	Activated by low virulence *B. pseudomallei* isolates but not by high virulence isolates	Feterl *et al.* (2008)
CD14	Cell surface and soluble	Gram-negative bacterial LPS	Co-receptor for TLR4	Wiersinga *et al.* (2007b); Wiersinga *et al.* (2008)
NOD2	Cytoplasmic	Bacterial cell wall derived muramyl-dipeptide, LPS and other unknown ligands	Activates NF-κB and MAP kinases signalling; deficiency in NOD2 correlates with impaired control of *B. pseudomallei* replication in mice	Myers *et al.* (2014)
NLRP3	Cytoplasmic	Lipoligosaccharides and DAMP	Inflammasome formation, mediates the production of IL-18 and IL-1β	Ceballos-Olvera *et al.* (2011)
NLRC4	Cytoplasmic	T3SS proteins and bacterial flagellin	Inflammasome formation, mediates pyroptosis of *B. pseudomallei*-infected cells	Miao *et al.* (2010); Ceballos-Olvera *et al.* (2011)
DC-SIGN	Cell surface	Terminal mannose residues	Adhesion, phagocytosis, DC trafficking and T-cell synapse formation, not utilised by *B. pseudomallei*	Charoensap *et al.* (2008)

Abbreviations: CD – cluster of differentiation; DC – dendritic cell; DC-SIGN - DC-specific intercellular adhesion molecule-3-grabbing non-integrin; IL – interleukin; LPS – lipopolysaccharide; NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells; NLRC4 – NOD-like receptor (NLR) family CARD domain-containing protein 4; NLRP3 - NLR family pyrin domain containing protein 3; NOD - nucleotide-binding oligomerisation domain; TLR – Toll-like receptor

Studies have predominantly investigated the role of toll-like receptors (TLR) and nucleotide-binding oligomerisation domain (NOD) like receptors (NLR) in recognising PAMP of *B. pseudomallei* (Table 2.8). The TLR family consists of 11 transmembrane receptors expressed on the cell surface or within internal cell compartments, such as endosomes, where they recognise different pathogen products. This triggers the activation of phagocytes and the secretion of chemokines and cytokines. Based on the type of PAMP that are recognised by different members of the TLR family, TLR1, 2, 4, 5 and 9 have the potential to recognise and initiate immune responses against *B. pseudomallei* (Medzhitov, 2001; Gan, 2005). Wiersinga *et al.* (2007b) confirmed that patients with septicaemic melioidosis have up-regulated expression of TLR1, 2 and 4 on peripheral blood monocytes. Additionally, the study used transfected human embryonic kidney (HEK) 293 to provide evidence that TLR2 contributes to cellular immune responses of the host to *B. pseudomallei* (Wiersinga *et al.*, 2007b). Although LPS of Gram-negative bacteria typically signal via TLR4, Wiersinga *et al.* (2007b) reported that TLR2 was able to recognise the LPS of *B. pseudomallei*. However, the ability of *B. pseudomallei* LPS to activate TLR2 appeared to be detrimental rather than protective since mice lacking TLR2 receptors demonstrated increased survival (Wiersinga *et al.*, 2007b). Wiersinga *et al.* (2007b) also reported that TLR4 was not involved in the recognition of *B. pseudomallei* LPS unlike other Gram-negative bacteria. To determine the ability of TLR4 to recognise *B. pseudomallei* or its LPS Wiersinga *et al.* (2007b) used HEK 293 cells transfected to express CD14 and TLR4. However, recognition and subsequent signalling via TLR4 requires both CD14 and MD2 co-receptors. Consequently, the results reported by Wiersinga *et al.* (2007b) regarding TLR4 may not represent the true signalling capacity of TLR4 in response to *B. pseudomallei* or its LPS due to the absence of the co-receptor MD2 in the study.

Evidence that *B. pseudomallei* does indeed activate TLR4 when both co-receptors are available was demonstrated in two subsequent studies (Hii *et al.*, 2008; West *et al.*, 2008). Both Hii *et al.* (2008) and West *et al.* (2008) used HEK 293 cells transfected with TLR4 along with CD14 and MD2 to demonstrate that in response to *B. pseudomallei* the expression of TLR4 is up-regulated. Hii *et al*. (2008)

demonstrate that in response to *B. pseudomallei* the expression of TLR2, 4 and 5 are up-regulated. However, Hii *et al*. (2008) also found that the downstream activation of NF-κβ and IL-8 secretion can be induced following the internalisation of *B. pseudomallei* independently of these three TLR (Hii *et al.*, 2008). West *et al*. (2008) also demonstrated that the macrophage cytokine response to *B. pseudomallei* was dependent on signalling via TLR4 and this response was augmented in the absence of signalling via TLR2.

Activation of TLR2, 4, 5 and 9 on macrophages (macrophage cell line RAW 246.7 and peritoneal macrophages from BALB/c mice) infected with *B. pseudomallei* isolates of varied virulence has also been compared (Feterl *et al.*, 2008). Feterl *et al*. (2008) demonstrated similar trends in TLR activation for RAW 246.7 macrophages and BALB/c peritoneal macrophages. Furthermore, *B. pseudomallei* virulence did not appear to affect the expression of TLR2, 4 or 5. However, expression of TLR9, the PRR for unmethylated CpG, was reduced in response to *B. pseudomallei* isolates of high virulence compared to isolates of low virulence (Feterl *et al.*, 2008). West *et al*. (2013) have since demonstrated that TLR5, the PRR known to recognise bacterial flagellin, is activated by *B. pseudomallei*. Furthermore, defects in the TLR5 protein was associated with enhanced protection against death and organ failure in patients with melioidosis (West *et al.*, 2013; Chantratita *et al.*, 2014). This appears to correlate with decreased IL-10 and IL-6 production by macrophages in individuals with TLR5 defects (West *et al.*, 2013).

The NLR family are cytosolic PRR that play a role in regulating inflammation by either providing a platform for caspase-1 inflammasome formation or by activating alternative inflammatory signalling cascades (Zhong *et al.*, 2013). Inflammasome forming NLR include NLR pyrin domain containing protein 3 (NLRP3) and NLR caspase recruitment domain (CARD) containing protein 4 (NLRC4). The formation of NLRP3 inflammasome is triggered by microbial PAMP such as bacterial lipoligosaccharides and also by danger-associated molecular patterns (DAMP) while NLRC4 is triggered by bacterial flagellin or T3SS proteins (Miao *et al.*, 2010; Zhao *et al.*, 2011; Ferrand and Ferrero, 2013). Ceballos-Olvera *et al.* (2011) demonstrated that both NLRP3 and NLRC4

inflammasomes are important for mediating resistance in mice infected with *B. pseudomallei*. In particular, NLRC4 inflammasomes triggered pyroptosis of host infected cells, while NLRP3 inflammasomes mediated the production of IL-18 and IL-1β. Although production of IL-18 by NLRP3 inflammasomes is beneficial to the host by inducing IFN-γ production, the production of IL-1β by NLRP3 inflammasomes correlated with excessive neutrophil infiltration and was detrimental in *B. pseudomallei*-infected mice (Ceballos-Olvera *et al.*, 2011). Other NLR family members, such as NOD2, regulate inflammation by activating nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPKs), and IFN regulatory factors (IRF) signalling cascades (Zhong *et al.*, 2013). In a study by Myers *et al*. (2014), *B. pseudomallei* was demonstrated to activate cytosolic NOD2 in transfected HEK 293 cells. In addition, NOD2-deficient mice were found to be less efficient at controlling *B. pseudomallei* replication and dissemination (Myers *et al.*, 2014). Our understanding of the role that other PRR play in response to *B. pseudomallei* infection and whether *B. pseudomallei* is able to avoid recognition by these receptors or interfere with the signalling cascades they activate is yet to be elucidated.

2.9.5 Phagocytosis and intracellular survival of *B. pseudomallei*

Phagocytic cells continually survey tissues for potential pathogens using mechanisms termed endocytosis, macropinocytosis and phagocytosis (Mayor and Pagano, 2007; Bohdanowicz and Grinstein, 2013). Phagocytosis is a ligand dependent process utilised by phagocytic cells to internalise large pathogens, such as bacteria or apoptotic pathogen-infected host cells, following recognition via PRR such as scavenger, complement or Fc receptors. The process of phagocytosis begins with recognition and engagement of the bacterium at the cell surface (Kinchen and Ravichandran, 2008; Steinberg and Grinstein, 2008). Upon recognising a bacterium as foreign, rearrangement of the actin cytoskeleton network is initiated to form a phagocytic cup with protruding pseudopodia which surround the bacterium then pinch off to form a plasma membrane-derived vacuole termed a phagosome (Mayor and Pagano, 2007; Steinberg and Grinstein, 2008; Swanson, 2008).

Harley *et al.* (1998) published the first study to provide visual evidence of *B. pseudomallei* phagocytosis by macrophages (Figure 2.6). The transmission electron microscopy (TEM) images produced show macrophages extend pseudopodia around the bacteria and internalise the bacteria into plasma membrane-derived intracellular vacuoles termed phagosomes. Unlike *Legionella pneumophila* which is internalised by coiling phagocytosis, *B. pseudomallei* is internalised by conventional phagocytosis (Harley *et al.*, 1998b).

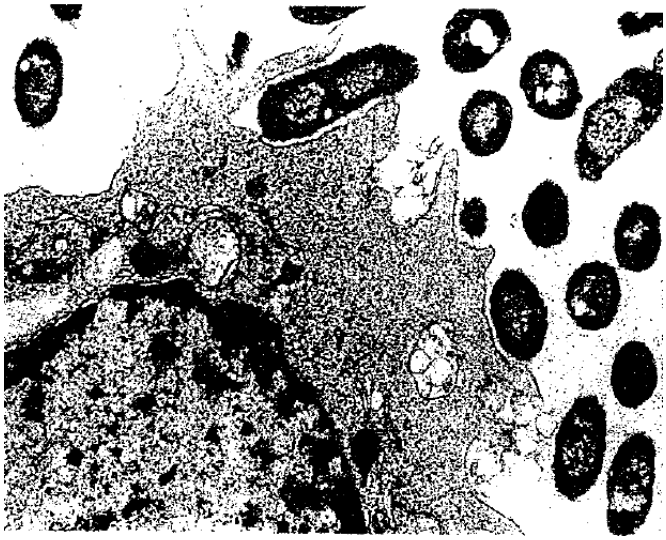

Figure 2.6 Phagocytosis of *B. pseudomallei*
The first TEM image of a macrophage (RAW 246) using conventional phagocytosis to internalise *B. pseudomallei* (Adapted from Harley *et al.*, 1998b).

Following the formation of a phagosome, maturation from an innocuous membrane bound organelle to a microbicidal phagolysosome is required to enable killing and degradation of the internalised bacterium (Kinchen and Ravichandran, 2008). Phagosomal maturation process involves sequential recruitment of different components of the endocytic pathway, which progressively matures the phagosomes to an early phagosome, intermediate phagosome, late phagosome and finally a phagolysosome after fusing with a lysosome. Throughout the maturation pathway, the internal environment of the phagosome becomes increasingly acidic which, along with the acquisition of hydrolases, enables the killing and degradation of the internalised bacterium (Flannagan *et al.*, 2009). In addition to providing a mechanism for eliminating invading pathogens, degradation of the pathogen following phagocytosis also generates peptides for antigen presentation

typically via major histocompatibility complex (MHC) class II, a process that drives the activation of adaptive immune responses (Kinchen and Ravichandran, 2008).

Successful intracellular pathogens have acquired one or more specialised mechanisms that enable them to evade phagolysosomal killing. Such mechanisms include developing resistance to antimicrobial peptides, interfering with the production of antimicrobial peptides, escaping from the phagosome or arresting the maturation thereby preventing the formation of a phagolysosome. The ability of intracellular pathogens to use host cells as a niche for intracellular replication and persistence by escaping from phagosomes or interfering with phagosome maturation has been extensively investigated (Table 2.9).

Studies indicate that *B. pseudomallei* uses mechanisms that enable early escape from phagosomes, followed by uncontrolled replication in the cytosol rather than inhibition of phagosome maturation (Harley *et al.*, 1998b; Kespichayawattana *et al.*, 2000; Stevens *et al.*, 2002; Stevens and Galyov, 2004; Wiersinga *et al.*, 2006). Visualisation of *B. pseudomallei* exiting endocytic vesicles demonstrated that endosomal escape of the bacteria occurs as quickly as 15 minutes following phagocytosis (Harley *et al.*, 1998b). The secretion of T3SS3 proteins, BsaZ and BipD have been found to be important for lysis of the endosomal membrane by *B. pseudomallei*. By disrupting specific genes within the T3SS3 gene cluster, Stevens *et al*. (2002) created *B. pseudomallei* mutants that were unable to secrete BsaZ and BipD and consequently unable to escape from within endocytic vesicles. These mutants failed to proliferate in the J774.2 murine macrophage-like cell line, indicating that endocytic vesicle escape is required for proliferation of *B. pseudomallei* within the cell (Stevens *et al.*, 2002).

Table 2.9 Partial list of mechanisms that intracellular bacteria use to escape from or inhibit phagosome maturation

Bacteria	Mechanism	Reference
Burkholderia pseudomallei	Escapes from phagosome by secreting T3SS3 proteins (BsaZ and BipD) to lyse the endosomal membrane then replicates in the cytoplasm	Harley *et al.* (1998b); Stevens *et al.* (2002); Kespichayawattana *et al.* (2004); Stevens and Galyov (2004); Wiersinga *et al.* (2006)
Shigella flexneri	Escapes from phagosome by secreting invasion protein antigen B, forms cation selective ion channels that allows potassium influx causing vacuolar destabilisation	Paetzold *et al.* (2007); Senerovic *et al.* (2012)
Salmonella typhimurium	Uses T3SS effector proteins to hijack the phagosome to form a *Salmonella*-containing vacuole where the bacteria survive and replicate	Ochman *et al.* (1996); Richter-Dahlfors *et al.* (1997); Agbor and McCormick (2011); Malik-Kale *et al.* (2011)
Listeria monocytogenes	Escapes from phagosome by secreting toxins that lyse the vacuole membrane, listeriolysin O and two phospholipases, PlcA and PlcB	Portnoy *et al.* (2002); Birmingham *et al.* (2008); Eylert *et al.* (2008)
Mycobacterium tuberculosis	Survives in non-lysosomal phagosomes by using effector molecules, including the lipids phosphatidylinositol mannoside and lipoarabinomannan, and the phosphatidylinositol-3-phosphate phosphatase SapM to arrest phagosome maturation	Fratti *et al.* (2003); Vergne *et al.* (2003); Vergne *et al.* (2005); Majlessi *et al.* (2007)
Legionella pneumophila	Survives within phagosomes by promoting fusion with endoplasmic reticulum-derived membranes instead of lysosomes	Tilney *et al.* (2001); Ninio *et al.* (2009); Hubber and Roy (2010); Joshi and Swanson (2011)
Coxiella burnetti	Survives within phagosomes by delaying maturation and promoting fusion with autophagocytic vesicles bearing LC3	Voth and Heinzen (2007); Pan *et al.* (2008)
Francisella tularensis	Unopsonised bacteria escape from the phagosome within 1 hr while opsonised bacteria are restricted within phagolysosomes	Chong *et al.* (2008); Geier and Celli (2011)

Within the cytoplasm of the host cell, unchecked *B. pseudomallei* replication precedes cell-to-cell spread or cell-to-cell fusion leading to multinuclear giant cell formation (Wiersinga *et al.*, 2006). Cell-to-cell spread of *B. pseudomallei* is facilitated by the formation of actin based membrane protrusions

(Kespichayawattana *et al.*, 2000). Polymerisation of actin, in an end-on-end fashion at one pole of *B. pseudomallei* propels the bacteria forward creating a protrusion in the host cell membrane (Stevens *et al.*, 2002). Extension of the membrane protrusion continues until it projects into or is phagocytised by an adjacent host cell. Although Stevens *et al.* (2002) were not able to identify the effector responsible, they noted that the mechanism for inducing actin tail formation at the pole of *B. pseudomallei* appeared to be unique from that reported for *Shigella* and *Listeria* actin nucleation (Stevens *et al.*, 2002). In a subsequent study, Stevens *et al*. (2005) demonstrated that the *B. pseudomallei* T3SS3 secreted protein BimA, similar to the *Yersinia* autosecreted adhesion YadA, played a critical role in the actin-based motility of *B. pseudomallei*. BimA was found to be located at the bacterial pole where actin polymerisation occurs and defects in BimA abolished actin-based motility of *B. pseudomallei*. Furthermore, purified BimA was shown to bind actin and stimulate actin polymerisation *in vitro* (Stevens *et al.,* 2005).

Other mechanisms that facilitate the intracellular survival of *B. pseudomallei* have been suggested but not yet proven. The ability of *B. pseudomallei* to induce multinucleated giant cell formation in phagocytic cells *in vitro* and *in vivo* his thought to be controlled by the *B. pseudomallei* global regulatory factor, RpoS (Utaisincharoen *et al.*, 2006). Studies suggest that *B. pseudomallei* also uses RpoS functions to evade killing and cause apoptosis of macrophages (Utaisincharoen *et al.*, 2006; Lengwehasatit *et al.*, 2008). The *B. pseudomallei* virulence factor TssM is a deubiquitinase involved in immune suppression, specifically by inhibiting NF-kB signalling and type I IFN production in macrophages, which is independent of but appears to be regulated by the T3SS3 (Sun and Gan, 2010; Tan *et al.*, 2010). The T3SS3 protein BopA, which shares homology with the *Shigella* effector protein involved in the lysis of cell membranes, may play a role in mediating *B. pseudomallei* cell-to-cell spread (Utaisincharoen *et al.*, 2006). In an attempt to identify the genes involved in the intracellular persistence of *B. pseudomallei*, Chieng *et al.* (2012) compared the gene expression profiles of intracellular bacteria to extracellular bacteria. The changes in gene expression suggest that T3SS3 genes are involved in *B. pseudomallei* invasion, while T6SS1 genes appeared to be involved in

intracellular persistence (Chieng *et al.*, 2012). As this conflicts with earlier studies which reported that the T3SS3 proteins BsaZ and BipD facilitated phagosome escape, further investigations on the intracellular persistence and cell-to-cell spread are required to elucidate the mechanisms employed by *B. pseudomallei* to establish infection while concealing itself from the host's immune responses. Overall, the ability of *B. pseudomallei* to evade killing by host cells is mediated by virulence factors largely controlled by the T3SS3 which enable the bacteria to escape from the phagosome, inhibit the production of host antimicrobial mediators followed by the induction of host cell apoptosis. However, our understanding of the putative roles for *B. pseudomallei* T3SS3, and other T3SS and T6SS gene clusters, is largely based on extrapolation of findings shown for homologous counterparts of the T3SS for *Salmonella* and *Shigella*. Consequently, further studies to confirm the putative mechanisms and how they are regulated specifically for *B. pseudomallei* is required.

2.10 Innate Immune Responses Against *B. pseudomallei*

The first line of host defence against pathogens are non-specific responses which are important for recognising and controlling pathogens as they enter the body and for presenting antigens for activation of adaptive immune responses, leading to the development of pathogen-specific immunity (Murphy *et al.*, 2011). Non-cellular components of the innate immune response include complement, cytokines, enzymes and secreted PRR, which target extracellular pathogens causing agglutination, neutralisation, activation of complement cascades, promotion of phagocytosis by opsonisation and regulation of inflammatory responses (Bottazzi *et al.*, 2010). Extracellular *B. pseudomallei* have been shown to activate the alternative complement pathway, resulting in the deposition of C3 on the bacteria, although some *B. pseudomallei* strains appear to be resistant to serum bactericidal killing (Ismail *et al.*, 1988; Egan and Gordon, 1996). Reckseidler-Zenteno *et al.*, (2005) demonstrated that encapsulated *B. pseudomallei* strains have increased resistance to serum bactericidal killing and reduced complement cascade activation. Through actively promoting internalisation by both non-phagocytic and phagocytic cells of the host, *B. pseudomallei* is capable of evading these components of the innate immune

system. Ultimately, cells of the innate immune response, such as macrophages, neutrophils, NK cells and DC, are key to limiting the spread of *B. pseudomallei* infection until antigen presenting cells activate protective adaptive immune responses.

Pro-inflammatory and anti-inflammatory cytokine networks regulate inflammatory responses to infection by supporting innate and adaptive immune cells to provide resistance and develop protection in the host (Beutler, 2004). Pro-inflammatory cytokines promote inflammation by recruiting and activating innate immune cells (i.e. macrophages, neutrophils and NK cells) to contain the infection. Cytokines such as IL-1β, IL-6, IL-8, IL-12, IFN-γ and TNF-α are involved in initiating inflammation and also provide feedback signalling which maintains the inflammatory state by promoting the continued production of pro-inflammatory cytokines (Dinarello, 2000). However, hyper or prolonged pro-inflammatory cytokine signalling can result in excessive immune cell infiltration and activation causing collateral pathological damage to host tissue and potentially culminating in the development of fatal septic shock. Anti-inflammatory cytokines counteract pro-inflammatory cytokines by promoting tissue repair and down regulating pro-inflammatory cytokine production to prevent excessive or prolonged inflammation from damaging host tissue. However, excessive production of anti-inflammatory cytokines, such as IL-4, IL-10 and IL-13, can drive a state of immune suppression impairing the host's ability to control the infection (Dinarello, 2000; Opal and DePalo, 2000). The level of pro-inflammatory cytokines produced during *B. pseudomallei* infection has been shown to contribute to the development of resistance or susceptibility in the host (Santanirand *et al.*, 1999; Ulett *et al.*, 2000a). In particular, a moderate elevation in IFN-γ production, which influences a number of immune responses including the activation of macrophages and NK cells, promotes antigen presentation and the differentiation of T_h1 T cells, has been shown to play an important role in signalling appropriate immune cell responses to *B. pseudomallei* infection (Ulett *et al.*, 2000a; Liu *et al.*, 2002). Comparison of cytokine gene profiles produced in the liver and spleen of BALB/c and C57BL/6 mice demonstrated that the hyper production of IFN-γ by BALB/c mice during *B. pseudomallei* infection is associated with the development of acute melioidosis (Ulett *et al.*, 2000b; Ulett *et*

al., 2000a). Following *B. pseudomallei* infection, BALB/c mice also demonstrate increased expression of TNF-α, IL-1β and IL-6 compared to C57BL/6 mice (Ulett *et al.*, 2000b; Ulett *et al.*, 2000a). Koo and Gan (2006) also demonstrated that BALB/c splenocytes infected with *B. pseudomallei in vitro* produced significantly higher levels of TNF-α, IL-1β and IL-6 compared to C57BL/6 splenocytes, supporting the findings of Ulett *et al*. (2000a and b). IL-12 production, typically by macrophages and dendritic cells, is important for the induction of IFN-γ production by immune cells such as T cells and NK cells and thus has an indirect influence on host susceptibility to *B. pseudomallei* (Santanirand *et al.*, 1999; Koo and Gan, 2006). The association between high IFN-γ, TNF-α, IL-1β and IL-6 cytokine expression and the development of acute melioidosis in susceptible BALB/c mice indicates that hyper-activation of immune responses by pro-inflammatory cytokines can be detrimental to the host rather than protective against *B. pseudomallei* infection (Liu *et al.*, 2002; Koo and Gan, 2006).

Similar to the murine studies, a link between disease severity and pro-inflammatory cytokine levels in the serum of patients with melioidosis has also been observed. Lauw *et al.* (1999) found that compared to healthy controls, serum concentrations of IFN-γ, IL-12, IL-15 and IL-18 were significantly elevated in patients with melioidosis. Furthermore, patients with bacteraemic melioidosis had significantly higher serum pro-inflammatory cytokines levels compared to patients with non-bacteraemic melioidosis (Lauw *et al.*, 1999). Wiersinga *et al.* (2007a) used gene profiling to compare the extent of inflammation activation between healthy controls and patients with melioidosis sepsis and found that the expression of IL-1β, IL-6, IL-15, IFN-γ, TNF-α and TNF-β were all significantly upregulated in leukocytes from patients with melioidosis sepsis compared to healthy controls. Appreciation for the ability of cytokines, such as IL-6 and type I IFN, to play dual roles by contributing to both pro-inflammatory and anti-inflammatory cytokine networks is growing (Scheller *et al.*, 2011; Gonzalez-Navajas *et al.*, 2012). Type I IFN (IFN-α and IFN-β) are produced by specialised IFN-producing cells called plasmacytoid DC (pDC), and to a lesser extent by other leukocytes, have been shown to both induce and suppress inflammation while activating innate and adaptive immune responses (Gonzalez-Navajas *et al.*,

2012). Recently, profiling of gene expression signatures from whole blood of patients with melioidosis demonstrated that type I IFN responses were prominent (Koh *et al.*, 2013a). However, further studies are required to determine how type I IFN production is contributing to the balance between pro-inflammatory and anti-inflammatory states and whether this is beneficial or detrimental to the host during *B. pseudomallei* infection. In Chapter 4 of this thesis, the type I IFN response of pDC infected with *B. pseudomallei* has been investigated.

Effector cells of the innate immune response, such as macrophages, neutrophils and natural killer (NK) cells play a crucial role in the early control of *B. pseudomallei* replication and dissemination. The functional capacity of macrophages has been associated with innate susceptibility or resistance of a host toward *B. pseudomallei* infection (Leakey *et al.*, 1998; Ulett *et al.*, 1998; Breitbach *et al.*, 2006; Koo and Gan, 2006). Using the acute (BALB/c) and chronic (C57BL/6) murine models of melioidosis, studies have demonstrated that macrophages derived from *B. pseudomallei*-resistant C57BL/6 mice were more efficient at internalising and killing *B. pseudomallei* in comparison to macrophages derived from *B. pseudomallei*-susceptible BALB/c mice (Leakey *et al.*, 1998; Ulett *et al.*, 1998; Breitbach *et al.*, 2006; Koo and Gan, 2006). Furthermore, C57BL/6 mice become highly susceptible to *B. pseudomallei* infection following the depletion of their macrophages (Barnes *et al.*, 2008). Puthucheary and Nathan (2006) also found that macrophages from patients with melioidosis demonstrated poor phagocytosis and bacterial killing of *B. pseudomallei* compared to macrophages from healthy individuals. Studies have shown that *B. pseudomallei* is not only able to evade macrophage killing by inhibiting the production of antimicrobial mediators, it can also direct cytotoxic activity against the macrophages that ensures bacterial survival (Utaisincharoen *et al.*, 2003; Utaisincharoen *et al.*, 2004; Sun *et al.*, 2005). Interestingly, the killing efficiency of macrophages is dependent on IFN-γ, as macrophages treated with IFN-γ demonstrate enhanced *B. pseudomallei* killing (Miyagi *et al.*, 1997). Therefore, the bactericidal activity of macrophages is key for the early control of *B. pseudomallei* infection however, the capacity for macrophages to allow uncontrolled intracellular replication until the cell ruptures, indicates that other immune cells providing bacterial killing and pro-inflammatory cytokine

production are important as well (Nathan and Puthucheary, 2005; Puthucheary and Nathan, 2006).

Other innate immune cells that contribute to the control of *B. pseudomallei* but have been less studied included NK cells, neutrophils and DC. NK cells are cytotoxic lymphocytes which provide innate immune responses by producing pro-inflammatory cytokines and eliciting cytotoxic activity against stressed, pathogen-infected host cells (Vivier *et al.*, 2008). During *B. pseudomallei* infection, NK cells have been shown to rapidly produce IFN-γ which supports the activation and killing capacity of macrophages (Lertmemongkolchai *et al.*, 2001). Neutrophils are polymorphonuclear leukocytes that are typically the first responders recruited to the site of *B. pseudomallei* infection where they provide early defence by internalising and killing opsonised *B. pseudomallei* (Jones *et al.*, 1996; Easton *et al.*, 2007; Laws *et al.*, 2011). The internalisation and killing capacity of neutrophils is dependent on the deposition of complement C3 on *B. pseudomallei* (Jones *et al.*, 1996; Ramsay *et al.*, 1999; Woodman *et al.*, 2012). However, the polysaccharide capsule of *B. pseudomallei* significantly decreases the deposition of C3 enabling the bacteria to escape clearance by neutrophils (Woodman *et al.*, 2012). Neutrophils can also mediate extracellular killing of *B. pseudomallei* via neutrophil extracellular traps (NET). Riyapa *et al.* (2012) demonstrated that *B. pseudomallei* activation of neutrophils caused the release of NET, formed from chromatin and granular proteins that entrap and damage the bacteria. However, *B. pseudomallei* virulence factors such as the type III secretion system and capsule, may enable *B. pseudomallei* to avoid the activation and release of NET by neutrophils (Riyapa *et al.*, 2012). DC are innate immune cells that are described as professional antigen presenting cells. Although their role during *B. pseudomallei* infection is largely unknown, they are more likely to play a role in modulating other innate immune cells and activating adaptive immune responses.

2.11 Dendritic Cells Link the Innate and Adaptive Immune Responses

Ralph Steinman and Zanvil Cohn first described the morphology and distribution of DC while studying murine splenocytes in 1973 (Steinman and Cohn, 1973).

Subsequent studies by Steinman and colleagues demonstrated that DC mature and activate T cells *in vitro* and later described the maturation phenotype of DC (Steinman and Witmer, 1978; Schuler and Steinman, 1985). DC are highly specialised antigen presenting cells that provide an important link between the innate and adaptive immune responses through pathogen recognition, antigen processing and presentation to T cells (Banchereau *et al.*, 2000). In comparison to other phagocytic cells, DC express higher levels of MHC class II and I receptors and have a unique antigen presenting cell function (Mellman and Steinman, 2001; Wilson and O'Neill, 2003). The versatility of DC also includes the tailoring of selective immune responses necessary to clear different classes of pathogens (Gad *et al.*, 2003; deJong *et al.*, 2005). Although DC play a central role in the development of protective adaptive responses, their function in response to *B. pseudomallei* infection is poorly understood and formed the major focus of the work presented in this thesis.

2.11.1 Pathogen recognition triggers dendritic cell maturation

Dendritic cells reside in all peripheral tissues including the skin, airway epithelium and afferent lymph, where they capture foreign antigens and pathogens as they enter the body using one of three pathways; macropinocytosis, receptor-mediated endocytosis via C-type lectin receptors (CLR) or phagocytosis (Banchereau *et al.*, 2000). The large repertoire of phagocytic receptors, such as CD14, scavenger receptor-A, Fc receptor (FcγR) 1 and FcγR2b, Toll-like receptors and lectins, enables DC to efficiently recognise and phagocytose pathogens (Wallet *et al.*, 2005; Savina and Amigorena, 2007). The various combinations of receptors on DC create subpopulations capable of selective pathogen recognition, phagocytosis and tailored effector function (Banchereau *et al.*, 2000; Ueno *et al.*, 2007).

Dendritic cell maturation is triggered following recognition of pathogen molecules. During the maturation process, DC increase expression of surface molecules for antigen presentation and T cell co-stimulation to enable efficient activation and potential for initiation of pathogen-specific adaptive immune responses (Mellman and Steinman, 2001). The terms ‘immature’ and ‘mature’

DC describe the activation state of DC (Villadangos and Schnorrer, 2007). Immature DC are defined as motile cells with a high phagocytic capacity that enables the recognition and ingestion of pathogens. The interaction between DC and pathogen, LPS, nucleic acids or pro-inflammatory cytokines initiates maturation of DC (Figure 2.7). During maturation, DC demonstrate a reduction in phagocytic capacity, migration to lymphoid tissue and develop a mature phenotype with up-regulated expression of surface markers such as MHC molecules and CD40, along with increased production of cytokines and chemokine receptor expression (Mellman and Steinman, 2001; Villadangos and Schnorrer, 2007; Heath and Carbone, 2009).

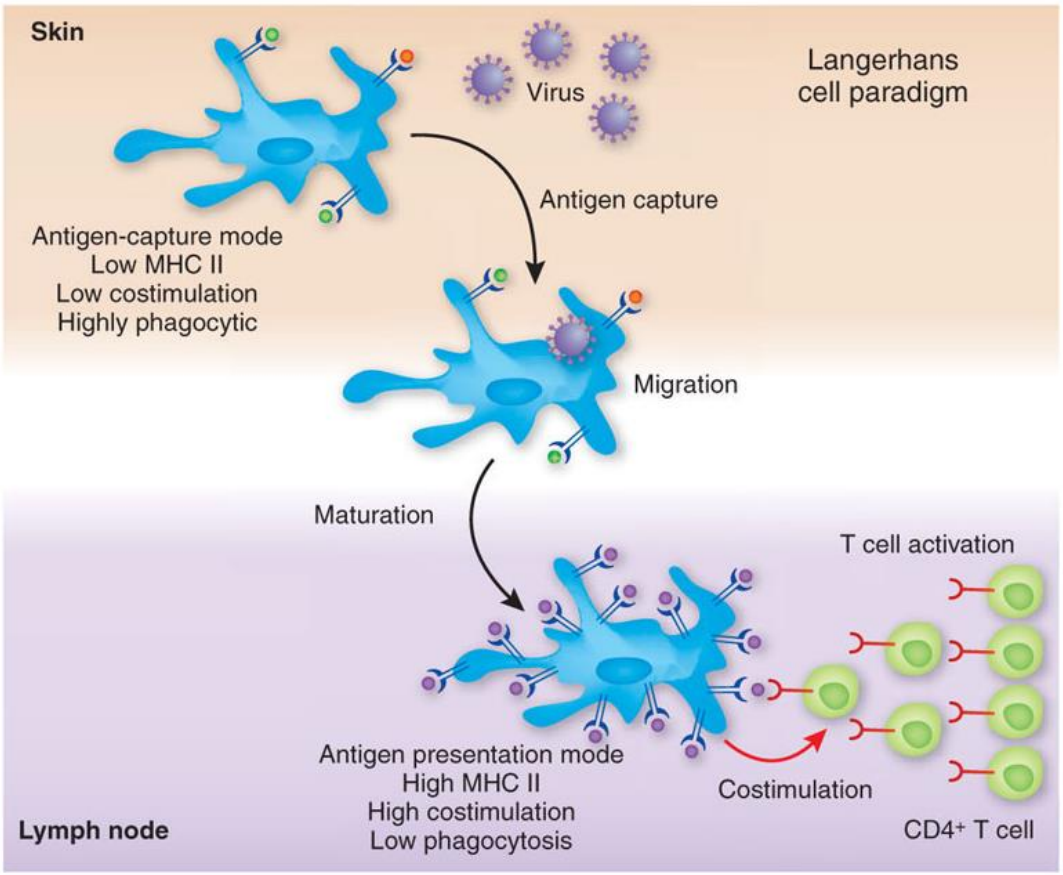

Figure 2.7 DC maturation pathway
The Langerhans cell paradigm describes the maturation of immature DC to a mature DC with high expression of antigen presenting molecules and T cell co-stimulatory molecules. Mature DC migrate from the site of infection to secondary lymphoid tissue to activate naïve T cells (Adapted from Heath and Carbone, 2009).

Maturation of DC in response to intracellular bacteria such as *L. monocytogenes* has been demonstrated (Howard and Inglis, 2003; Brzoza *et al.*, 2004). Using

wild type *L. monocytogenes* and a haemolysin gene-deficient mutant which is unable to produce the cytoplasmic invasion mediator, Listeriolysin (LLO), Brzoza *et al*. (2004) demonstrated that DC maturation and cytokine production is enhanced following cytoplasmic invasion by *L. monocytogenes.* A subsequent study by Westcott *et al*. (2007) compared DC to macrophages during *L. monocytogenes* infection and found that unlike macrophages, the internal milieu of DC does not provide a suitable niche for *L. monocytogenes* replication and survival. Compared to macrophages, DC demonstrated an increased capacity to restrict *L. monocytogenes* within vacuoles and enhanced bactericidal killing (Westcott *et al.*, 2007). In contrast, *Brucella suis*, an intracellular pathogen which causes brucellosis in humans and animals, is able to efficiently invade and replicate within DC, without stimulating DC maturation. The functional implications of *B. suis*-infected DC that fail to mature include an inability to provide efficient antigen presentation to and activation of naïve T cells and the inability to secrete appropriate cytokines such as TNF-α. DC maturation can be restored by introducing exogenous TNF-α to cultures of *B. suis*-infected DC (Billard *et al.*, 2007). *B. suis* is therefore one example of an intracellular bacterium that uses immature DC to evade the host immune response to permit chronic infection.

A few studies have since investigated the host-pathogen interactions between DC and live *B. pseudomallei*. Williams *et al*. (2008) used murine bone marrow-derived DC (BMDC) to demonstrate the internalisation and killing of *B. pseudomallei* by DC, indicating that DC do not provide an intracellular niche for the persistence of *B. pseudomallei*. In response to *B. pseudomallei* infection, BMDC developed a mature phenotype demonstrated by increased expression of antigen presenting molecules (MHC class II) and T cell co-stimulatory molecules (CD86) and significantly increased cytokine gene expression (IL-12). Williams *et al*. (2008) also demonstrated that DC-*B. pseudomallei* interactions are not influenced by *B. pseudomallei* virulence. However, comparison of *B. pseudomallei*-infected BMDC isolated from *B. pseudomallei*-resistant C57BL/6 mice and *B. pseudomallei*-susceptible BALB/c mice demonstrated that DC are an additional cell-mediator contributing to the altered immune responses

which underlie the susceptibility of BALB/c mice toward *B. pseudomallei* (Williams et al., 2008).

A second study by Charoensap *et al.* (2009) observed the survival and replication of *B. pseudomallei* (*Bp*-844) within human monocyte derived DC (MDC), contradictory to the findings of Williams *et al*. (2008). However, in the presence of IFN-γ, human MDC demonstrated increased killing of intracellular *B. pseudomallei* (Charoensap *et al.*, 2009). In 2012, Horten *et al.* (2012) demonstrated internalisation and killing of *B. pseudomallei* (MSHR520) by human MDC. However, sporadic wells demonstrated intracellular survival of *B. pseudomallei* within MDC. Because of the discordant findings published by Williams *et al*. (2008) and Charoensap *et al*. (2009), Horton *et al*. (2012) investigated a number of factors in an attempt to explain the contrasting findings, such as the multiplicity of infection and comparison of the antibiotic, kanamycin against imipenim, used in the protection assay. However, the contrasting results and sporadic persistence of *B. pseudomallei* within MDC could not be explained by the factors tested by Horton *et al.* (2012). Despite these studies, our knowledge of DC-*B. pseudomallei* interactions remains limited, especially with regard to other DC functions, such as migration and antigen presentation, and the role of different DC subsets in the development of protective immune responses to *B. pseudomallei* infection.

2.11.2 Dendritic cell migration

The differences in the migratory behaviour of immature and mature DC are critical for their function. Immature DC are responsive to signals which co-ordinate their migration to a site of infection where they capture antigen. In contrast mature DC respond to migratory cues that co-ordinate their homing to secondary lymphoid organs for antigen presentation (Banchereau *et al.*, 2000; Martin-Fontecha *et al.*, 2009). The migration of immature and mature DC is co-ordinated by a range of CC-chemokine (CCR), CXC-chemokine (CXCR) and CX_3C-chemokine receptors (CX_3CR) expressed on the cell surface. These receptors enable DC to sense and follow chemokines which direct the migration of DC to reach sites of infection or home DC to T cell rich regions in secondary

lymphoid tissue to establish interactions with naïve T cells (Banchereau *et al.*, 2000; Alvarez *et al.*, 2008; Martin-Fontecha *et al.*, 2009).

The migration of immature DC from within blood vessels into inflamed and/or infected tissues involves a multi-step process facilitated by a variety of adhesion molecules which together with chemokine receptors, such as CCR1, CCR2, CCR5 and CXCR1, provide immature DC with specialised properties that enable them to traverse along and through vessel walls (Banchereau *et al.*, 2000; Merad *et al.*, 2002; Yamagami *et al.*, 2005). Tethering and rolling of DC along venular epithelium occurs via P- and E- selectins (Pendl *et al.*, 2002). The endothelial intercellular adhesion molecule-2 (ICAM-2) has also been identified to facilitate the transendothelial migration of immature DC (Wethmar *et al.*, 2006). After entering inflamed or infected tissues, pathogen-activated maturation of DC and/or cytokine signalling via TNF-α or IL-1β initiates the mobilisation of DC from the site of infection to secondary lymphoid tissues using lymphatic or haematogenous pathways (Wang *et al.*, 1999; Martìn-Fontecha *et al.*, 2003). The migration of DC to secondary lymphoid tissue is a complex multistep process that requires enzymatic digestion to enable DC to traverse through tissues to reach lymphatic or blood vessels and chemokine signalling to direct DC movement (Randolph *et al.*, 2008; Martin-Fontecha *et al.*, 2009). Mature DC use the lymphatic system to migrate to secondary lymphoid tissues but can also enter the circulatory system and migrate throughout the body to secondary lymphoid tissue and other organs, such as the spleen (Figure 2.8).

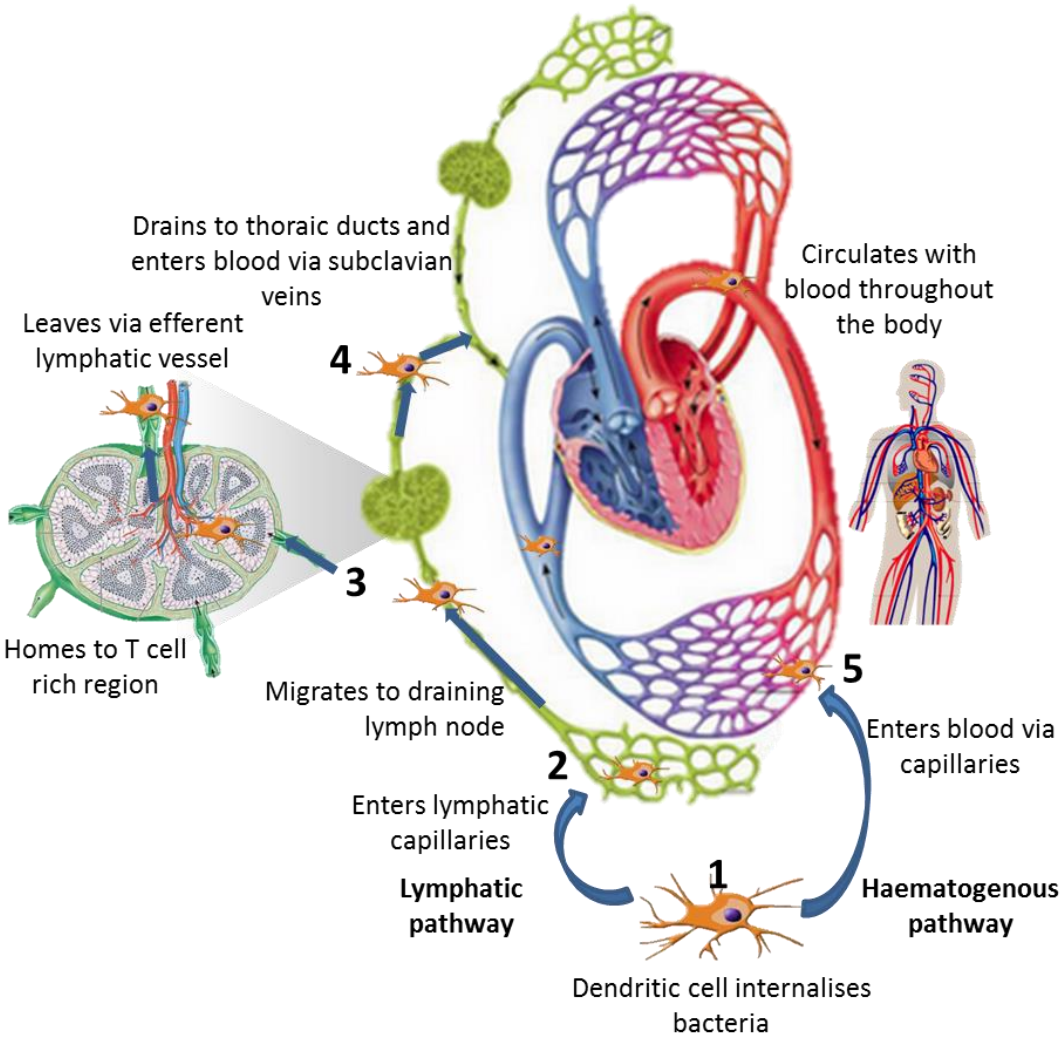

Figure 2.8 Migration pathways of mature DC throughout the body
After internalising bacteria (1), DC undergo maturation and migrate throughout the body by entering lymphatic capillaries (2) and migrating to draining lymph nodes, where they home to T cell rich regions (3) or leave via efferent vessels to enter the blood system via the subclavian veins (4). Alternatively, mature DC can migrate from the tissue and directly enter the circulatory system via blood capillaries (5).

The migratory behaviour of DC changes during maturation, which triggers up-regulation of CCR7, maintenance of CXCR4 expression and down-regulation of CCR1, CCR2, CCR5 and CXCR1. Consequently, mature, antigen-loaded DC expressing CCR7 sense and move toward the ligands, CCL19 and CCL21 which co-ordinate their migration to secondary lymphoid tissue (Saeki *et al.*, 1999; Comerford *et al.*, 2013). At the site of infection, CCL21 expression on the surface of endothelial cells initiates the migration of mature DC through flexible junction 'portals' between the basement membrane and into the lymphatic or blood vessels (Martin-Fontecha *et al.*, 2009; Pflicke and Sixt, 2009). Mature DC traverse the

lymphatics with lymph flow and drain into the subcapsular sinus of lymph nodes or circulate within the blood to the spleen. The process of inward migration of DC to T cell rich zones within the paracortex of lymph nodes or periarteriolar lymphoid sheaths (PALS) of the spleen, also occurs in a CCR7/CCL21/CCL19 dependent manner (Comerford *et al.*, 2013). Within secondary lymphoid organs, fibroblastic reticular cells and stromal cells express membrane-bound CCL21 and soluble CCL19. Mature DC respond to CCL21 expression on the surface of fibroblastic reticular cells while soluble CCL19 is essential for homing of DC toward T cell rich zones to enable activation of pathogen-specific T cell responses (Turley *et al.*, 2010). Similarly, the extravasation of haematogenous mature DC into secondary lymphoid tissues is also co-ordinated by CCR7 ligands, in particular CCL21 which is expressed on high endothelial venules in lymph nodes (Martin-Fontecha *et al.*, 2009). There is also evidence for the transport of soluble perivascular CCL19 to the surface of high endothelial venules suggesting that CCL19 plays an additional role in co-ordinating extravasation of mature DC from the blood into lymph nodes (Baekkevold *et al.*, 2001).

The expression of CXCR4 on DC is maintained during maturation. CXCR4 is known to bind CXCL12, which together play a role in facilitating the immigration of circulating immature DC into peripheral tissues. However, the role of CXCR4 in co-ordinating the migration of mature DC is largely unknown (Kabashima *et al.*, 2007; Ricart *et al.*, 2011). Studies suggest that mature DC exhibit weak migration toward CXCR4 ligand compared to strong migration toward CCR7 ligands (Saeki *et al.*, 1999; Ricart *et al.*, 2011). Using competitive migration assays, Ricart *et al.* (2011) found that CCL19 is more potent at attracting DC migration than CCL21 and CXCR12. However, CXCR4/CXCL21-mediated migration appeared to compensate for absent CCR7/CCL19/CCL21-mediated migration. Furthermore, CXCL12 is expressed in different regions of lymphoid tissues compared to CCL19 and CCL21, suggesting CXCR4/CXCL12 may contribute to differential homing of DC within lymphoid tissue compared to CCR7CCL19/CCL21 (Ohl *et al.*, 2003; Kabashima *et al.*, 2007).

The importance of the CCR7/CCL19/CCL21 chemokine axis in the generation of adaptive immune responses to bacterial infections has been demonstrated by

selectively depleting CCR7 in mice. In response to *L. monocytogenes* and *M. tuberculosis* infection, $CCR7^{-/-}$ mice demonstrated impaired DC migration and failure to activate $CD8^+$ T cells, resulting in increased bacterial burden and susceptibility to infection (Kursar *et al.*, 2005; Olmos *et al.*, 2010). In contrast, a study by Eppert *et al*. (2010) found that the accumulation of DC and T cells at the site of infection in CCR7 deficient mice infected with *Pseudomonas aeruginosa* was beneficial. Eppert *et al.* (2010) found that CCR7 deficiency resulted in higher expression of CD80 and CD86 on DC, increased production of IL-12/23p40, IFN-γ and IL-1α, increased neutrophil respiratory burst and ultimately, increased clearance of *P. aeruginosa*.

Successful intracellular pathogens can directly influence the migratory behaviour of DC and in doing so can utilise DC as a vehicle for dissemination or subvert DC migration in order to impede T cell activation. The systemic dissemination of bacteria such as *L. monocytogenes* and *F. tularensis* is facilitated by DC migration from infected tissues to secondary lymphoid tissue (Pron *et al.*, 2001; Bar-Haim *et al.*, 2008). Alternatively, intracellular bacteria such as *Yersinia pestis* and *B. suis*, interfere with the development of pathogen-specific CMI responses by directly inhibiting DC migration (Velan *et al.*, 2006; Billard *et al.*, 2007). The migratory response of *B. pseudomallei*-infected DC has not previously been investigated and therefore is the focus of the research presented in Chapter 5 and 6 of this Thesis.

2.11.3 Antigen presentation

An important effector function of mature DC is antigen presentation and provision of potent activation of naïve T cells (Banchereau *et al.*, 2000). During maturation, DC increase their expression of MHC class II and I molecules to which antigens processed from captured pathogens are loaded (Mellman and Steinman, 2001). Consequently, upon reaching the lymphoid tissue, mature DC are equipped with high levels of antigen-loaded MHC class II and I molecules for presentation to $CD4^+$ and $CD8^+$ T cells, respectively (Mellman and Steinman, 2001; Gad *et al.*, 2003). Mature DC also provide T cell co-stimulation through expression of B7 co-stimulatory molecules CD80 and CD86, which trigger CD28 on the T cell (Kapsenberg, 2003). Depending on the pathogen encountered, DC produce

different cytokines that drive the polarisation of $CD4^+$ T cell subsets; T helper (T_h) 1, T_h2, T_h17, T follicular helper (T_{fh}) or T regulatory (T_{reg}) cells (Figure 2.9; O'Shea and Paul, 2010).

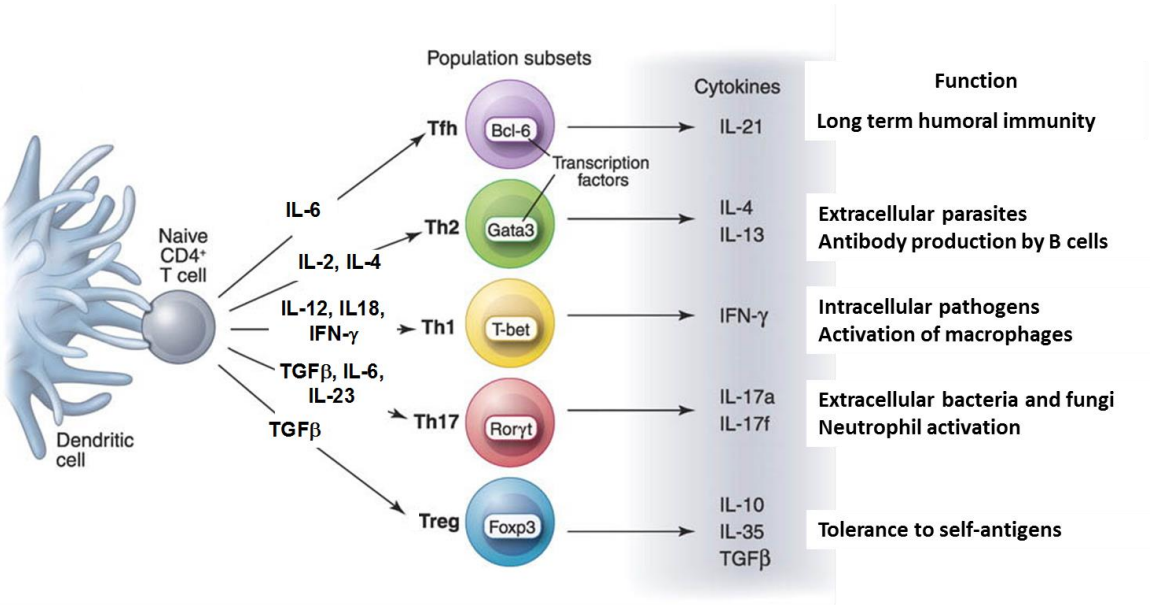

Figure 2.9 DC polarise $CD4^+$ T cell subsets
Immune responses mediated by $CD4^+$ T cells is polarised by the cytokines produced by DC in response to different pathogens (Adapted from O'Shea and Paul, 2010).

Brzoza *et al*. (2004) found that DC play an important role during *L. monocytogenes* infection through efficient activation of naïve $CD8^+$ T cells that are necessary to clear the bacterial infection. *S. typhimurium* is another facultative intracellular bacterium that activates DC maturation following infection (Yrlid *et al.*, 2001a; Yrlid *et al.*, 2001b). Yrlid *et al.* (2001a) demonstrated that DC containing live or heat-killed *S. typhimurium* efficiently activated naïve $CD4^+$ and $CD8^+$ T cells. The same group also demonstrated *Salmonella*-encoded antigens were presented by DC directly on either MHC class II or I, thus are key antigen presenting cells in the immune response to *Salmonella* (Yrlid *et al.*, 2001b).

The importance of adaptive CMI responses involving T cells for recovery from *B. pseudomallei* infection has been demonstrated (Ketheesan *et al.*, 2002; Barnes *et al.*, 2004; Haque *et al.*, 2006b; Barnes and Ketheesan, 2007). Therefore, it is plausible that DC would play a central role in the development of protective T cell responses against *B. pseudomallei*. However, there are limited studies investigating the role of DC in the development of protective CMI responses

against *B. pseudomallei*. Early DC-*B. pseudomallei* studies investigated the use of DC as a vaccine vector to provide protection against *B. pseudomallei.* Healey *et al.* (2005) and Elvin *et al.* (2006) demonstrated that DC derived from the bone marrow of BALB/c mice could be used as an immunisation vector in BALB/c mice. Immunisation of mice with DC pulsed with heat-killed *B. pseudomallei* stimulated the activation of *B. pseudomallei*-specific T cell responses, although low antibody levels were detected. When mice immunised with DC pulsed with heat-killed *B. pseudomallei* were challenged with virulent *B. pseudomallei,* partial protection was demonstrated (Healey *et al.*, 2005). Elvin *et al*. (2006) also investigated the use of CpG oligodeoxynucleotides (ODN) in conjunction with DC pulsed with heat-killed *B. pseudomallei*. Increased protection against virulent *B. pseudomallei* was demonstrated when the immunisation regime of DC pulsed with heat-killed *B. pseudomallei* included CpG ODN (Elvin *et al.*, 2006). Chareonsap *et al*. (2008) used human MDC to demonstrate that paraformaldehyde (PFA)-fixed *B. pseudomallei* do not bind to the C-type lectin receptor, DC-SIGN, indicating that *B. pseudomallei* does not target DC-SIGN to hijack DC function and facilitate persistence. In addition, human MDC stimulated with PFA-fixed *B. pseudomallei* induced T_h1 polarisation of naïve T cells (Charoensap *et al.*, 2008). The ability of human MDC to present *B. pseudomallei* antigen to *B. pseudomallei*-specific memory T cells from patients who have recovered from melioidosis has been demonstrated (Tippayawat *et al.*, 2011). Human MDC stimulated with heat-killed *B. pseudomallei* or potential vaccine antigens derived from *B. pseudomallei*, re-activated *B. pseudomallei*-specific $CD4^+$ T cells *in vitro*. The reactivation *B. pseudomallei*-specific memory T cells required direct contact with MDC (Tippayawat *et al.*, 2011). However, the antigen presenting capacity of DC exposed to live *B. pseudomallei* and their subsequent role in the development of protection during active *B. pseudomallei* infection is unknown.

2.11.4 Dendritic cell subsets

Since the discovery of DC, multiple DC subsets have been defined. The family of DC are defined by the development from a common DC precursor into immature DC that express CD11c and MHC class II and undergo maturation to acquire a mature phenotype, capable of activating naïve T cells (Shortman and Naik, 2007; Alvarez *et al.*, 2008). During steady state, the two main categories described are conventional DC and plasmacytoid DC (pDC). An additional DC subset, monocyte-derived DC is only observed during inflammation. The conventional DC are further divided into multiple subsets with unique phenotype and functions, although there is apparent overlapping and redundancy between DC subsets (Table 2.10; Shortman and Liu, 2002; Banchereau *et al.*, 2009; Heath and Carbone, 2009; Hespel and Moser, 2012).

Resident DC within the skin includes Langerhans cells (LC), dermal DC ($CD11b^+$) and skin associated $CD103^+$ DC. Dermal DC and skin associated $CD103^+$ DC reside in the dermis of the skin and express additional C-type lectin receptors (CLRs) not observed on LC: DC-SIGN, DEC-205, LOX-1, CLEC-6 and Dectin-1 (Banchereau *et al.*, 2009; Heath and Carbone, 2009). LC are the archetypal DC, which reside in the epidermis of the skin and are easily identified by the expression of langerin and intracytoplasmic organelles called Birbeck granules (Merad *et al.*, 2008; Banchereau *et al.*, 2009). As the only haematopoietic cell in the epidermis, LC are the first immunological barrier encountered by potential pathogens as they breach the skin. Importantly, LC are capable of inducing a robust pathogen-specific T cell responses by both naïve $CD4^+$ and $CD8^+$ T cells (Ueno *et al.*, 2007). Furthermore, cytotoxic $CD8^+$ T cells activated by LC are more potent and more efficient at killing a target cell in comparison to $CD8^+$ T cells primed by dermal DC ($CD14^+$). However, dermal DC appear to have a functional advantage for activating strong pathogen-specific humoral responses compared to LC. Dermal DC are classically $CD11b^{high}$, preferentially stimulate naïve $CD4^+$ T cells to develop into follicular helper T cells (T_{fh}) which promote increased IgM antibody production in naïve B cells and can also signal switching antibody production to IgG or IgA (Ueno *et al.*, 2007; Banchereau *et al.*, 2009; Heath and Carbone, 2009). The skin associated

$CD103^+$ DC create additional complexity to the DC subset as these DC also express langerin, are $CD11b^{low}$ and reside in additional sites to the skin such as the lung, liver and lymph nodes draining the lungs, liver and kidneys (Heath and Carbone, 2009).

Typical resident DC in secondary lymphoid tissue are described as either $CD8a^+$ DC or $CD8a^-$ DC. The expression of CD4 can be used to further divide $CD8a^-$ DC into $CD8a^-$ $CD4^+$ (referred to as $CD4^+$ DC in Table 2.10) and $CD8a^-$ $CD4^-$ DC (referred to as double negative, DN, in Table 2.10; Heath and Carbone, 2009; Elpek *et al.*, 2011). These DC subsets reside in different anatomical locations within lymphoid tissue, have additional differential surface marker phenotype and drive different adaptive immune responses. $CD8a^+$ DC are found in the T cell zones of the spleen, demonstrate specialised capacity to cross-present antigen supported by preferential expression of MHC class I, activate T_h1 responses and induce antigen-specific $CD8^+$ T cell. In contrast, $CD8a^-$ DC are found within the red pulp and marginal zone of the spleen, express MHC class II, induce antigen-specific $CD4^+$ T cells and tend to drive T_h2 responses (Dudziak *et al.*, 2007). These DC subsets can be identified in all secondary lymphoid organs with the exception of the thymus (only contains $CD8a^+$ DC). In the lymph nodes, $CD8a^-$ DC are predominantly $CD4^-$ ($CD8a^-CD4^-$DC; Heath and Carbone, 2009). Furthermore, tentative site-associated differences in the transcriptional and functional roles of each lymphoid DC subset has been reported, although the implications of such site-associated differences is unknown (Elpek *et al.*, 2011).

Table 2.10 Defined DC subsets

DC subset	Phenotype		Functions
	Mouse	Human	
Plasmacytoid 	$CD11c^{int}$, MHC class II, CD317 (BST2, PDCA-1), CD45R (B220), Siglec-H, Ly6C, GR-1/Ly6G	CD4, CD123, CD303 (BDCA-2), CD304 (BDCA-4), HLA-DR, MHC class II, CD85k (ILT3)	Involved in innate protection
Langerhans cell 	CD45, CD11b, CD11c, MHC class II, CD205, CD207 (Langerin), E-cadherin, CD326 (EpCAM)	CD45, HLA- DR, MHC class II, CD1a, CD207, E-cadherin, CD326	Only DC in epidermis, migratory, activate $CD4^+$ and $CD8^+$ T cells
$CD11b^+$ Dermal DC 	CD45, $CD11b^{hi}$, CD11c, MHC class II, CD205	$CD1c, CD1a^{+/-}$, CD45, HLA-DR, CD11b, CD11c, MHC class II	Dermis and other tissues, migratory, activate $CD4^+$ T cells
$CD103^+$ Skin associated 	CD45, $CD11b^{low}$, CD11c, CD103, MHC class II, CD205, CD207	CD1c, CD45, HLA-DR, CD11b, CD11c, CD14, MHC class II, CD209 (DC-SIGN)	Dermis and other tissues, migratory, activate $CD8^+$ T cells
$CD103^+$ $CD11b^+$ Gut-associated 	CD45, $CD11b^{hi}$, $CD11c^{hi}$, CD103, MHC class II, $CD8^-$	Unconfirmed	Novel DC subset in intestinal lamina propria and draining lymph nodes
$CD8^+$ 	CD8, CD11c, MHC class II, CD205, CD207, CD1a, CLEC9a, XCR1	$CD11c^{low}$, CD141, XCR1, HLA-DR	Lymphoid tissue, activate $CD8^+$ T cells
$CD4^+$ 	CD4, $CD11c^{hi}$, CD11b, CD172a MHC class II	$CD11c^{hi}$, CD11b, CD1c, HLA-DR	Lymphoid tissue, activate $CD4^+$ T cells
Double negative (DN) 	$CD11c^{low}$, CD11b, CD172a, $CD8^-$, $CD4^-$, CCR2, CX_3CR1^{hi}	Unconfirmed	Lymphoid tissue, activate $CD4^+$ T cells
Monocyte derived 	CD11c, CD11b, CX_3CR1, CD206, CD209, Ly6C, MAC	CD14, CD11b, CX_3CR1, CD209, HLA-DR	Activates effector/memory T cells at inflammation site

(Adapted from Heath and Carbone, 2009).

Inflammatory DC, also known as monocyte-derived DC are not observed during steady state; rather inflammation or infection initiates their differentiation from a monocyte precursor (Shortman and Naik, 2007; Hespel and Moser, 2012). Studies indicate that monocytes are recruited from the bone marrow to a site of infection or inflammation where they are activated to differentiate into inflammatory DC (Hespel and Moser, 2012). At the site of infection, inflammatory DC produce TNF-α and nitric oxide to promote clearance of intracellular bacteria. Although initial studies demonstrated the role of inflammatory DC to provide innate immune response to pathogens, they have since been shown to activate adaptive T_h1 and T_h2 immune responses. Furthermore, migration of inflammatory DC to secondary lymphoid organs is not co-ordinated by the CCR7/CCL19/CCL21 axis. Therefore, in studies where DC migration is blocked by depletion of CCL19 and CCL21, inflammatory DC can activate sufficient pathogen-specific adaptive immune responses, providing redundancy (Hespel and Moser, 2012).

Plasmacytoid DC (pDC) are strikingly different from other DC subsets due to their resting state morphology and specialised ability to rapidly produce large quantities of type I IFN (Colonna *et al.*, 2004). Like other DC, pDC arise from a common DC precursor which is dependent on the cytokine Fms-like tyrosine kinase 3 ligand (FLT-3L) but diverge away to develop into an immature pDC with migratory behaviour and morphology similar to antibody secreting plasma cells (Gilliet *et al.*, 2002; Villadangos and Young, 2008; Sathe *et al.*, 2013; Shortman *et al.*, 2013). Furthermore, pDC have an unusual phenotype in comparison to other DC. Defined as $CD11c^{int}$ and MHC class II^{+}, pDC also express the B cell maker CD45R (B220) along with Ly6C, GR-1/Ly6G and pDC antigen 1 (PDCA-1, also known as CD317; Shortman and Liu, 2002). Although the activation of pDC via TLR7 and TLR9 is well described, pDC also express a number of other PRR including I-type lectin receptors (Siglec-H and Siglec-5), C-type lectin receptors (BDCA2, DNGR and MBL) along with IFN and TNF receptors (CD40 and OXL40; Reizis *et al.*, 2011b). Importantly, stimulation of pDC initiates maturation that causes pDC to develop a DC morphology with pseudopods and dendrites, changes in gene expression profile and the up-regulation of antigen presenting and T cell co-stimulatory molecules which together, enables mature

pDC to function similar to other DC (Grouard *et al.*, 1997; Siegal *et al.*, 1999). The observation that pDC acquire a mature phenotype capable of activating naïve T cells supports their inclusion in the DC family (O'Doherty *et al.*, 1994).

Although pDC develop a mature phenotype capable of activating naïve T cells they are not considered professional antigen presenting cells. Rather, pDC are primarily considered immunomodulating cells that are involved in the innate response to pathogens. The main effector function described for pDC is a strong type I IFN response that activates NK cells and cytotoxic $CD8^+$ T cells (Liu, 2005). While pDC are not the only cell type to produce type I IFN, pDC are characterised by their ability to rapidly produce large quantities of type I IFN including IFN-α and IFN-β following stimulation. Plasmacytoid DC are superior type I IFN produces because they constitutively express high levels of the IRF7, which allows rapid assembly of the multiprotein signal transduction complex for producing IFN-α and IFN-β (Liu, 2005). In contrast, other cell types require IFN-β feedback to up-regulate IRF expression to produce type I IFN (Decker *et al.*, 2005).

The protective type I IFN response of pDC following stimulation with RNA or DNA viruses via TLR7 or TLR9 respectively, is well established. An important effector function of pDC and type I IFN is the activation of NK and $CD8^+$ T cell cytolytic activity against viral infected cells and the production of IFN-γ (Colonna *et al.*, 2004; Gerosa *et al.*, 2005). Other benefits of type I IFN production by pDC in response to viral infections include enhancing DC functions, supporting T_h1 cell differentiation and promoting antibody production by B cells (Colonna *et al.*, 2004; Liu, 2005). Consequently, pDC and type I IFN were considered effectors of anti-viral immunity. Devitt *et al*. (1996) challenged the dogma that type I IFN only mediate anti-viral responses by demonstrating that the LPS of *Chlamydia* species induced type I IFN production and subsequently activated nitric oxide production which acted as a bactericidal defence mechanism (Devitt *et al.*, 1996). Since then, type I IFN production during bacterial infections has been shown to mediate a range of immunomodulatory effects (Figure 2.10), which can have both beneficial and detrimental impacts on the host's resistance to infection (Table 2.11).

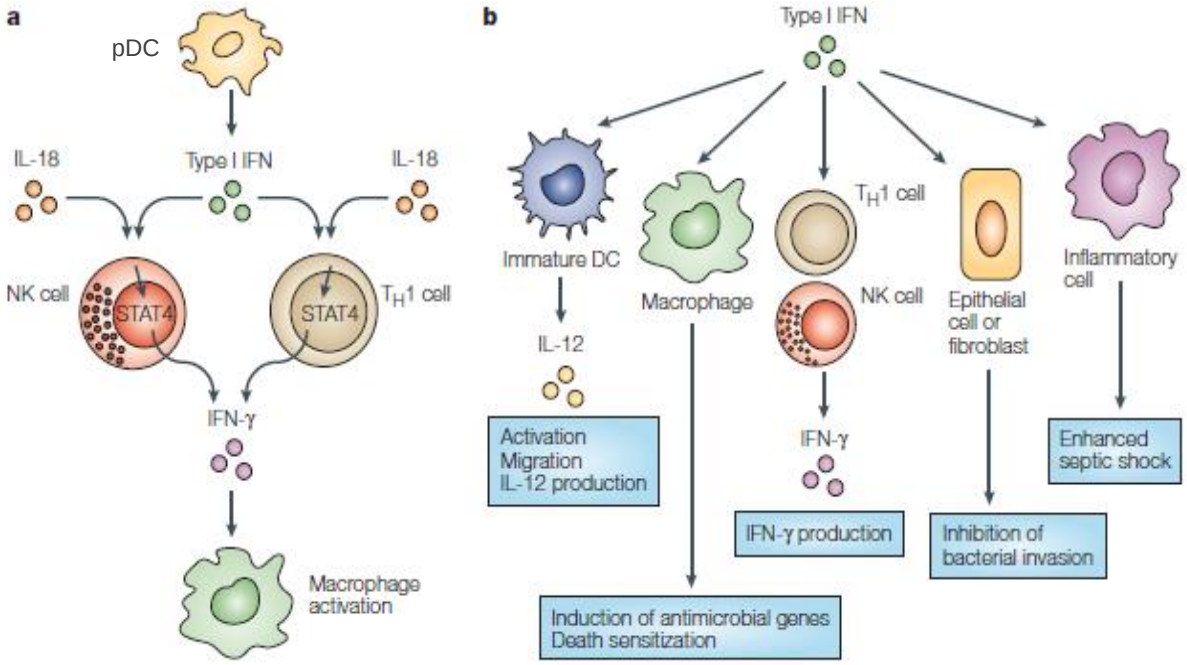

Figure 2.10 Type I IFN production by pDC during bacterial infections
Stimulation of pDC with bacteria activates type I IFN production. A) Production of type I IFN by pDC activates cytolytic activity and IFN-γ production by NK and cytotoxic $CD8^+$ T cells. B) Type I IFN also promote the function of DC, macrophages, inflammatory DC and epithelial cells/fibroblasts (Adapted from Decker *et al*., 2005).

Table 2.11 Immunomodulatory effects of type I IFN responses to bacterial infections

Bacteria	Effect of type I IFN	Ref
Chlamydia trachomatis	Protective, supports nitric oxide production and killing by macrophages	Devitt *et al.* (1996)
Legionella pneumophila	Protective, inhibits intracellular growth	Schiavoni *et al.* (2004)
Listeria monocytogenes	Detrimental, sensitises/induces death in effector cells	Auerbuch *et al.* (2004); Stockinger *et al.* (2004)
Mycobacterium tuberculosis	Detrimental, correlation between virulence and type I IFN production	Bouchonnet *et al.* (2002); Bond *et al.* (2012)
Salmonella typhimurium	Protective, reduces bacterial invasion and supports IL-12 dependent IFN-γ production	Bukholm *et al.* (1984); Freudenberg *et al.* (2002)
Streptococcus pneumoniae	Protective, enhances antibody production	Weigent *et al.* (1986)

The role of pDC during bacterial infections has been underappreciated. However, evidence is accumulating that pDC also respond to bacterial infections by producing type I IFN which together mediate conflicting beneficial and detrimental immunomodulatory effects in the host (Decker *et al.*, 2005). Ang *et al.* (2010) demonstrated that pDC are rapidly recruited to the lungs of *L. pneumophila*-infected C57BL/6 mice and that these infiltrating pDC were bactericidal against *L. pneumophila.* Furthermore, depletion of pDC corresponded with increased bacterial burden and disease severity in *L. pneumophila*-infected C57BL/6 mice (Ang *et al.*, 2010). The ability of pDC and type I IFN production to drive beneficial and detrimental anti-bacterial responses was highlighted in a study using mice infected with *Chlamydia pneumoniae.* Crother *et al.* (2012) demonstrated that boosting pDC numbers by injecting FLT-3L into mice was detrimental as the increased pDC response caused excessive inflammation in the lungs of mice. Conversely, depletion of pDC was detrimental resulting in impaired bacterial clearance that leads to severe and prolonged infection (Crother *et al.*, 2012). Although *Staphylococcus aureus* has been shown to stimulate maturation of human pDC and the production of type I IFN, TNF-α and IL-6, these mature *S. aureus*-infected pDC are in fact detrimental to the host as they drive the development of a B cell-mediated tolerance that is ineffective at clearing the *S. aureus* infection (Michea *et al.*, 2013; Parcina *et al.*, 2013). The functional response of pDC toward *B. pseudomallei* has not been investigated and therefore was the focus of the research presented in Chapter 4 of this Thesis.

2.12 Adaptive Immune Responses to *B. pseudomallei*

The activation of the adaptive immune responses is crucial for providing pathogen-specific effector function to neutralise the pathogen, destroy infected host cells and develop immunological memory. B cells contribute to eliminating pathogens by producing antibodies which coat the surface of extracellular pathogens, triggering the classical complement pathway and promoting phagocytosis and killing of the pathogen by innate immune cells (Dempsey *et al.*, 2003). Evidence of *B. pseudomallei*-specific antibodies in patients with melioidosis led to the development of antibody detection assays such as the IHA

(Ashdown, 1981; Ashdown, 1987). However, *B. pseudomallei* is able to evade humoral immune responses by invading host cells where they survive and replicate within the cytoplasm (Jones *et al.*, 1996; Harley *et al.*, 1998b). Thus the activation of CMI responses for targeted detection and killing of infected host cells, by $CD8^+$ and $CD4^+$ T cells, is critical for the control of intracellular pathogens, such as *B. pseudomallei*.

The T cells of the adaptive immune system are divided broadly into $CD8^+$ and $CD4^+$ T cells. Cytotoxic $CD8^+$ T cells are activated by antigen presented via MHC class I and elicit targeted killing of pathogen-infected host cells (Murphy *et al.*, 2011). $CD4^+$ T cells are activated by antigen presented via MHC class II and differentiate in to T_h1, T_h2, T_h17, T_{fh} or T_{reg} cells, with the subsets providing a range of flexible effector functions (Figure 2.9). The importance of the development of T cell responses in determining disease progression and providing resistance during *B. pseudomallei* infection, has been demonstrated in both humans and in murine models (Ketheesan *et al.*, 2002; Barnes *et al.*, 2004; Haque *et al.*, 2006b; Barnes and Ketheesan, 2007). Comparison of T cells derived from patients who had recovered from melioidosis and healthy individuals demonstrated that when T cells were stimulated with *B. pseudomallei* antigens, proliferation and IFN-γ production was significantly higher in cells from patients compared to controls. The response of both $CD4^+$ and $CD8^+$ T cell subsets was higher in convalescent patients following *B. pseudomallei* infection in comparison to healthy controls (Ketheesan *et al.*, 2002). Similarly, Tippayawat *et al.* (2009) also observed that Thai patients who have recovered from melioidosis develop *B. pseudomallei*-specific $CD4^+$ and $CD8^+$ T cell memory, primarily terminally differentiated T effector memory cells which produce IFN-γ upon rechallenge with *B. pseudomallei* antigen *in vitro*.

In the chronic murine model of melioidosis it has been suggested that T cell responses are important for providing protection against *B. pseudomallei* infection. It was found that antigen-specific T cells were important for providing resistance to *B. pseudomallei* infection during later stages of infection (Haque *et al.*, 2006b; Barnes and Ketheesan, 2007). Investigation of the role of adaptive immune responses against re-infection with *B. pseudomallei* by Haque *et al*.

(2006b) was conducted by using BALB/c mice immunised with a live attenuated mutant of *B. pseudomallei*, 2D2. Immunisation of BALB/c mice with 2D2 led to the activation of $CD4^+$ T cells rather than $CD8^+$ T cells and provided significant but incomplete protection against a subsequent lethal challenge (Haque *et al.*, 2006a). Barnes and Ketheesan (2007) investigated the ability of lymphocytes to confer immunity to naïve mice by transferring *B. pseudomallei*-specific T cells generated in C57BL/6 mice to naïve C57BL/6 mice. While the recipient mice developed a significant delayed-type hypersensitivity (DTH) reaction, they were not protected against subsequent challenge with a highly virulent *B. pseudomallei* isolate (Barnes and Ketheesan, 2007). Further investigation into the activation of protective immune responses by Barnes and Ketheesan (2007) demonstrated that *B. pseudomallei* antigen composition affects the development of protection. Susceptible BALB/c mice immunised with low-dose live *B. pseudomallei* developed significant DTH response and demonstrated significant protection and lower bacterial loads in the spleen following subsequent lethal challenge in comparison to BALB/c mice immunised with heat-killed *B. pseudomallei*, heat-killed *B. pseudomallei* with cultured filtrate antigen or *B. pseudomallei* lysate (Barnes and Ketheesan, 2007). These studies provide evidence that adaptive immune responses are activated following infection with *B. pseudomallei*.

2.13 Conclusion

Burkholderia pseudomallei is a resilient bacterium that employs a number of mechanisms to resist killing and facilitate its intracellular survival. Despite improved understanding of the host's immune response to *B. pseudomallei* infection over the last 100 years, there are still large gaps in our knowledge of host-pathogen interactions. Investigations of the interactions between the host and *B. pseudomallei* have found that co-ordinated and robust innate immune responses, namely the efficient function of macrophages and appropriate activation of adaptive immune responses involving $CD4^+$ and $CD8^+$ T cells during the later stages of infection contribute to the development of immunity against *B. pseudomallei.*

Dendritic cells are important antigen presenting cells which provide one of the major links between the innate and adaptive immune responses. However, there have been limited investigation of the role of DC during *B. pseudomallei* infections. Thus, important aspects of DC functionality, including the role of different DC subsets and the migration capacity of DC infected with *B. pseudomallei* remain unclear. Furthermore, diagnosis and management of patients with melioidosis is affected by the lack of accurate and reliable methods for detecting active *B. pseudomallei* infection and monitoring the development of protective CMI responses. Investigating host-pathogen interactions is paramount to define the pathways leading to the development of protective immunity. Such knowledge will be instrumental in the development of improved assays for the diagnosis and management of patients with melioidosis and also for the strategic development and delivery of new therapeutic and vaccine candidates.

CHAPTER 3
GENERAL MATERIALS AND METHODS

3.1 Mice

Eight to twelve week old C57BL/6 and BALB/c mice were sourced from the James Cook University Small Animal Breeding Facility. Institutional ethics approval was obtained for this study (A1601). All animal investigations were conducted in accordance with the National Health and Medical Research Council (NHMRC) guidelines for the care and use of animals for scientific purposes.

3.2 Peripheral Blood Collection from Human Participants

James Cook University Human Ethics Committee provided institutional ethical approval for the use of human peripheral blood in this study (Approval #H4470). Queensland Health Townsville Hospital and Health Service also granted ethical approval for the collection of human peripheral blood from patients who have recovered from melioidosis (Approval # 71/04). In accordance with the NHMRC National statement on the Ethical Conduct of Human Research, all participants were respectfully informed of the purposes of the study and requirements for participation. After obtaining the participant's voluntary consent, peripheral blood was collected by venepuncture into lithium heparin or serum clot activator vacutainers (BD Biosciences, North Ryde, Australia) by a trained phlebotomist. For isolation of human plasmacytoid dendritic cells (pDC; Chapter 4), 50 ml of peripheral blood was collected into lithium heparin vacutainers. For isolation of peripheral blood mononuclear cells (PBMC) and serum (Chapter 7), 20 ml and 5 ml of peripheral blood was collected into lithium heparin and serum clot activator vacutainers, respectively.

3.3 Burkholderia pseudomallei

Burkholderia pseudomallei is classified as a risk group 3/category B pathogen, which is deemed to have the potential to be used in biological warfare (Rotz *et al.*, 2002). Therefore, all investigations requiring the manipulation, culturing or handling of *B. pseudomallei* were conducted at the JCU Physical Containment level 3 (PC3) laboratory within PC2 Biocontainment hoods, according to JCU PC3 Facility standard operation procedures (SOP). Animals experimentally exposed to *B. pseudomallei* were housed within an ISOcage Air Handling Unit (Techniplast S.p.a, Buguggiate, Italy) inside the JCU PC3 Facility. All contaminated biohazard waste was autoclaved and disposed of according to the JCU PC3 Facility SOP.

3.3.1 Isolates for characterisation of DC function

Two *B. pseudomallei* isolates, NCTC 13178 (high virulence) and NCTC 13179 (low virulence), were used to investigate the functional responses of pDC (Chapter 4) and conventional DC (Chapter 5 and 6) toward *B. pseudomallei.* Both NCTC 13178 and NCTC 13179 are clinical isolates originally cultured from two patients diagnosed with melioidosis at the Townsville Hospital. These two isolates have been characterised, sequenced and the virulence determined by calculating the LD_{50} doses for these isolates in BALB/c and C57BL/6 mice using the Reed and Muench method (Table 2.6 in Chapter 2; Reed and Muench, 1938; Barnes and Ketheesan, 2005).

3.3.2 Isolates cultured from IHA-negative patients

Six *B. pseudomallei* isolates originally cultured from the six patients who participated in the study described in Chapter 7, were obtained from the Townsville Hospital collection. The six patients, who had recovered from culture confirmed melioidosis, were selected based on persistently negative serology (minimum 1 month post-discharge, the longest being to 18 years post-discharge) for *B. pseudomallei* antibodies according to the indirect haemagglutination assay (IHA). These clinical isolates which had been cultured from each IHA-negative

patient during their admission were added to the long term cryopreserved cultures stored in the JCU *B. pseudomallei* isolate library and used for the preparation of *B. pseudomallei* lysates described below (Section.3.3.6).

3.3.3 Preparation of *B. pseudomallei* glycerol stocks

Working stocks of the two *B. pseudomallei* isolates, NCTC 13178 and NCTC 13179, were prepared from the long term cryopreserved cultures stored in the JCU *B. pseudomallei* isolate library. Each isolate was first cultured on blood agar (Appendix 1) and Ashdown agar (Appendix 1) for 48 hr at 37 °C. Following confirmation of purity and characteristic *B. pseudomallei* colony morphology, colonies were harvested from the plate then washed and resuspended in phosphate buffered saline (PBS, pH 7.2, Appendix 1). BALB/c mice were infected with $1xLD_{50}$ intraperitoneally with NCTC 13178 (n=5) and NCTC 13179 (n=5) according to Barnes and Ketheesan (2005). Two days post-infection, the mice were killed by CO_2 asphyxiation and the spleens harvested. Each spleen was macerated in 2 ml PBS and plated on Ashdown agar. After 48 hr of culture, the colonies of each plate were resuspended (1 ml PBS/plate) to create a pooled suspension for each isolate. The bacteria were centrifuged (4000 G, 10 min) and the supernatant discarded. The bacterial pellets were resuspended in PBS and the wash step repeated twice. After the final wash, the bacterial pellets were resuspended in 50 % glycerol in PBS. The glycerol stocks were aliquoted into microfuge tubes and frozen at -80 °C.

3.3.4 Preparation of log phase *B. pseudomallei*

As required, a fresh vial of *B. pseudomallei* glycerol stock (NCTC 13178 and/or NCTC 13179) was defrosted then cultured on blood agar and Ashdown agar for 48 hr at 37 °C to confirm pure cultures (Barnes and Ketheesan, 2005). One colony of each isolate from the blood agar cultures was then used to inoculate 10 ml of tryptic soy broth (TSB; Acumedia, Cell Biosciences Pty Ltd, Heidelberg, Australia) and incubated overnight with shaking (100 rpm) at 37 °C. Overnight TSB cultures were diluted 1:10 and incubated for 3 hr with shaking at 37 °C until *B. pseudomallei* reached log phase. Log phase *B. pseudomallei* were washed with

PBS and adjusted to an optical density (OD, wavelength 600 nm) of 0.150 in PBS. At OD 0.150, the bacteria are at an approximate concentration of 10^8 CFU/ml and were used for experimental infection. The optimal conditions for infecting DC cultures with *B. pseudomallei* have previously been determined (Williams *et al.*, 2008; Williams *et al.*, 2011). Infectious doses were retrospectively determined by serially diluting (10-fold) the bacteria and plating 10 µl aliquots of each dilution in triplicate on Ashdown Agar. Plates were incubated for 48 hr at 37 °C, colonies were then enumerated and the original bacterial concentration calculated.

3.3.5 Preparation of heat-killed *B. pseudomallei*

Log phase *B. pseudomallei* (NCTC 13178 and NCTC 13179) prepared as previously described (Section 3.3.4) were diluted to 10^7 CFU/ml. Aliquots (5 ml) were placed into 10 ml tubes and incubated in a water bath at 80 °C for 1 hr then stored at 4 °C for one week. Prior to heat treatment, one aliquot was reserved for retrospective determination of the bacterial concentration as previously described (Section 3.3.4). To confirm that the heat-killed (Hk)-*B. pseudomallei* were sterile, 1 ml of Hk-*B. pseudomallei* was plated on blood agar and Ashdown agar and incubated for 48 hr at 37 °C then checked to ensure there was no bacterial growth.

3.3.6 Preparation of *B. pseudomallei* lysates

Bacterial lysates were prepared from NCTC 13178 and NCTC 13179 together with each of the isolates derived from IHA-negative patients. For each isolate, cultures were grown on Ashdown agar at 37 °C for 48 hr then colonies were resuspended in 30 ml of sterile PBS. The bacterial wet weight was estimated and an equal weight of 0.1 mm diameter glass beads (Daintree Industries Pty Ltd, St. Helens, Australia) was added. The bacteria and beads were then resuspended in breaking buffer (Appendix 1) and sonicated on ice using four 10 min bursts (S-4000 Sonicator Ultrasonic Processor, Misonix Inc, Daintree Scientific, St Helens, Australia), with 10 min breaks between each burst. The suspension was centrifuged (4500 G, 12 min) and the supernatant filtered using 0.22 µm filters (Merck Millipore, Bayswater, Australia). The lysates were dialysed and re-passed through 0.22 µm filters. Sterility was confirmed by plating 200 µl aliquots on

Ashdown agar and blood agar then incubating the plates at 37 °C for 48 hr. Protein concentrations of the lysates were determined using a bicinchoninic acid (BCA) protein assay kit (Progen Pharmaceuticals Ltd, Darra, Australia) as per manufacturer's instructions. Lysates were diluted to 5 mg/ml and stored at -80 °C.

3.4 Cell Culture

3.4.1 Culture of bone marrow derived DC

Culture of BMDC was carried out according to previously published methods (Lutz *et al.*, 1999; Williams *et al.*, 2008). The hind legs were removed from C57BL/6 and BALB/c mice and cleaned of all soft tissue. The bone marrow from the femur and tibia was removed by flushing RPMI 1640 (Invitrogen, Mulgrave, Australia) through the bone using a syringe and 27 gauge needle. Isolated BM was filtered through 63 μm mesh to produce a single cell suspension. Bone marrow cells ($2x10^6$ cells/ml) were cultured in petri dishes containing 10 ml of DC media (RPMI-1640 with 10% HI-FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 50 μM 2-Mercaptoethanol; Appendix 1) supplemented with 10 % GM-CSF culture supernatant prepared as described below (Section 3.4.2). On day 10 of culture, non-adherent BMDC were harvested by gentle pipetting.

3.4.2 Preparation of GM-CSF supernatant

Culture supernatant containing GM-CSF was prepared using Ag8653 myeloma cells transfected with the gene encoding murine GM-CSF (kindly provided by B. Stockinger, MRC National Institute for Medical Research, Mill Hill, London, UK). A cryovial of Ag8653 myeloma cells was quickly thawed in a 37 °C water bath. Cells were washed twice with 10 ml RPMI 1640 then seeded into a culture flask ($4x10^5$ cells/ml) containing Ag8653 myeloma culture media (Appendix 1) and incubated at 37 °C in 5 % CO_2. Once cell growth was nearly confluent (exponential phase, approximately day 2-3), cells were harvested by pipetting and transferred to a 50 ml conical tube (BD Biosciences, North Ryde, Australia),

centrifuged (500 G, 10 min) and resuspended in culture media to set up fresh growth flasks. This was repeated until the cell numbers were sufficiently up scaled to 10^8 cells. Fresh culture flasks were seeded with cells (10^5 cells/ml) and cultured at 37 °C in 5 % CO_2 for 6 days until cell viability was reduced to 50 % (determined by trypan blue exclusion staining). At this time, the supernatant containing GM-CSF was harvested, filtered through low protein binding 0.22 μm filters and stored at -80 °C.

3.4.3 Isolation of human peripheral blood mononuclear cells

Peripheral blood was collected in lithium heparin vacutainers (BD Biosciences, North Ryde, Australia) as described in Section 3.2. To isolate the peripheral blood mononuclear cells (PBMC), blood was diluted 1:1 with RPMI-1640 (Gibco®, Life Technologies Australia Pty Ltd, Mulgrave, Australia) and layered over 3 ml of Ficoll-paque PLUS (GE Healthcare Australia Pty Ltd, Rydalmere, Australia) using sterilised glass Pasteur pipettes. The tubes were centrifuged (500 G, 20 min) and the PBMC within the buffy coat removed from the interface between the Ficoll-paque and RPMI-1640 using glass transfer pipettes and gently transferred to a fresh tube. The isolated PBMC were washed twice with RPMI-1640 (500 G, 10 min) and the cell pellet resuspended at 10^6 cells/ml in RPMI-1640.

3.5 Flow Cytometry Acquisition and Analysis

Cells were prepared for acquisition as described in the Methods and Materials Sections of Chapter 4, 5, 6 and 7. Immediately prior to acquisition, cells were filtered through 63 μm mesh and 10 μl of Countbright™ absolute counting beads (Invitrogen, Life Technologies Australia Pty Ltd, Mulgrave, Australia) was added. Then samples were acquired using a FACS Calibur with Cell Quest software (BD Biosciences, North Ryde, Australia). Post-acquisition analysis was performed using Flow Jo software (Tree Star Inc, Ashland, USA). Countbright™ absolute counting beads are a calibrated suspension of microspheres with a known concentration that enabled determination of absolute cell count, cell concentration and volume of samples analysed by flow cytometry. During flow cytometric

analysis, the beads were gated in the top left hand corner of forward against side scatter FACS plots (Figure 3.1). A minimum of 1000 bead events were acquired to ensure statistically significant determination of cell concentrations according to the manufacturer's instructions. The number of beads counted was then used to determine the cell concentrations (below, other formulas in Appendix 3):

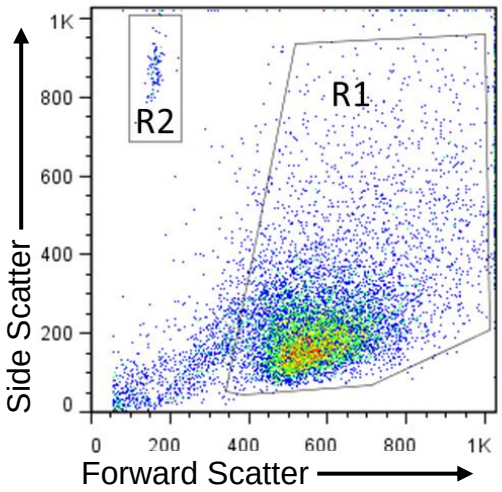

Figure 3.1 Gating of Countbright™ beads for calculating cell concentration
The example FACS plot shows the gating of target cell population (R1) and Countbright™ beads (R2) from which event numbers are recorded and used to determine cell concentration.

Analysed cell concentration (cells/ml) =

$$\frac{\text{Number of cell events counted (R1)}}{\text{Number of bead events counted (R2)}} \times \frac{\text{Number of beads added (see TDS)}}{\text{Volume of sample (ml)}}$$

Original cell concentration (cells/ml) =

$$\frac{\text{Analysed cell concentration (cells/ml)} \times \text{Volume of sample analysed (ml)}}{\text{Volume of original cells used (ml)}}$$

CHAPTER 4
INTERACTIONS BETWEEN PLASMACYTOID DENDRITIC CELLS AND *BURKHOLDERIA PSEUDOMALLEI*

4.1 Introduction

In Australia and Thailand, 46-60 % and 100 % of patients with acute melioidosis, respectively become bacteraemic triggering rapid progression of the disease from a localised infection to a disseminated sepsis involving multiple organs and significantly increasing the patient's risk of developing fatal septic shock (Suputtamongkol *et al.*, 1994; Currie *et al.*, 2000b; Malczewski *et al.*, 2005). Early diagnosis and treatment is imperative to prevent mortality associated with this severe form of the disease. Despite clinician awareness in areas endemic for *B. pseudomallei*, melioidosis sepsis is often fatal. The mortality rate of melioidosis in Australia and Thailand is 14-25 % and 26-68 %, respectively although following the development of septic shock this increases dramatically to 50 % and 77 %, respectively (Currie *et al.*, 2010; Limmathurotsakul *et al.*, 2010). Consequently, melioidosis is the third most common cause of death from an infectious disease in Thailand, after AIDS and tuberculosis (Limmathurotsakul *et al.*, 2010).

Sepsis caused by melioidosis is characterised by dysregulated immune cell activation, significantly increased pro-inflammatory cytokine production and the development of multiple organ failure (Wiersinga *et al.*, 2007a). Investigations of immune cell interactions during *B. pseudomallei* infection have found that macrophages and neutrophils provide important early defence against *B. pseudomallei* (Leakey *et al.*, 1998; Barnes *et al.*, 2008; Hodgson *et al.*, 2011; Woodman *et al.*, 2012). However, the signalling pathways driving melioidosis sepsis are undefined. Recently, profiling of gene expression signatures from the whole blood of patients with melioidosis demonstrated that type I interferon (IFN) responses were prominent, although the implications of these findings are yet to be investigated (Koh *et al.*, 2013a). While type I IFN signalling can be beneficial, excessive type I IFN signalling during infection can drive inflammatory

syndromes that lead to pathological damage in the host (Decker *et al.*, 2005; Trinchieri, 2010).

Plasmacytoid dendritic cells (pDC) are capable of rapid type I IFN production however, their contribution to the immune responses against *B. pseudomallei* has not been explored. pDC are a unique subset of dendritic cells (DC) that provide innate immune responses to pathogens and share characteristics with both conventional DC (cDC) and B cells (Liu, 2005). The development of pDC is similar to cDC however, pDC morphology and migratory behaviour resembles B cells. Following interaction with a pathogen, pDC develop a dendritic morphology and acquire a mature phenotype with elevated expression of antigen presenting molecules and T cell co-stimulatory molecules, similar to cDC (Liu, 2005). Although pDC develop a mature phenotype capable of antigen presentation, unlike cDC, they are not considered efficient antigen presenting cells. Consequently, pDC are often considered to be an effector cell of the innate immune response, providing immune modulating signals such as type I IFN (Villadangos and Young, 2008).

In comparison to other type I IFN producing cell such as cDC, macrophages and epithelial cells, pDC are considered specialised IFN-producing cells capable generating systemic responses by rapidly producing large quantities of type I IFN (Decker *et al.*, 2005; Trinchieri, 2010). In fact, pDC have been found to account for 95 % of type I IFN produced by circulating peripheral blood mononuclear cells (PBMC; Siegal *et al.*, 1999; Liu, 2005). pDC are able to rapidly produce large quantities of type I IFN due to constitutive high expression levels of IFN regulatory factor (IRF) 7, which allows rapid assembly of the multiprotein signal transduction complex necessary for inducing type I IFN production (Reizis *et al.*, 2011a). In contrast, other cell types such as macrophages use a IRF3/IFN-β/IFNα/β receptor (IFNAR)/IRF7 positive feedback pathway to increases IRF7 expression levels in order to produce type I IFN (Decker *et al.*, 2005; Trinchieri, 2010).

Historically, pDC and type I IFN were thought to only potentiate 'anti-viral' immunity. It is now recognised that pDC and type I IFNs are capable of orchestrating diverse immune responses to bacterial infections that can be both

beneficial or detrimental to the host (Schiavoni *et al.*, 2004; Decker *et al.*, 2005; Meyer-Wentrup *et al.*, 2008; Parcina *et al.*, 2013). While the importance of pDC and type I IFN is well described for viral infections, the role that pDC play during bacterial infections is poorly defined and underappreciated. Although limited, studies on bacteria and pDC interactions have demonstrated that pDC are indeed capable of responding to bacterial RNA/DNA by up regulating type I IFN production (Eberle *et al.*, 2009; Poth *et al.*, 2010). During pulmonary infection of mice with *Legionella pneumophila*, pDC are rapidly recruited to the lung where they play an active role in controlling bacterial burden (Ang *et al.*, 2010). In contrast, *Staphylococcus aureus* has been shown to suppress immune responses by exploiting pDC and type I IFN production to establish a B cell-mediated immune tolerance (Parcina *et al.*, 2013).

Production of type I IFN appears to be the main effector function of pDC. However, defining the role of pDC during bacterial infections is complicated by this feature since type I IFN signalling is known to drive conflicting beneficial anti-bacterial and detrimental immune suppressive functions in different bacterial infections (Shankar *et al.*, 1996; Schiavoni *et al.*, 2004; Decker *et al.*, 2005; Trinchieri, 2010). Type I IFN signalling can directly inhibit the bacterial replication of *Chlamydia* species and induce intracellular killing of bacteria (Ishihara *et al.*, 2005). Treatment of murine macrophages with type I IFN *in vitro*, was found to be protective and completely inhibited the intracellular growth of *L. pneumophila* (Schiavoni *et al.*, 2004). In contrast, *Listeria monocytogenes* infection has been shown to stimulate type I IFN production which is detrimental to the host, sensitising host cells to *L. monocytogenes*-induced cell death (Stockinger and Decker, 2008). Type I IFN treatment of human monocytes/macrophages suppressed their normal macrophage mycobacteriostatic activity, demonstrated by unrestricted intracellular replication of *Mycobacterium bovis* BCG (Bouchonnet et al 2002). Similarly, the ability of *M. tuberculosis* strains to induce type I IFN by the host correlated with bacterial virulence (Simmons *et al.*, 2010).

Bacterial-induced type I IFN production also indirectly affects other antibacterial defence mechanisms. The lipopolysaccharide (LPS) of *Chlamydia* species has

been shown to induce type I IFN production, which can subsequently activate nitric oxide production. The release of nitric oxide is an important effector mechanism that regulates both pathogenic and protective responses of macrophages to chlamydial infection (Devitt *et al.*, 1996). Type I IFN mediated production of nitric oxide is important for activating protective responses by macrophages and NK cells against *Chlamydia* species however, in excess can enhance the adverse effects of early innate immune responses and suppress adaptive immunity by inhibiting T cell activation and inducing apoptosis of activated T cells (Huang *et al.*, 2002). Furthermore, downstream signalling of type I IFN has also been found to positively and negatively influence the secretion of the type II IFN, IFN-γ, a well-documented anti-bacterial cytokine (Trinchieri, 2010). Following *Salmonella typhimurium* infection, type I IFN signalling is required for IFN-γ production, which enhances antigen presentation by DC and activation of macrophages to kill intracellular pathogens (Yaegashi *et al.*, 1995; Frucht *et al.*, 2001; Freudenberg *et al.*, 2002). However, following infection with *Mycobacterium leprae* the production of type I IFN, specifically IFN-β and downstream signals such as interleukin (IL) 10 secretion, caused significant inhibition of IFN-γ signalling and correlated with disseminated and progressive lepromatous lesions (Teles *et al.*, 2013).

Type I IFN also have complex and often opposing effects in controlling susceptibility and lethality in various models of sepsis or endotoxic shock (Trinchieri, 2010). Deficiency in type I IFN or IFN-α receptor (IFNAR) correlate with reduced lethality in models of LPS-induced endotoxic shock and septic peritonitis (Karaghiosoff *et al.*, 2003; Weighardt *et al.*, 2006). Significantly increased peritoneal neutrophil recruitment and activation was observed in $IFNAR^{-/-}$ mice with septic peritonitis. In contrast, in an alternative murine model of sepsis using cecal ligation and puncture, $IFNAR^{-/-}$ mice demonstrated increased mortality, which was correlated with decreased neutrophil recruitment (Kelly-Scumpia *et al.*, 2010). These studies further highlight the complex and conflicting downstream effects of type I IFN signalling.

Together, pDC and type I IFN present a complex relationship that can drive beneficial or detrimental processes following bacterial infection. The contribution

of pDC to clearance or persistence of *B. pseudomallei* infection has not been investigated. Therefore, this study aimed to investigate the ability of pDC to internalise and kill *B. pseudomallei,* undergo maturation and produce type I IFN. Both human pDC isolated from PBMC and murine bone marrow (BM) derived pDC were utilised. Whilst investigation of B. *pseudomallei* interactions with human cells is more physiologically relevant, human pDC represent a small fraction of circulating human PBMC, resulting in low cell yield and placing limitations on the experimental design. Therefore, the current study included murine pDC generated from BM cell cultures due to the high yields of pDC produced using this method.

The culture of murine BM cells *in vitro* with Fms-like tyrosine kinase 3 ligand (FLT-3L) supplementation has been reported to generate a heterogeneous cell population that includes pDC (Brasel *et al.*, 2000; Gilliet *et al.*, 2002). Murine pDC were generated from C57BL/6 and BALB/c mouse strains, which have been characterised for their innate resistance and susceptibility to *B. pseudomallei* infection respectively (Leakey *et al.*, 1998; Barnes and Ketheesan, 2005; Breitbach *et al.*, 2011). The innate resistance and susceptibility of C57BL/6 and BALB/c mice correlates with distinct differences in functional responses of macrophages and DC toward *B. pseudomallei* (Leakey *et al.*, 1998; Williams *et al.*, 2008; Breitbach *et al.*, 2011). In addition, C57BL/6 and BALB/c mice are known to possess contrasting genetic capacity for type I and II IFN production. While C57BL/6 mice are high type I and II IFN producers that bear the high expression allele for *If-1* IFN regulatory locus (*If-1*^{h}), BALB/c mice bear the *If-1*^{l} (low) expression allele and demonstrate consistently poor type I and II IFN production in response to bacterial infection (Daigneault *et al.*, 1988; Shankar *et al.*, 1996). In the current study, the interactions of murine pDC with two characterised and sequenced *B. pseudomallei* clinical isolates of high (NCTC 13178) and low (NCTC 13179) virulence were investigated to determine if *B. pseudomallei* virulence correlates with the ability to induce type I IFN production, similar to *M. tuberculosis* (Barnes and Ketheesan, 2005; Simmons *et al.*, 2010; Breitbach *et al.*, 2011). The type I IFN response of human pDC was determined following exposure to the high virulence *B. pseudomallei* isolate only

as it was predicted that the yield of human pDC would be insufficient to compare their response to isolates of both high and low virulence.

Therefore the specific aims of this study were to:

i) Assess the ability of human pDC exposed to highly virulent *B. pseudomallei* to
    a. Internalise the bacteria and
    b. Produce type I IFN
ii) Compare the ability of murine pDC derived from C57BL/6 and BALB/c mice exposed to *B. pseudomallei* isolates of high and low virulence to
    a. Internalise and kill the bacteria
    b. Produce type I IFN and
    c. Undergo maturation
iii) Compare the internalisation and killing capacity of murine pDC against cDC derived from C57BL/6 and BALB/c mice

4.2 Materials and Methods

4.2.1 Enrichment and culture of human pDC from peripheral blood

4.2.1.1 Collection of human peripheral blood

Peripheral blood (5 ml) was collected in lithium heparin vacutainers (BD Biosciences, North Ryde, Australia), as previously described (Section 3.2; James Cook University (JCU) Human Ethics approval H4470) from six healthy individuals (2 females and 4 males). The percentage of pDC in PBMC and the potential yield of pDC was estimated for each of the six healthy individuals. Four of these six healthy individuals (4 males) with high yields (greater than 0.7 %) were then selected as human pDC donors. Peripheral blood (50 ml) was collected in lithium heparin vacutainers from these four donors for all subsequent experiments.

4.2.1.2 Enrichment of human pDC from peripheral blood

Peripheral blood mononuclear cells were isolated as previously described (Section 3.4.3) from human peripheral blood by density gradient centrifugation using Ficoll-paque PLUS (GE Healthcare Australia P/L, Rydalmere, Australia). Enrichment of pDC from PBMC was achieved by using a negative selection human pDC isolation kit (Stemcell EasySep™, Stemcell Technologies Inc, Tullamarine, Australia) to deplete non-pDC according to the manufacturer's instructions. Briefly, PBMC were first incubated with anti-human CD32 (Fcγ RII) blocker to prevent non-specificFc/FcR interactions from lowering the purity of human pDC yielded by negative selection. PBMC were then incubated with human pDC enrichment cocktail, a combination of specific tetrameric antibody complexes directed against cell surface antigens of myeloid DC and non-pDC (note: exact cell surface antigens targeted were not specified by the supplier). These tetrameric antibody complexes are formed by linking an anti-cell surface antibody to an anti-dextran antibody, which enables the complex to bind both cell and dextran (Figure 4.1). Dextran-coated magnetic iron particles were added to bind to the unwanted non-pDC. The PBMC were then exposed to a strong magnetic force (BD iMag™ magnet, BD Biosciences, North Ryde, Australia) which physically separated the magnetically labelled non-pDC from the sample to leave untouched, enriched human pDC.

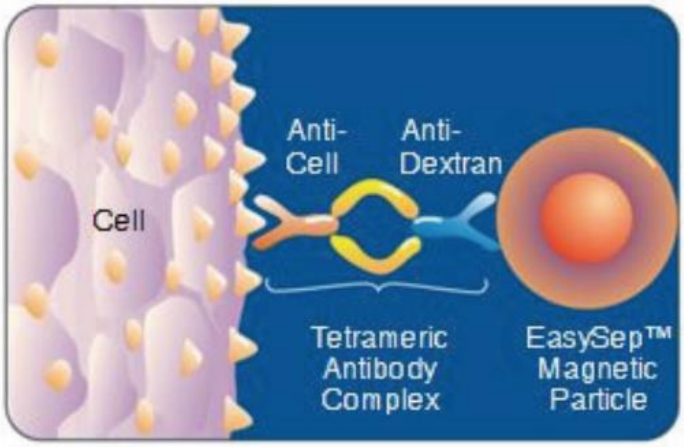

Figure 4.1 Schematic of the Easysep™ tetrameric antibody complex
A tetrameric antibody complex achieves the binding of human cells and dextran coated magnetic particles. The complex is formed by linking an anti-cell surface antibody to an anti-dextran antibody (STEMCELL Technologies, 2014).

4.2.1.3 Assessment of enriched human pDC purity

To assess the purity of the enriched human pDC, a sample of the pDC isolated from each individual was analysed by flow cytometry using a human pDC identification kit (IMGENEX, Jomar Bioscience P/L, Stepney, Australia) according to manufacturer's instructions. Briefly, cells were labelled with a mix of human lineage marker antibodies (hLMAX; CD3, CD14, CD16, CD19, CD20 and CD50 fluorescein isothiocyanate (FITC) conjugated), human leukocyte antigen (HLA)-DR peridinin-chlorophyll proteins (PerCP) with cyanine (Cy) 5.5 (PerCP-Cy5.5) conjugated and CD123 Alexa Fluor® (AF) 647 conjugated or mouse IgG_1 AF647 conjugated. The CD123 marker, also known as the IL-3 receptor α-chain, is highly expressed on pDC and important for IL-3 supported survival of pDC (Grouard *et al.*, 1997). Although CD123 is expressed on other myeloid cells, such as mast cells and basophils, the $hLMAX^-$, $HLA\text{-}DR^+$ and $CD123^+$ combination is distinctive for human pDC. All flow cytometry and calculations of pDC purity were performed as previously described (Section 3.5, Appendix 3).

4.2.2 Culture and enrichment of murine pDC

4.2.2.1 Culture of murine pDC from bone marrow using FLT-3L supplementation

To generate pDC from C57BL/6 and BALB/c mice (Section 3.1), bone marrow cells were flushed from the femur and tibia with RPMI 1640 (Invitrogen, Mulgrave, Australia), filtered through 63 μm mesh and centrifuged (400 G, 10 min). Red blood cells were lysed by resuspending the bone marrow cells in tris-ammonium chloride buffer and incubated at 37 °C for 5 min, then washed with RPMI 1640. Murine BM cells were cultured *in vitro* with FLT-3L supplementation to generate a heterogeneous cell population that included pDC, using previously published methods (Brasel *et al.*, 2000; Gilliet *et al.*, 2002). Washed BM cells were resuspended to 10^6 cells/ml in pDC media (RPMI-1640 with 10 % heat-inactivated foetal bovine serum (HI-FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 50 μM 2-Mercaptoethanol, 2.5 mM HEPES buffer; Appendix 1) supplemented with FLT-3L (150 ng/ml,

Peprotech, Abacus ALS P/L, East Brisbane, Australia). A total of $3x10^6$ bone marrow cells were seeded into each well of a 6 well culture plate (Nunc, Thermo Fisher Scientific P/L, Scoresby, Australia). On day 5 of culture, 50 % of the culture media was replaced and cultured until day 10.

4.2.2.2 Enrichment of murine pDC from FLT-3L supplemented bone marrow cell cultures

Commercial kits for positively selecting murine pDC are available, which target magnetic particles to the pDC antigen 1 (PDCA-1, also known as CD317 or bone marrow stromal cell antigen 2 [BST2]) to enable magnetic separation of murine pDC. Due to the high number of BM cells that required processing to enrich the PDCA-1^+ murine pDC, the current study used a combination of biotinylated anti-PDCA-1 antibody and streptavidin conjugated magnetic nanoparticles (BD IMag™ Streptavidin Particles Plus - DM) to magnetically separate PDCA-1^+ cells from the heterogeneous BM cell cultures generated by FLT-3L supplementation. On day 10 of culture, FLT-3L supplemented BM cell cultures from C57BL/6 and BALB/c mice were harvested, washed with buffer (1x BD IMag™ Buffer; BD Biosciences, North Ryde, Australia), resuspended to 10^7 cells/ml and incubated with biotinylated anti-mouse PDCA-1 antibody (1 μg/10^7 cells; eBioscience, Jomar Bioscience P/L, Stepney, Australia) on ice for 15 min. The cells were washed with buffer to remove excess unbound PDCA-1 antibody then the cell pellet was resuspended in streptavidin conjugated magnetic nanoparticles (20 μl/10^7 cells, BD IMag™ Streptavidin Particles Plus – DM; BD Biosciences, North Ryde, Australia) and incubated at 4 °C for 30 min. The volume was adjusted to 1 ml in buffer to give approximately 2-8$x10^7$ cells/ml then the cells were exposed to a strong magnetic force (BD iMag™ magnet, BD Biosciences, North Ryde, Australia) to physically separate the murine pDC (PDCA-1^+) with bound magnetic nanoparticles, from the non-pDC (PDCA-1^-). The purity of the murine pDC was improved by resuspending the isolated cells in 1 ml buffer and repeating the magnetic separation step twice. After completing the magnetic isolation steps, an aliquot of murine pDC were used for phenotype and purity assessment (Section 4.2.2.3). The remaining cells were resuspended in 10 ml RPMI 1640 and centrifuged (500 G, 10 min). The cells were washed again with

10 ml RPMI 1640 then 10^5 murine pDC were seeded into each well of a 96 well plate (Nunc, Thermo Fisher Scientific P/L, Scoresby, Australia) in pDC media and incubated at 37 °C in 5 % CO_2.

4.2.2.3 Assessment of murine pDC phenotype

Flow cytometric analysis of murine pDC generated from FLT-3L supplemented BM cultures was used to evaluate the phenotype and to assess the purity of murine pDC following magnetic selection of PDCA-1^+ cells. Cells were prepared for analysis by transferring 10^6 cells to a FACS tube (BD Biosciences, North Ryde, Australia). Following centrifugation (400 G, 5 min), cell pellets were washed with 2 ml sodium azide buffer (SAB; Appendix 1) prior to being resuspended in 20 µl of diluted primary antibodies or appropriate isotype control antibodies (Table 4.1) and incubated on ice for 30 min. To remove excess unbound primary antibody, cells were washed with 2 ml SAB, then incubated with streptavidin-APC (0.04 µg/10^6 cells; BD Biosciences, North Ryde, Australia) on ice for 30 min. Stained cells were washed with 2 ml SAB, then 2 ml of phosphate buffered saline (PBS, pH 7.2; Appendix 1). Washed cells were then resuspended in 300 µl PBS and analysed by flow cytometry as previously described (Section 3.5). Murine pDC generated in FLT-3L supplemented BM cultures were defined as PDCA-1^+/CD11c^+/B220$^+$/Ly-6c^+ cells. The purity of murine pDC following magnetic selection of PDCA-1^+ cells was calculated by determining the percentage of enriched cells that expressed PDCA-1 as described in Appendix 3 and expressed as the mean purity (%) ± SEM.

Table 4.1 Antibodies (anti-mouse) used for flow cytometric analysis of phenotype, purity and maturation of murine pDC

Antibody	Clone	Conjugate	Isotype	µg/10^6 cells
CD11c	HL3	Biotin	Armenian Hamster IgG_1	0.05
CD317 (PDCA-1)	eBio927	PE	Rat IgG_{2b}	0.1
CD317 (PDCA-1)	eBio927	Biotin	Rat IgG_{2b}	0.1
CD45R (B220)	RA3-6b2	PerCP-Cy5.5	Rat IgG_{2a}	0.05
Ly-6c	HK1.4	FITC	Rat IgG_{2c}	0.05
I-A/I-E (MHC class II)	M5/114.15.2	PE	Rat IgG_{2b}	0.04
CD86	GL1	PE	Rat IgG_{2a}	0.04

Note: Anti-mouse CD11c, I-A/I-E and CD86 antibodies were purchased from BD Biosciences, North Ryde, Australia. Anti-mouse CD45R, CD317 and Ly-6c antibodies were purchased from eBioscience, Jomar Bioscience Pty Ltd, Stepney, Australia. The optimal amount of antibody required for staining 10^6 cells in a final volume of 20 µl was determined by titration of the antibodies in preliminary experiments (data not shown).

4.2.3 *B. pseudomallei* infection of pDC and cDC

Plasmacytoid DC and cDC (prepared as previously described in Section 3.4.1) were infected with *B. pseudomallei* as previously described (Williams *et al.*, 2008; Williams *et al.*, 2011). Following enrichment and assessment of purity, $5x10^4$ human pDC or 10^5 murine pDC were seeded into each well of a 96 well plate in pDC media and incubated at 37 °C in 5 % CO_2. To enable comparison between murine pDC and cDC, 10^5 murine cDC/well (prepared as described in Section 3.4.1) were cultured in parallel with murine pDC. Two clinical *B. pseudomallei* isolates, NCTC 13178 (high virulence) and NCTC 13179 (low virulence), were grown to log phase as previously described (Section 3.3.4). Triplicate wells of pDC and cDC were then infected with *B. pseudomallei* NCTC13178 (high

virulence) and NCTC 13179 (low virulence, murine pDC and cDC only) at a multiplicity of infection (MOI) 1:1.

As a positive control for stimulating type I IFN production via the toll-like receptor (TLR) 9, CpG ODN 2216 (Sigma-Aldrich, Sydney, Australia) was added to triplicate wells at a final concentration of 3 μg/ml (Krug *et al.*, 2001). Cultures were incubated for 4 hr at 37 ºC in 5 % CO_2, at which time internalisation of *B. pseudomallei* by human pDC, murine pDC and murine cDC was assessed. Antibiotic protection of pDC was required to enable enumeration of intracellular bacteria only and to prevent uncontrolled extracellular bacterial replication from affecting cell viability. *B. pseudomallei* is resistant to a large range of antibiotics, including gentamicin which is typically used for antibiotic protection assays. The isolates used in this study are known to be sensitive to kanamycin (Feterl *et al.*, 2006; Williams *et al.*, 2008). Therefore, kanamycin (250 μg/ml; Sigma-Aldrich, Sydney, Australia; Appendix 1) was added to parallel co-cultures of DC and *B. pseudomallei* that required incubation for longer than 4 hr to kill extracellular bacteria. After a total of 24 hr co-culture, the following were assessed; i) human and murine pDC type I IFN cytokine production, ii) *B. pseudomallei* survival within murine pDC and cDC and iii) phenotypic maturation response of murine pDC.

4.2.4 Assessment of internalisation and intracellular survival of *B. pseudomallei* within pDC and cDC

Internalisation of *B. pseudomallei* by human pDC, murine pDC and murine cDC (infected as described above, Section 4.2.3) was assessed after 4 hr of culture and kanamycin (250 μg/ml) added to parallel cultures to kill extracellular *B. pseudomallei*. The intracellular survival of *B. pseudomallei* within murine pDC and cDC was assessed at 24 hr of culture. To determine the number of viable intracellular bacteria within pDC and cDC at both time points, the cells were harvested, washed twice with sterile PBS then resuspended in 0.1 % Triton-X (Appendix 1) to disrupt cells and release intracellular *B. pseudomallei.* The lysates were diluted serially (10-fold) in PBS and 10 μl samples of each dilution plated in triplicate on Ashdown Agar (Appendix 1). Plates were incubated for

48 hr at 37 °C then CFU were enumerated (minimum limit of detection was 20 CFU/5x10^4 human pDC, 20 CFU/10^5 murine pDC and 20 CFU/10^5 murine cDC). The percentage of *B. pseudomallei* internalised at 4 hr and the percentage of intracellular *B. pseudomallei* surviving at 24 hr was calculated (Appendix 3) and expressed as the mean internalisation (% ± SEM) and mean intracellular survival (% ± SEM), respectively.

4.2.5 Quantification of IFN–α and IFN–β produced by pDC infected with *B. pseudomallei*

Production of IFN-α and IFN-β by pDC in response to *B. pseudomallei* was determined by assessing the cytokine concentration in cell-free supernatants of uninfected and *B. pseudomallei*-infected pDC cultures. Cells were also stimulated with CpG ODN 2216 (3 μg/ml; Sigma-Aldrich, Sydney, Australia) as a positive control to demonstrate that the enriched human and murine pDC were capable of high IFN-α and IFN-β production (Krug *et al.*, 2001). Briefly, enriched human and murine pDC were infected with *B. pseudomallei* as previously described (Section 4.2.3). After 4 hr of culture, kanamycin (250 μg/ml) was added to each well to kill extracellular bacteria. The supernatant from each sample was collected after 24 hr of culture and stored at -80 °C. The concentrations of IFN-α and IFN-β in cell culture supernatants was quantified using human and mouse IFN-α ELISA kits (eBiosciences, Jomar Bioscience Pty Ltd, Stepney, Australia) and IFN-β ELISA kits (Sapphire Bioscience, Waterloo, Australia) according to manufacturer's instructions. For human pDC, the mean concentration of IFN-α and IFN-β in culture supernatants was expressed as the mean (± SEM) pg/ml. To enable comparison between murine pDC generated from high (C57BL/6) and low (BALB/c) type I IFN producing mice, the mean concentration of IFN-α and IFN-β in culture supernatants of murine pDC was determined then expressed as the fold increase (± SEM) above unstimulated (Appendix 3).

4.2.6 Assessment of MHC class II and CD86 expression on murine pDC infected with *B. pseudomallei* as markers of maturation

The level of MHC class II and CD86 expressed on uninfected and *B. pseudomallei*-infected murine pDC was analysed by flow cytometry to determine whether *B. pseudomallei* activated the maturation of murine pDC. Briefly, enriched murine pDC were infected with *B. pseudomallei* as previously described (Section 4.2.3). After 4 hr of culture, kanamycin (250 μg/ml) was added to each well to kill extracellular bacteria. After 24 hr of culture, pDC were harvested, transferred to FACS tubes (BD Biosciences, North Ryde, Australia) and centrifuged (400 G, 5 min). Cell pellets were washed with 2 ml SAB prior to being resuspended in 20 μl of diluted primary antibodies or appropriate isotype control antibodies (Table 4.1) and incubated on ice for 30 min. The cells were washed with 2 ml SAB then incubated with streptavidin-APC (0.04 μg/10^6 cells; BD Biosciences, North Ryde, Australia) on ice for 30 min. Stained cells were washed once with 2 ml SAB then fixed with 4 % paraformaldehyde (ProSciTech Pty Ltd, Kirwan, Australia) for 30 min at 4 °C. The cells were washed twice with 2 ml of PBS, resuspended in 300 μl PBS and analysed by flow cytometry as previously described (Section 3.5). The percentage of murine pDC that were PDCA-1^+/MHCclassII$^+$ and PDCA-1^+/CD86^+ was determined and expressed as the mean change (± SEM) above unstimulated (Appendix 3)

4.2.7 Statistical analysis

Statistical analysis of data was performed using GraphPad Prism 6 Software and details are reported in Appendix 2. For human pDC IFN-α production data, statistical significance between uninfected and infected human pDC data was tested using an ordinary 1way ANOVA with recommended Tukey's posthoc multiple comparison test. For murine pDC data, statistical significance was tested using 2way ANOVA with recommended Sidak's or Tukey's posthoc multiple comparisons test to compare mouse strains (C57BL/6 and BALB/c) and stimulation groups (uninfected, NCTC 13178, NCTC 13179 and CpG ODN 2216). Differences between tested groups were considered significant if the P

value ≤ 0.05. Significant differences are indicated in figures, * = P ≤ 0.05 and ** = P ≤ 0.01.

4.3 Results

4.3.1 Optimisation of enrichment and culture of pDC

4.3.1.1 Identification of circulating human pDC in peripheral blood

Plasmacytoid DC are a distinct subset of DC that are specialised IFN producers and readily distinguished from other DC and immune cells by their unique cell surface phenotype. The characterised phenotype of human pDC is $CD4^+$, $CD45RA^+$, IL-3Rα$(CD123)^+$, $ILT3^+$, $ILT1^-$ and lineage (CD3, CD14, CD16, CD19, CD20 and $CD56)^-$ cells. Circulating pDC in peripheral blood also express two additional markers, BDCA-2 and BDCA-4 (Colonna *et al.*, 2004). For the purpose of the current study, human pDC were defined as $lineage^-$, $HLA\text{-}DR^+$ and $CD123^+$ (Figure 4.2). As the reported percentage of pDC circulating within the peripheral blood of humans ranges from 0.2-2 % of PBMC (Liu, 2005), the percentage of pDC within PBMC isolated from the peripheral blood of six healthy individuals was compared. In the current study, the percentage of pDC within PBMC varied from 0.5-2.35 % and the potential yield of pDC was estimated to range between $8x10^3$ to $4.6x10^4$ pDC/ml of peripheral blood. The four individuals with the highest potential pDC yield were selected as donors for all subsequent investigations requiring human pDC.

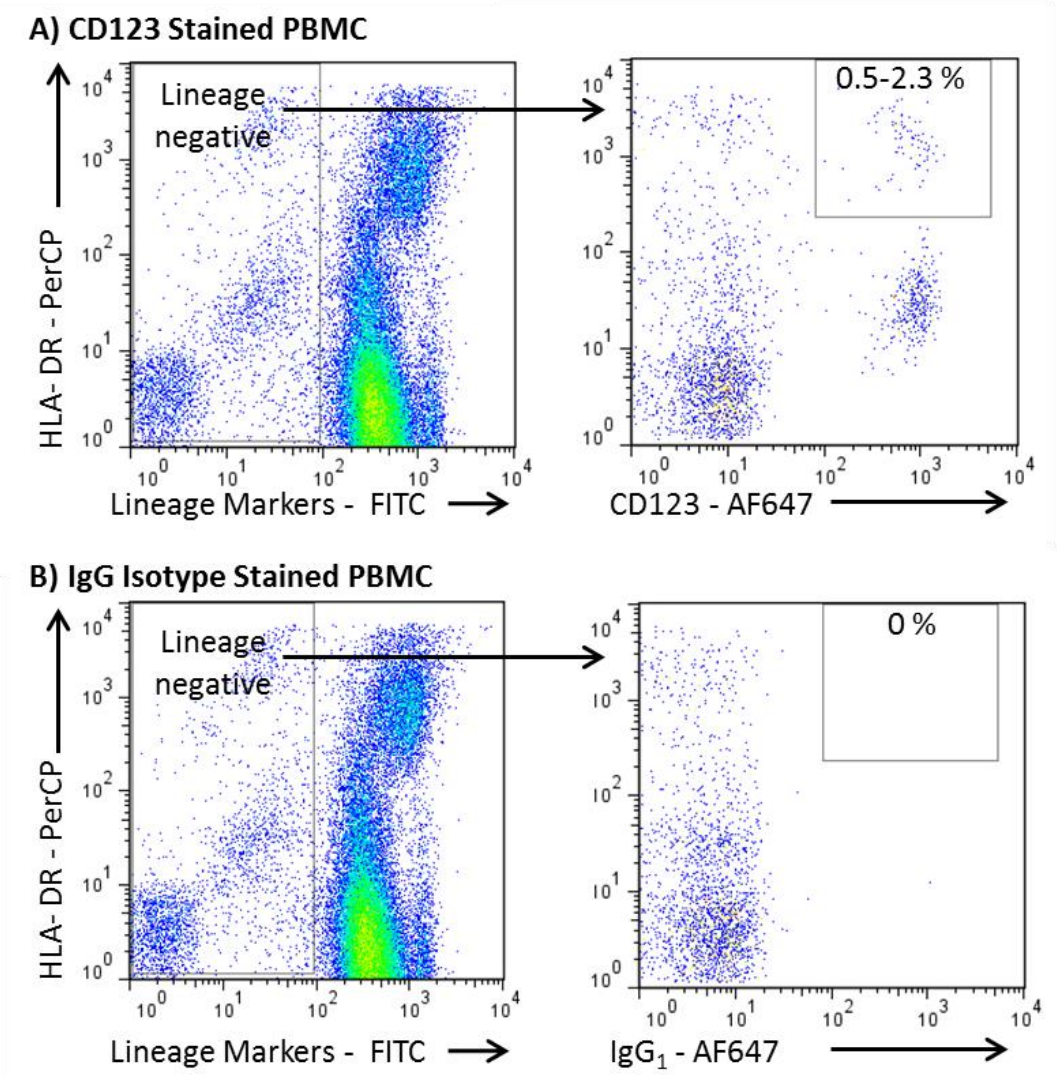

Figure 4.2 Flow cytometric identification of pDC within PBMC
Plasmacytoid DC within PBMC freshly isolated from the peripheral blood of six healthy individuals were identified by flow cytometry using a human pDC identification kit according to the manufacturer's protocol. The FACS plots shown are an example of one of the six healthy individuals assessed. A) The pDC identified are lineage negative (CD3, CD14, CD16, CD19, CD20 and CD56), HLA-DR positive and CD123 positive. B) Cells stained with IgG_1-AF647 isotype were used to confirm the specificity of CD123 staining of human pDC.

4.3.1.2 Enrichment of human pDC from peripheral blood

The ability of human pDC to internalise bacteria and produce type I IFN has previously been observed *ex vivo* by enriching human pDC from peripheral blood (Veckman and Julkunen, 2008; Michea *et al.*, 2013; Parcina *et al.*, 2013). The reported methods for achieving pure human pDC from peripheral blood differ between studies. In the previous section, the potential yield of human pDC from the peripheral blood of healthy donors was determined. Here, human pDC were enriched by negative selection from the PBMC of four individuals using a human pDC enrichment kit (Stemcell EasySep™) to magnetically deplete myeloid DC and non-pDC thus, following their removal, untouched human pDC remained. The purity of the enriched human pDC fractions was confirmed by flow cytometry using the human pDC identification kit (Imgenex) from the previous section, which defined human pDC as lineage$^-$/HLA-DR$^+$/CD123$^+$ (Figure 4.3). In addition to pDC, small population of basophils (CD123$^+$ cells that are lineage$^-$ but HLADR$^-$) and possibly conventional DC (lineage$^-$/HLA-DR$^+$/CD123$^-$) were observed (Stain *et al*., 1987; Agis *et al*., 1996; Olweus *et al.,* 1997). Overall, the purity of enriched human pDC was found to range between 88-97 % (Figure 4.3a). These enriched human pDC were then cultured in 96 well plates for subsequent experimental infection with *B. pseudomallei.*

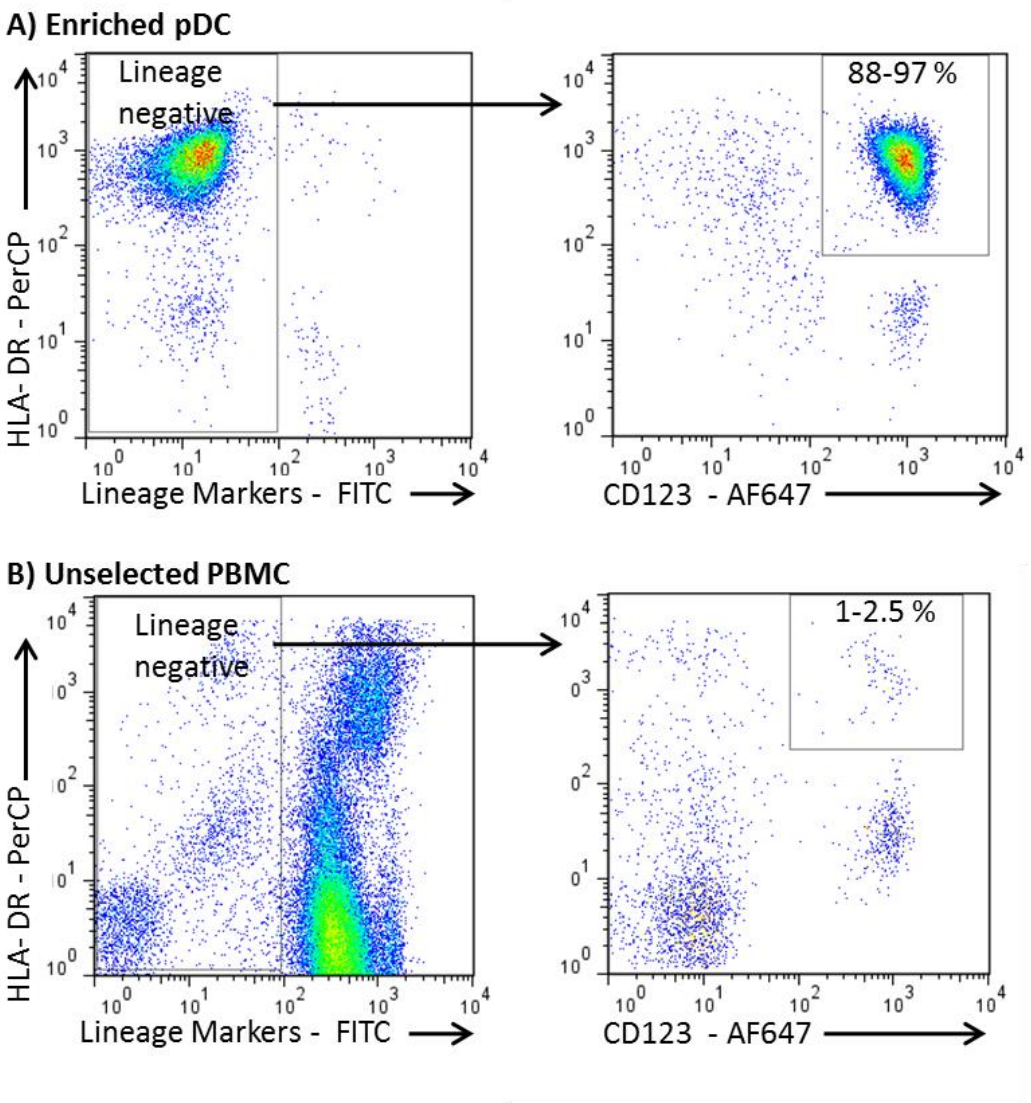

Figure 4.3 Purity of enriched human pDC assessed by flow cytometry
Human pDC were enriched from freshly isolated PBMC using a negative selection human pDC enrichment kit. A) Enriched pDC and B) unselected PBMC fractions were fluorescently labelled with antibodies from a human pDC identification kit then analysed by flow cytometry to identify human pDC in each fraction and determine the purity. Following enrichment of human pDC, the purity was determined to range between 88-97 %.

4.3.1.3 Characterisation of murine pDC phenotype generated from bone marrow cell cultures supplemented with FLT-3L

As human pDC represent a small fraction of circulating PBMC, it is difficult to achieve high yields of pDC, thus limiting the number of variables and functional assays that could be performed using human pDC. Consequently, the functional

responses of human pDC exposed to *B. pseudomallei* isolates of low and high virulence was not compared, nor was the ability of human pDC to kill *B. pseudomallei*. To allow for more detailed investigation of pDC and *B. pseudomallei* interactions and comparison between pDC and cDC, murine pDC were generated from the bone marrow of C57BL/6 and BALB/c mice. Although the published methods for using FLT-3L supplementation and the expected yield varies slightly, there is consensus about the phenotypical markers of murine pDC. Murine pDC can be identified by the expression of CD11c, B220 and Ly6c along with the pDC specific cell surface markers PDCA-1 and pDC-TREM (Brasel *et al.*, 2000; Gilliet *et al.*, 2002; Naik *et al.*, 2005; Blasius *et al.*, 2006; Watarai *et al.*, 2008). For the purpose of the current study, the bone marrow from C57BL/6 and BALB/c mice was cultured with FLT-3L supplementation (150 ng/ml) for up to 10 days and murine pDC were defined as PDCA-1^+, $CD11c^+$, $B220^+$ and $Ly6c^+$ by flow cytometry (Figure 4.4 and 4.5). It was noted that although C57BL/6 pDC were identified (Figure 4.4A) and demonstrated positive staining for PDCA-1 compared to controls (IgG isotype, Figure 4.4B), PDCA-1 staining was brighter on BALB/c pDC (Figure 4.5).

As previous studies used different lengths of culture periods with FLT-3L supplementation to generate murine pDC, the percentage of pDC in BM cell cultures was compared at day 8 and 10 of culture. The percentage of cells that with a pDC phenotype was higher in BM cultures supplemented with FLT-3L for 10 days (C57 – 74.3 ± 5.3 %, Figure 4.4; BALB/c – 83.4 ± 4.4 %, Figure 4.5) compared to cultures harvested on day 8 (C57 – 58.1 ± 1.7 %, BALB/c – 67.5 ± 4.7 %). For all subsequent investigations using murine pDC, BM cell cultures were supplemented with FLT-3L for 10 days prior to harvesting. While the majority of cells generated with FLT-3L supplementation for 10 days had a pDC phenotype, an enrichment step was required to increase the purity of pDC prior to experimental infection with *B. pseudomallei.*

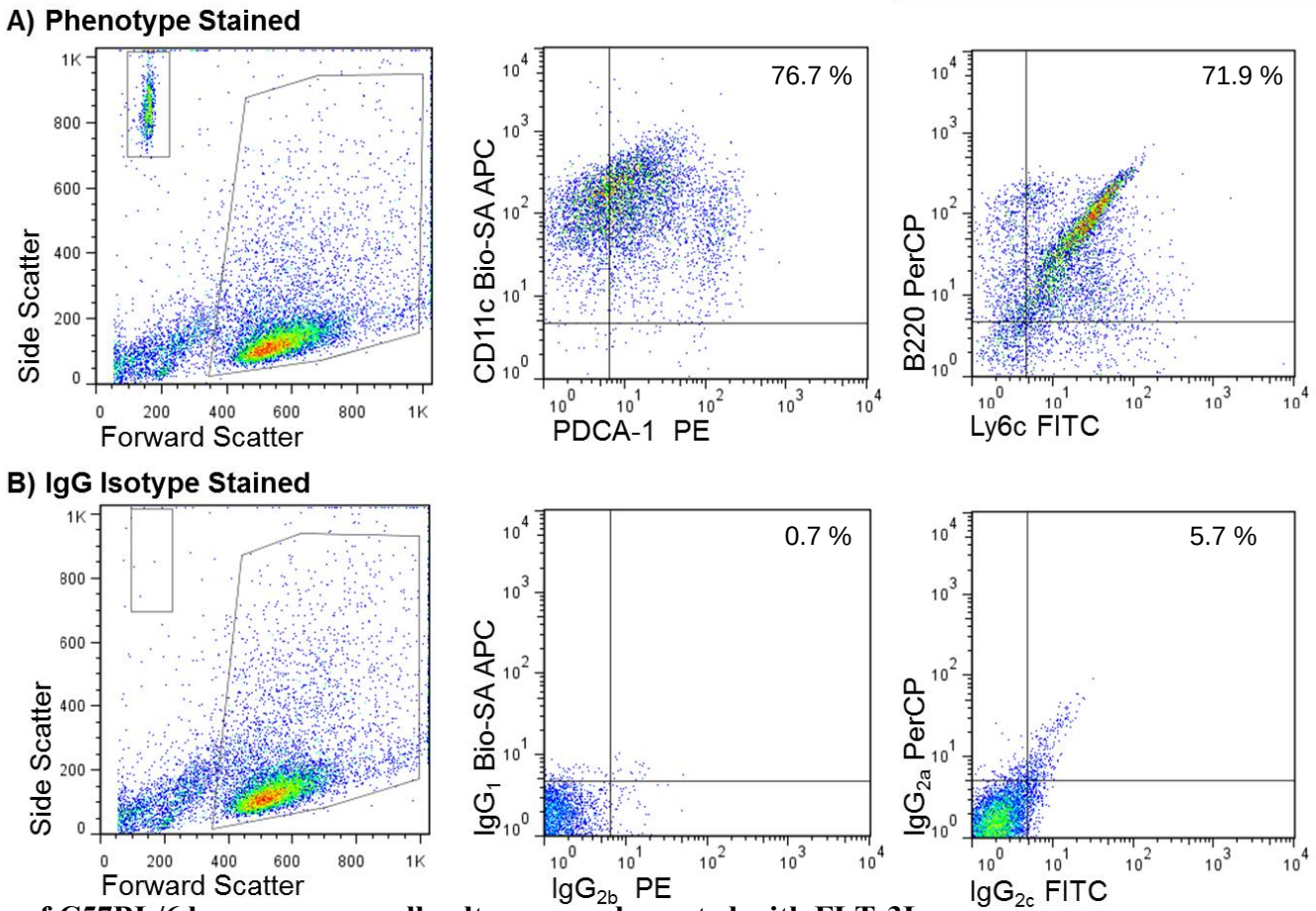

Figure 4.4 Phenotype of C57BL/6 bone marrow cell cultures supplemented with FLT-3L

The expression of PDCA-1, CD11c, B220 and Ly6c on BM cells from C57BL/6 mice supplemented with FLT-3L for 10 days was analysed by flow cytometry. The FACS plots are an example of phenotype stained and IgG isotype control stained cells and show the majority of cells present have a pDC phenotype ($PDCA\text{-}1^{+}/CD11c^{+}/B220^{+}/Ly6c^{+}$). Countbright™ counting were added to enable calculation of cell concentrations. On day 10 of culture, 74.3 ± 5.3 % of the C57BL/6 BM cells had a pDC phenotype.

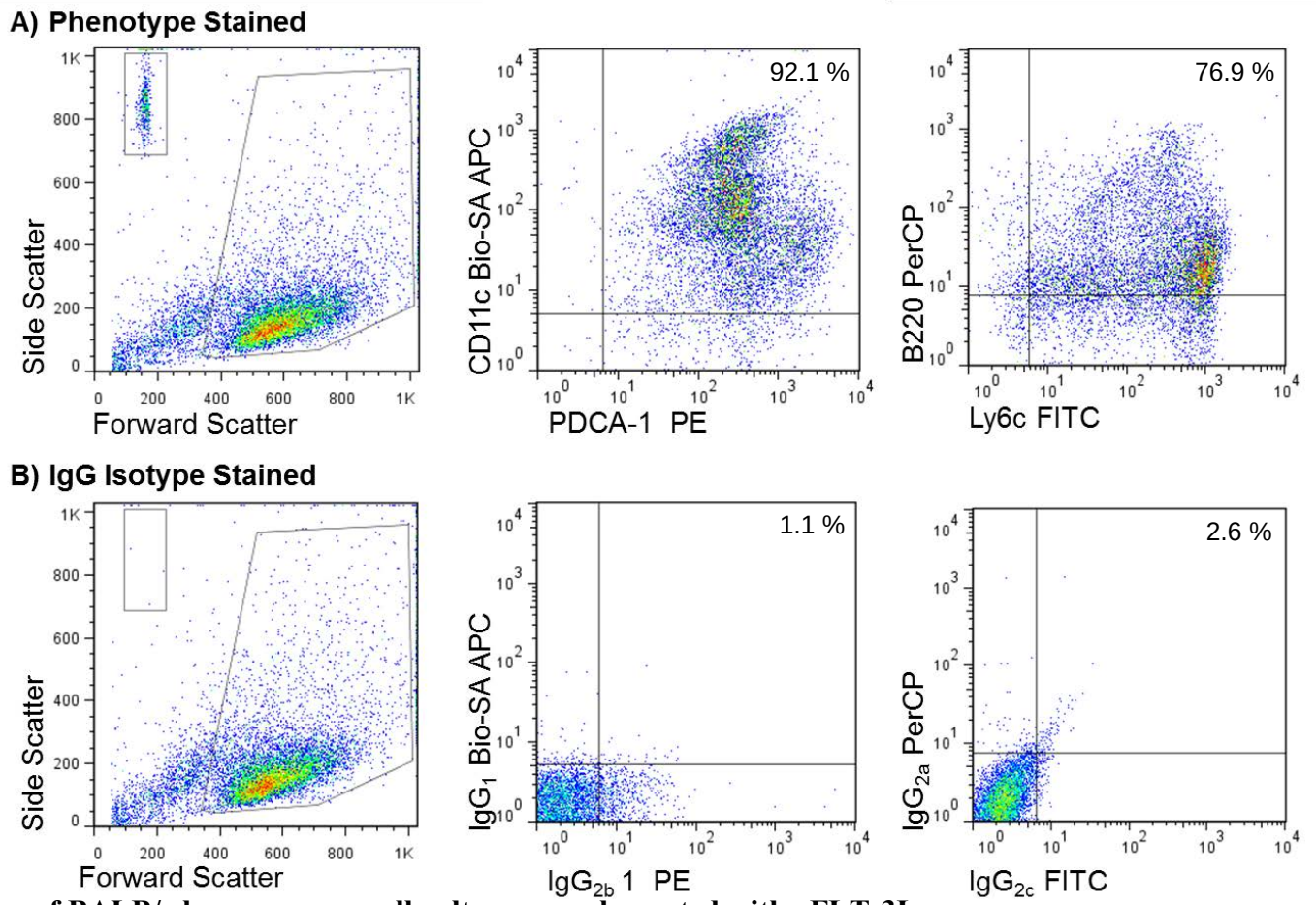

Figure 4.5 Phenotype of BALB/c bone marrow cell cultures supplemented with FLT-3L

The expression of PDCA-1, CD11c, B220 and Ly6c on BM cells from BALB/c mice supplemented with FLT-3L for 10 days was analysed by flow cytometry. The FACS plots are an example of phenotype stained and IgG isotype control stained cells and show the majority of cells present have a pDC phenotype (PDCA-1^+/CD$11c^+$/B220^+/Ly$6c^+$). Countbright™ counting were added to enable calculation of cell concentrations. On day 10 of culture, 83.4 ± 4.4 %of the BALB/c BM cells had a pDC phenotype.

4.3.1.4 Enrichment of murine pDC from bone marrow cell cultures supplemented with FLT-3L

In the previous section, characterisation of murine BM cell cultures supplemented with FLT-3L (150 ng/ml) for 10 days revealed that the majority of cells displayed a pDC phenotype. However, an enrichment step was required to increase the purity of murine pDC for experimental infection with *B. pseudomallei.* Therefore, murine pDC were enriched from the FLT-3L supplemented BM cell cultures on day 10 of culture by magnetically separating PDCA-1^+ cells. Enriched murine pDC (PDCA-1^+) fractions were then analysed for purity by flow cytometry and found to be 91 ± 2.8 % for C57BL/6 pDC (Figure 4.6A) and 90 ± 3.2 % for BALB/c pDC (Figure 4.7A). In comparison, the flow through supernatant predominantly contains unselected non-pDC that are PDCA-1^- (Figure 4.6B and 4.7B). The enriched murine pDC were cultured in 96 well plates for subsequent experimental infection with *B. pseudomallei.*

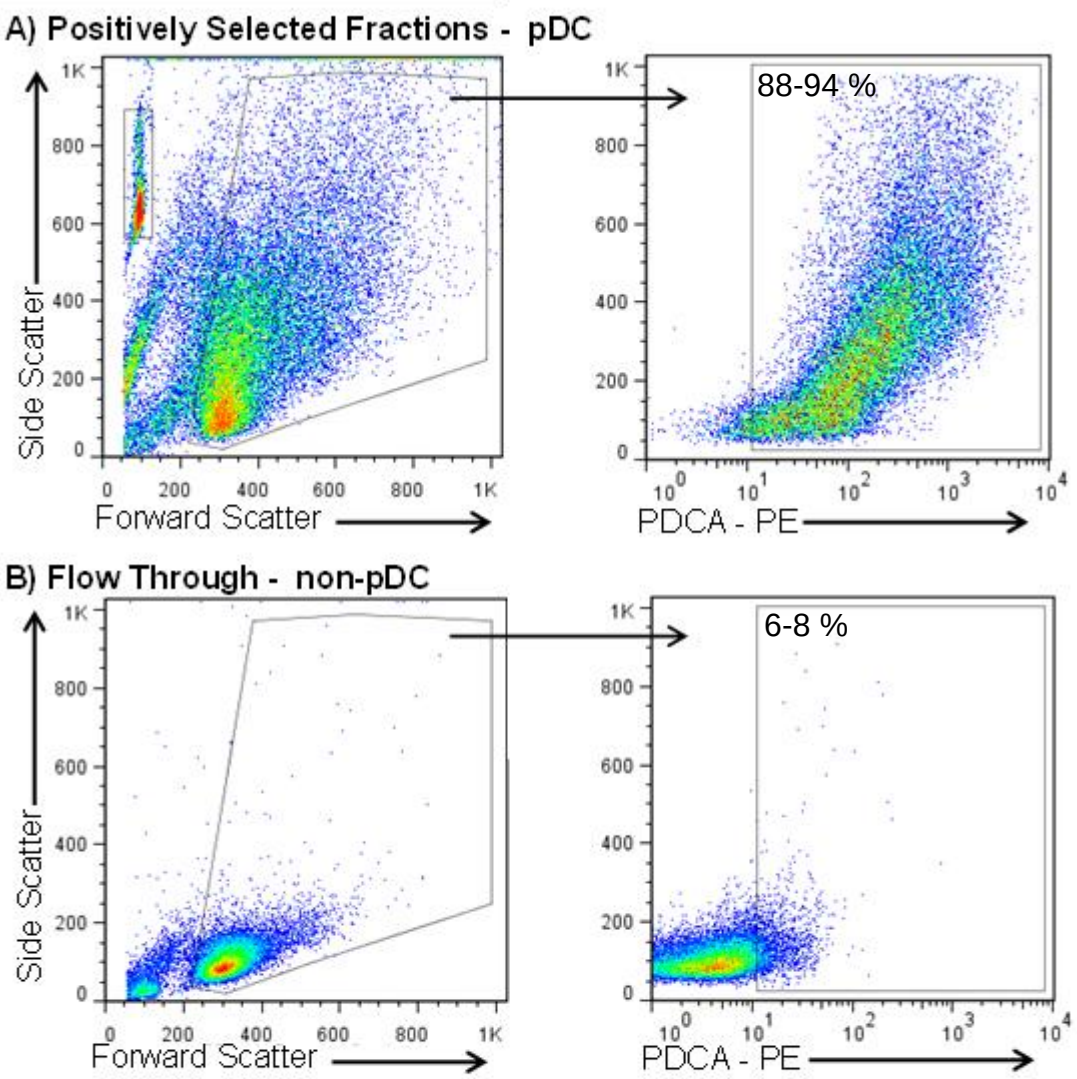

Figure 4.6 Purity of murine pDC from C57BL/6 mice following positive selection for PDCA-1$^+$ cells

C57BL/6 pDC (PDCA-1$^+$) were enriched from BM cells cultured with FLT-3L supplementation for 10 days. Flow cytometric analysis of PDCA-1 expression was used to determine the purity of murine pDC. The FACS plots are an example of pDC (PDCA-1$^+$) and non-pDC (PDCA-1$^-$) fractions generated from C57BL/6 mice. Countbright™ counting beads were added to enable calculation of cell concentrations and purity. Following enrichment of C57BL/6 pDC, the purity was determined to be 91 ± 2.8 %.

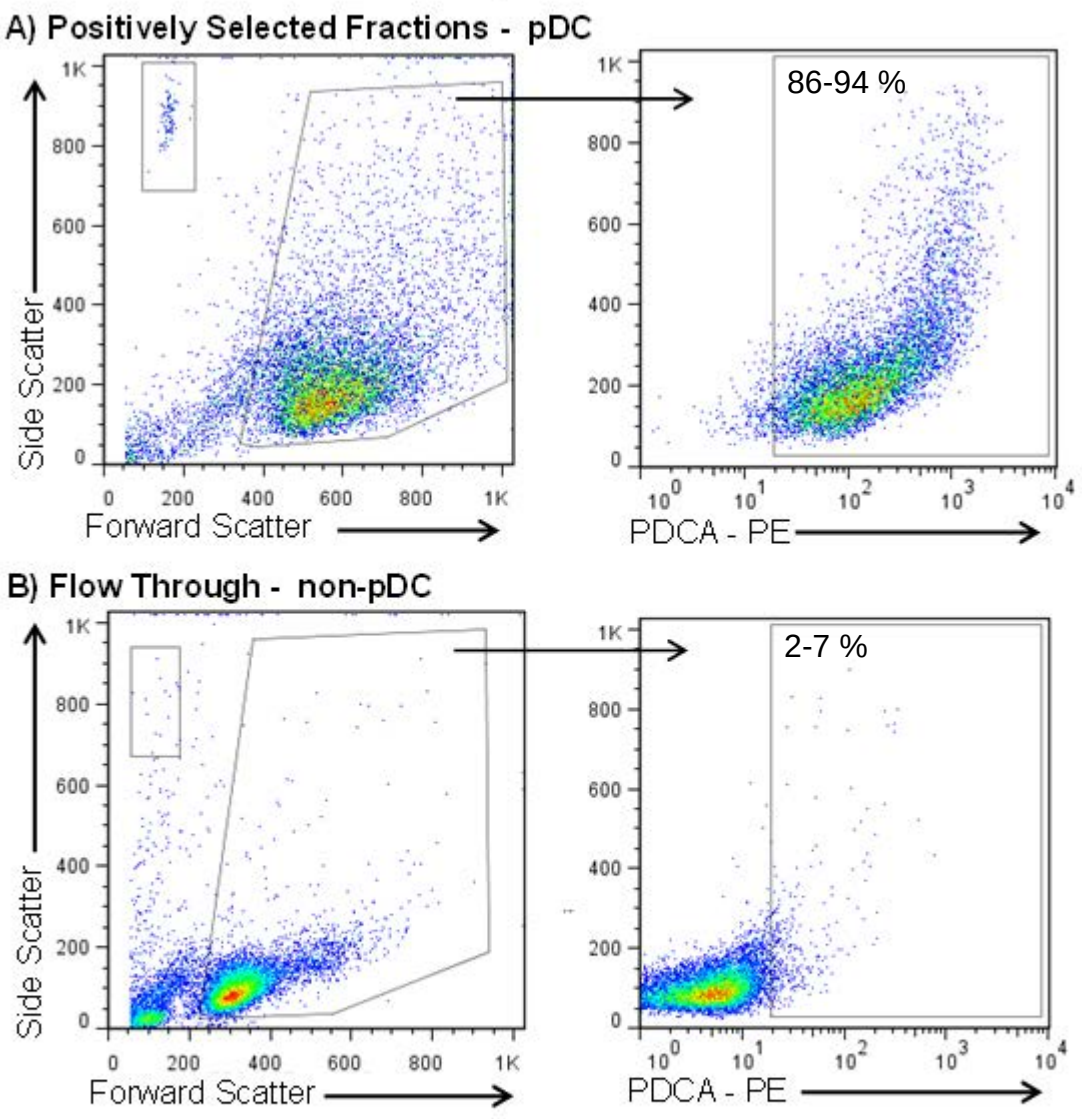

Figure 4.7 Purity of murine pDC from BALB/c mice following positive selection for PDCA-1$^+$ cells

BALB/c pDC (PDCA-1$^+$) were enriched from BM cells cultured with FLT-3L supplementation for 10 days. Flow cytometric analysis of PDCA-1 expression was used to determine the purity of murine pDC. The FACS plots are an example of pDC (PDCA-1$^+$) and non-pDC (PDCA-1$^-$) generated from BALB/c mice. Countbright™ counting beads were added to enable calculation of cell concentrations and purity. Following enrichment of BALB/c pDC, the purity was determined to be 90 ± 3.2 %.

4.3.2 Internalisation and killing of *B. pseudomallei* by pDC

4.3.2.1 Assessment of *B. pseudomallei* internalisation by human pDC

To investigate the *in vitro* functional responses of human pDC toward *B. pseudomallei*, the ability of human pDC to phagocytose a highly virulent isolate (NCTC 13178) was determined. Internalisation of *B. pseudomallei* by human pDC was demonstrated. After 4 hr, human pDC had internalised 2 ± 0.67 % of the bacteria added to the assay (Figure 4.8), an average of 1113 ± 342 CFU/$5x10^4$ pDC. Due to the low yield of human pDC from 50 ml of peripheral blood, the subsequent capacity of human pDC to kill intracellular *B. pseudomallei* was not able to be determined within the same assay system.

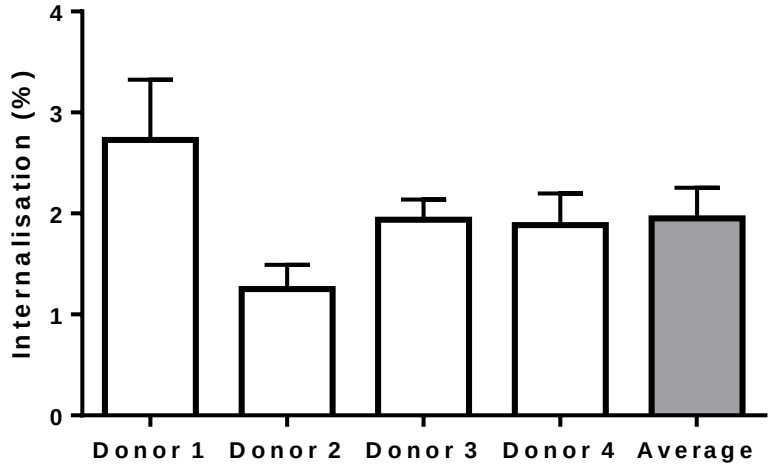

Figure 4.8 Internalisation of highly virulent NCTC 13178 *B. pseudomallei* by human pDC
Human pDC (n=4 donors) were infected *in vitro* with *B. pseudomallei* (NCTC 13178, high virulence). Viable intracellular *B. pseudomallei* were enumerated after 4 hr of co-culture and expressed as a percentage of bacteria internalised. Human pDC internalised 2 ± 0.67 % of the bacteria after 4 hr. Each bar represents human pDC isolated from one healthy donor or the average of all four donors. The mean ± SEM of duplicate experiments is displayed.

4.3.2.2 Assessment of *B. pseudomallei* internalisation and killing by murine pDC and cDC

The ability of murine pDC derived from *B. pseudomallei*-resistant C57BL/6 and *B. pseudomallei*-susceptible BALB/c mice to phagocytose *B. pseudomallei* isolates *in vitro* was compared (Figure 4.9). The percentage of *B. pseudomallei* internalised by C57BL/6 pDC after 4 hr of co-culture was comparable to BALB/c

pDC. When *B. pseudomallei* isolates of high and low virulence were compared, although internalisation of the highly virulent NCTC 13178 isolate was higher, no significant difference in uptake of *B. pseudomallei* by murine pDC was observed. Furthermore, internalisation of *B. pseudomallei* by murine pDC was assessed in parallel with murine cDC and demonstrated that murine pDC have a similar capacity to phagocytose *B. pseudomallei* as murine cDC (Figure 4.9).

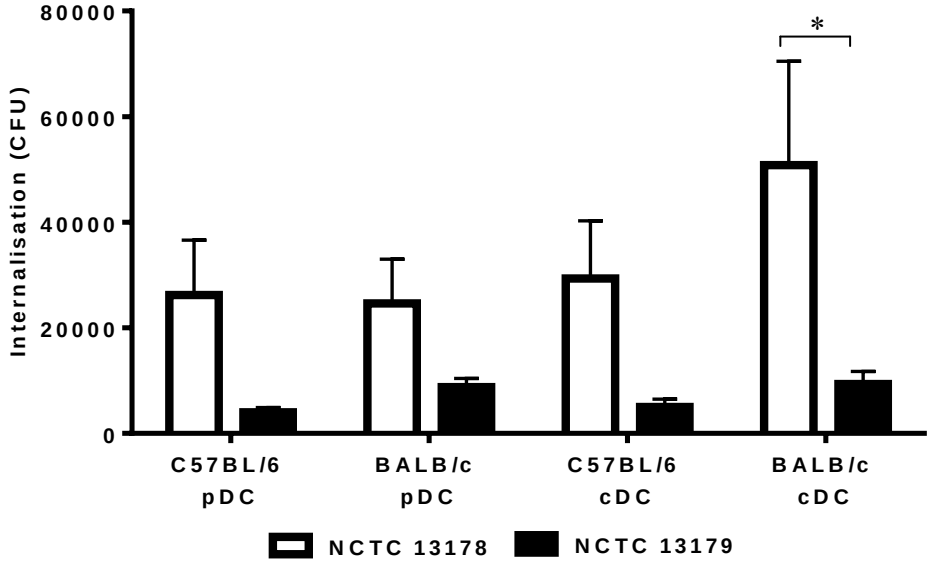

Figure 4.9 Internalisation of *B. pseudomallei* isolates of high and low virulence by murine pDC and cDC
Murine pDC and cDC were infected *in vitro* with *B. pseudomallei* isolates NCTC 13178 (high virulence) and NCTC 13179 (low virulence). Viable intracellular *B. pseudomallei* were enumerated after 4 hr of co-culture and expressed as a percentage of bacteria internalised. C57BL/6 and BALB/c pDC demonstrated similar capacity to internalise *B. pseudomallei* and this was not influenced by isolate virulence. Furthermore, murine pDC were as efficient as cDC at internalising *B. pseudomallei*. Bars depict the mean ± SEM of three experiments. * = P≤ 0.05 determined using a 2way ANOVA with multiple comparison tests.

Subsequent to assessing *B. pseudomallei* internalisation by murine pDC, the intracellular survival of *B. pseudomallei* within murine pDC derived from *B. pseudomallei*-resistant C57BL/6 and *B. pseudomallei*-susceptible BALB/c mice was assessed. The intracellular survival of *B. pseudomallei* was higher in BALB/c pDC compared to C57BL/6 pDC (Figure 4.10), suggesting that BALB/c pDC are not as efficient at killing *B. pseudomallei.* Although this trend was not statistically significant, it is similar to the significant trend seen for cDC (Figure 4.10). Of interest, murine pDC were more efficient at killing intracellular

B. pseudomallei compared to murine cDC. The intracellular survival of *B. pseudomallei* within murine pDC was lower than that observed within murine cDC however, this trend was only significant when the intracellular survival of NCTC 13178 (high virulence) within BALB/c pDC was compared to BALB/c cDC. The increased survival of *B. pseudomallei* in BALB/c cDC compared to C57BL/6 cDC is consistent with previously published data (Williams *et al.*, 2008).

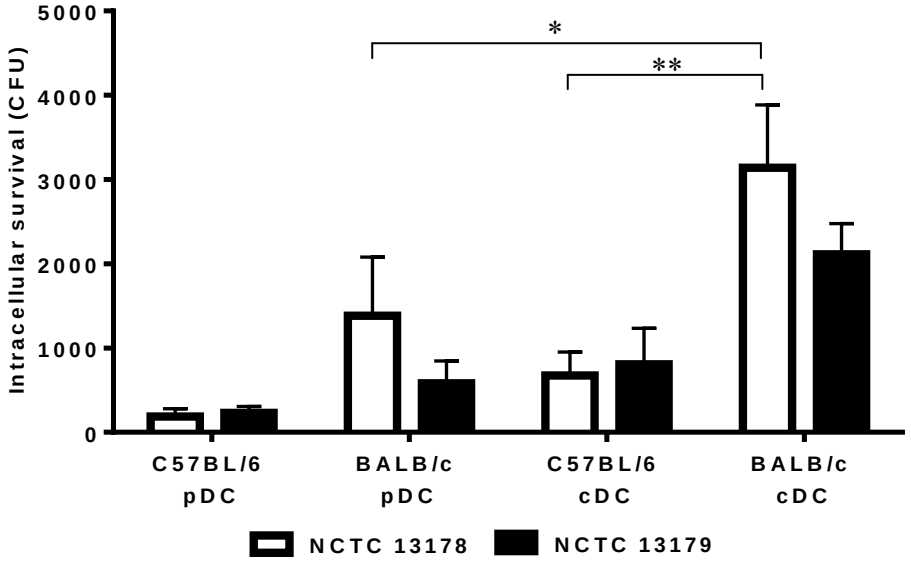

Figure 4.10 Intracellular survival of high and low virulence *B. pseudomallei* isolates within murine pDC and cDC
Murine pDC and cDC were infected with *B. pseudomallei* isolates NCTC 13178 (high virulence) and NCTC 13179 (low virulence). Kanamycin was added after 4 hr of co-culture to kill extracellular bacteria. The number of viable intracellular *B. pseudomallei* at 24 hr was enumerated and expressed as CFU of *B. pseudomallei.* Although murine pDC were able to kill intracellular *B. pseudomallei*, BALB/c pDC were less efficient than C57BL/6 pDC. Bars depict the mean ± SEM of three experiments. * = P≤ 0.05, ** = P≤ 0.01 determined using a 2way ANOVA with multiple comparison tests.

4.3.3 Type I IFN response by pDC infected with *B. pseudomallei*

4.3.3.1 Quantification of IFN-α and IFN-β produced by human pDC exposed to *B. pseudomallei*

The production of type I IFN is considered the main effector function of pDC. Therefore, the amount of type I IFN, IFN-α & IFN-β, produced by human pDC in response to *B. pseudomallei* infection was determined. Human pDC were infected with *B. pseudomallei* for 24 hr, then the concentration of IFN-α and

IFN-β in pDC culture supernatants was determined. To demonstrate that the enriched human pDC were capable of producing large quantities of type I IFN, CpG ODN 2216 was used to positively stimulate human pDC and resulted in high concentrations of IFN-α and IFN-β in culture supernatants (Figure 4.11). However, *B. pseudomallei*-infected pDC demonstrated low IFN-α and IFN-β production, 183 ± 34 pg/ml (Figure 4.11A) and 141 ± 25 pg/ml, respectively (Figure 4.11B). No significant difference was observed in the type I IFN response of *B. pseudomallei*-infected pDC compared to uninfected pDC.

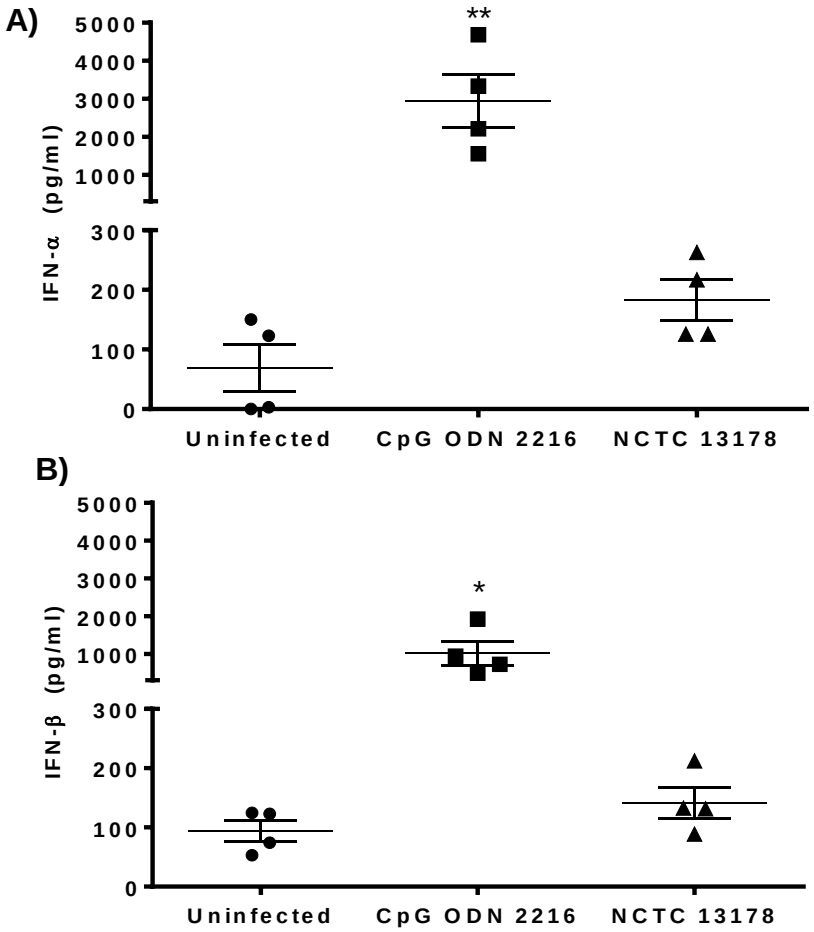

Figure 4.11 Production of type I IFN by human pDC in response to *B. pseudomallei*

Human pDC were infected *in vitro* with *B. pseudomallei* (NCTC 13178, high virulence) or stimulated with CpG ODN 2216. After 24 hr of co-culture, pDC culture supernatants were harvested and the quantity of A) IFN-α and B) IFN-β was evaluated by ELISA. Although human pDC were capable of high type I IFN production in response to CpG, low type I IFN production was observed in response to *B. pseudomallei*. Each data point represents the mean of triplicate wells of human pDC isolated from one healthy donor (assessed in duplicate). The mean of the four donors (± SEM) of duplicate experiments is also shown. * = P≤ 0.05, ** = P≤ 0.01 determined using a 1way ANOVA with multiple comparison tests.

4.3.3.2 Quantification of IFN-α and IFN-β produced by murine pDC infected with *B. pseudomallei*

The type I IFN response of murine pDC infected with *B. pseudomallei* was also determined. Murine pDC were infected with *B. pseudomallei* for 24 hr then the concentration of IFN-α and IFN-β in pDC culture supernatants was quantified. As uninfected BALB/c pDC demonstrated a reduced capacity to produce IFN-α compared to uninfected C57BL/6 pDC (Table 4.2), the increase in IFN-α and IFN-β above the baseline (uninfected pDC) was used for comparisons. Similar to the findings from the human pDC *in vitro* studies, high concentrations of IFN-α and IFN-β was observed in culture supernatants of C57BL/6 and BALB/c pDC stimulated with CpG ODN 2216 (Figure 4.12A and 4.12B respectively). The increase in IFN-α by *B. pseudomallei*-infected BALB/c pDC was significantly higher than that observed for *B. pseudomallei*-infected C57BL/6 pDC (Figure 4.12A). No differences in IFN-β production were observed for C57BL/6 and BALB/c pDC infected with NCTC 13178 (high virulence). However, BALB/c pDC infected with NCTC 13179 demonstrated significantly increased IFN-β production compared to C57BL/6 pDC (Figure 4.12B). The increase in IFN-α produced by pDC in response to *B. pseudomallei* isolates of high (NCTC 13178) and low (NCTC 13179) virulence was comparable. In contrast, *B. pseudomallei* virulence may differentially influence IFN-β production, although this trend was only observed for C57BL/6 pDC infected with NCTC 13178 compared to NCTC 13179. Overall, *B. pseudomallei*-infected BALB/c pDC demonstrated increased type I IFN production compared to *B. pseudomallei* C57BL/6 pDC.

Table 4.2 Concentrations of IFN-α and IFN-β in the culture supernatants of uninfected pDC from C57BL/6 and BALB/c mice

Mouse strain	IFN-α (mean ± SEM pg/ml)	IFN-β (mean ± SEM pg/ml)
Uninfected C57BL/6 pDC	114.86 ± 33.17	3.76 ± 1.61
Uninfected BALB/c pDC	15.11 ± 8.88	2.02 ± 1.02
P value (Mann Whitney test)	0.01	0.3

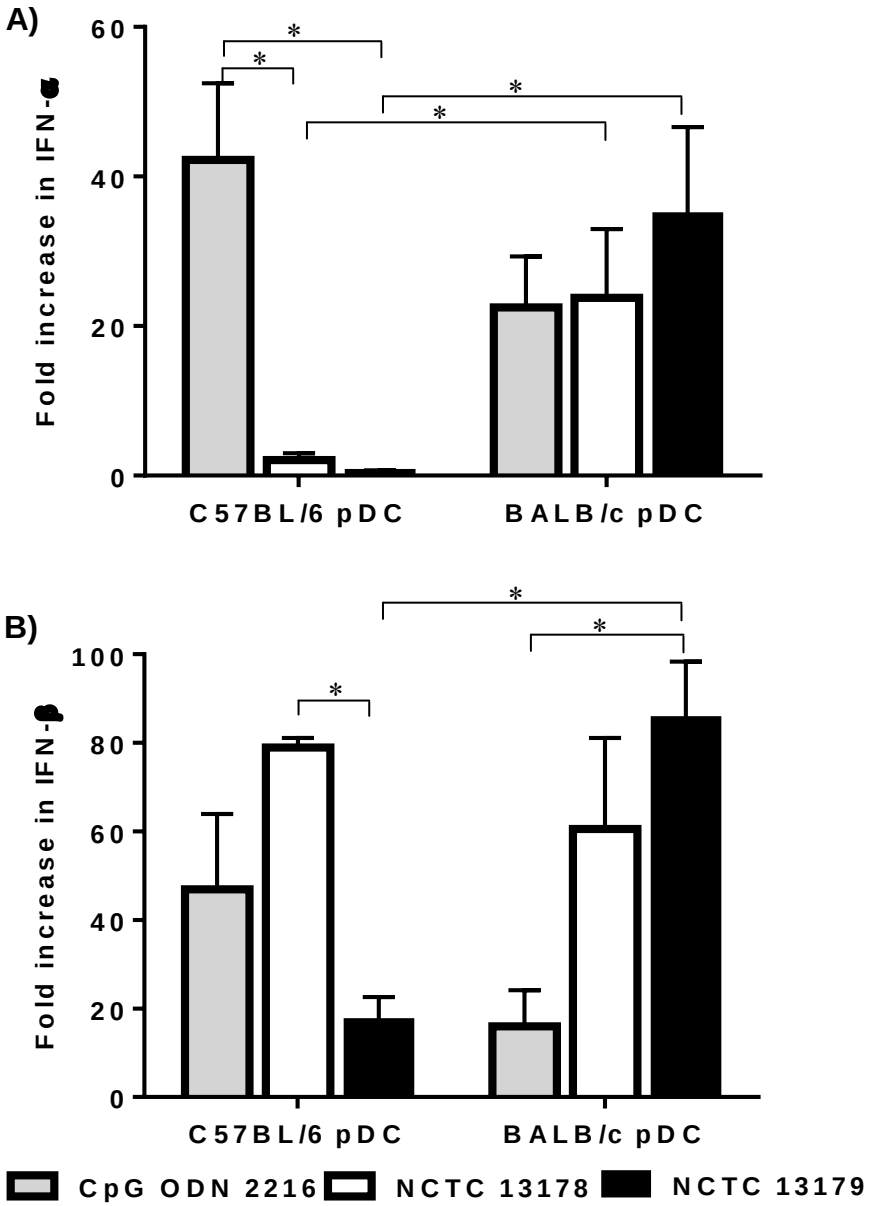

Figure 4.12 Type I IFN produced by murine pDC in response to *B. pseudomallei*

Murine pDC were infected *in vitro* with *B. pseudomallei* isolates NCTC 13178 (high virulence) and NCTC 13179 (low virulence) or stimulated with CpG ODN 2216. After 24 hr of co-culture, pDC culture supernatants were harvested and the quantity of A) IFN-α and B) IFN-β was evaluated by ELISA. The production of IFN-α and IFN-β was expressed as the fold increase of each cytokine in culture supernatants of stimulated murine pDC compared to uninfected murine pDC. A) BALB/c pDC demonstrated significantly increased IFN-α production compared to C57BL/6 pDC. B) The production of IFN-β by C57BL/6 pDC in response to NCTC 13179 (low virulence) was significantly lower compared to C57BL/6 pDC infected with NCTC 13178 (high virulence) and compared to BALB/c pDC infected with NCTC 13179 (low virulence). Bars depict the mean ± SEM of three independent experiments. * = P≤ 0.05 determined using a 2way ANOVA with multiple comparison tests.

4.3.4 Assessment of murine pDC maturation in response to *B. pseudomallei*

Current literature suggests that unlike cDC, pDC are immunomodulators rather than professional antigen presenting cells. There is evidence that pDC undergo maturation with increased MHC class II and CD86 expression. However, their efficiency as antigen presenting cells is still unconfirmed (Villadangos and Young, 2008). Since earlier investigations demonstrated that murine pDC were as efficient as murine cDC at internalisation and killing of *B. pseudomallei,* maturation of murine pDC was subsequently assessed. The expression of MHC class II and CD86 on uninfected and *B. pseudomallei*-infected pDC was compared after 24 hr by flow cytometry (Figure 4.13, 4.14 and 4.15). MHC class II expression decreased on C57BL/6 pDC and BALB/c pDC infected with *B. pseudomallei* compared to uninfected pDC (Figure 4.15A). In contrast, expression of CD86 increased on C57BL/6 pDC and BALB/c pDC infected with *B. pseudomallei* compared to uninfected pDC (Figure 4.15B). However, the changes observed were of a low magnitude and not significant due to high baseline MHC class II and CD86 expression on uninfected murine pDC; 77.2 ± 4.5 % and 71.7 ± 5.6 % respectively for C57BL/6 pDC, 70.1 ± 7.5 % and 72.1 ± 5.3 % respectively for BALB/c pDC (data not shown).

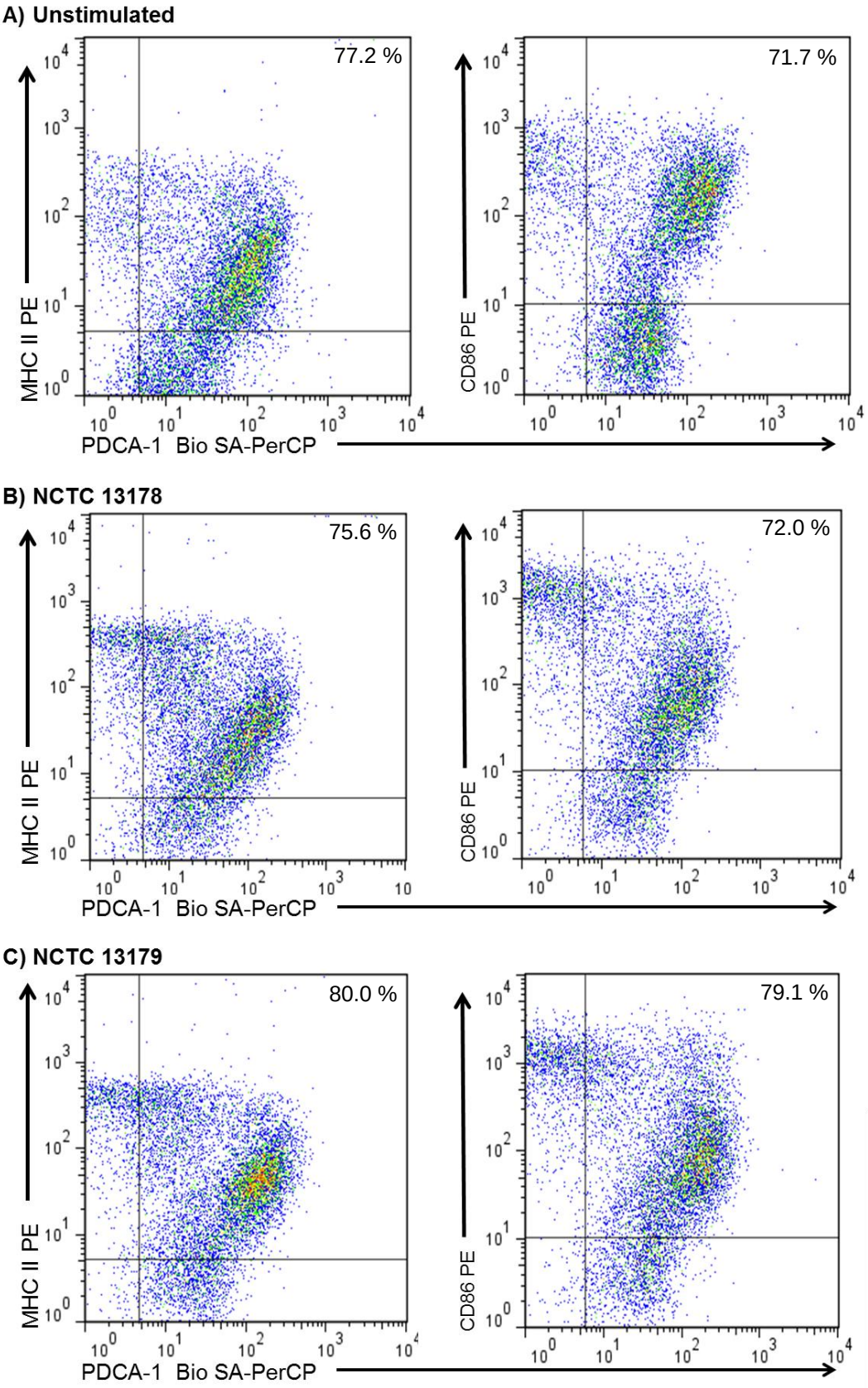

Figure 4.13 FACS analysis of MHC class II and CD86 on C57BL/6 pDC
Murine pDC generated from C57BL/6 mice were infected *in vitro* with *B. pseudomallei* isolates NCTC 13178 (high virulence) and NCTC 13179 (low virulence). After 24 hr of co-culture, the level of MHC class II and CD86 expression on murine pDC was determined. FACS plots shown are an example of MHC class II and CD86 expression on A) uninfected, B) NCTC 13178 (high virulence) and C) NCTC 13179 (low virulence) infected C57BL/6 pDC.

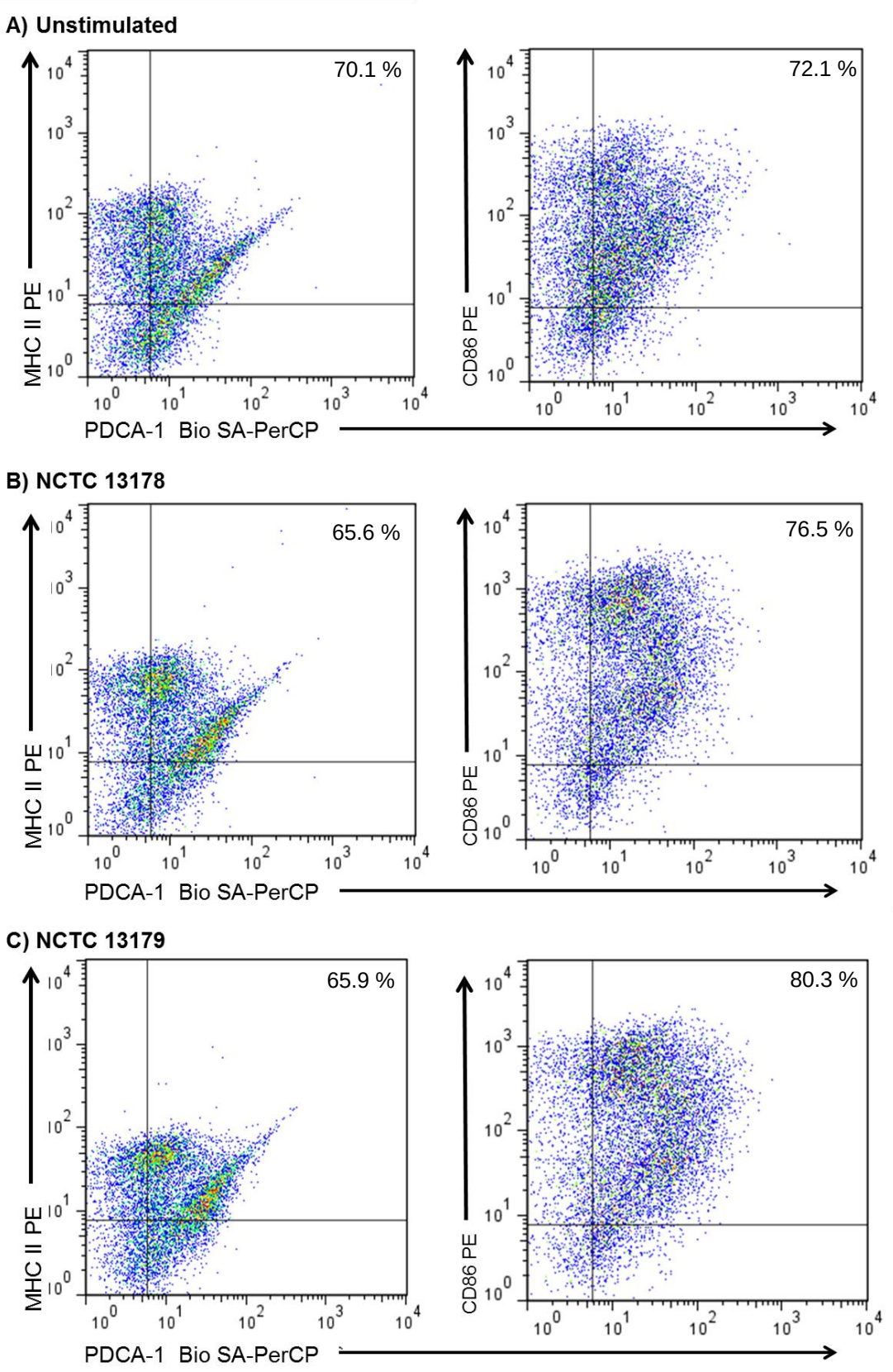

Figure 4.14 FACS analysis of MHC class II and CD86 on BALB/c pDC
Murine pDC generated from BALB/c mice were infected *in vitro* with *B. pseudomallei* isolates NCTC 13178 (high virulence) and NCTC 13179 (low virulence). After 24 hr of co-culture, the level of MHC class II and CD86 expression on murine pDC was determined. FACS plots shown are an example of MHC class II and CD86 expression on A) uninfected, B) NCTC 13178 (high virulence) and C) NCTC 13179 (low virulence) infected BALB/c pDC.

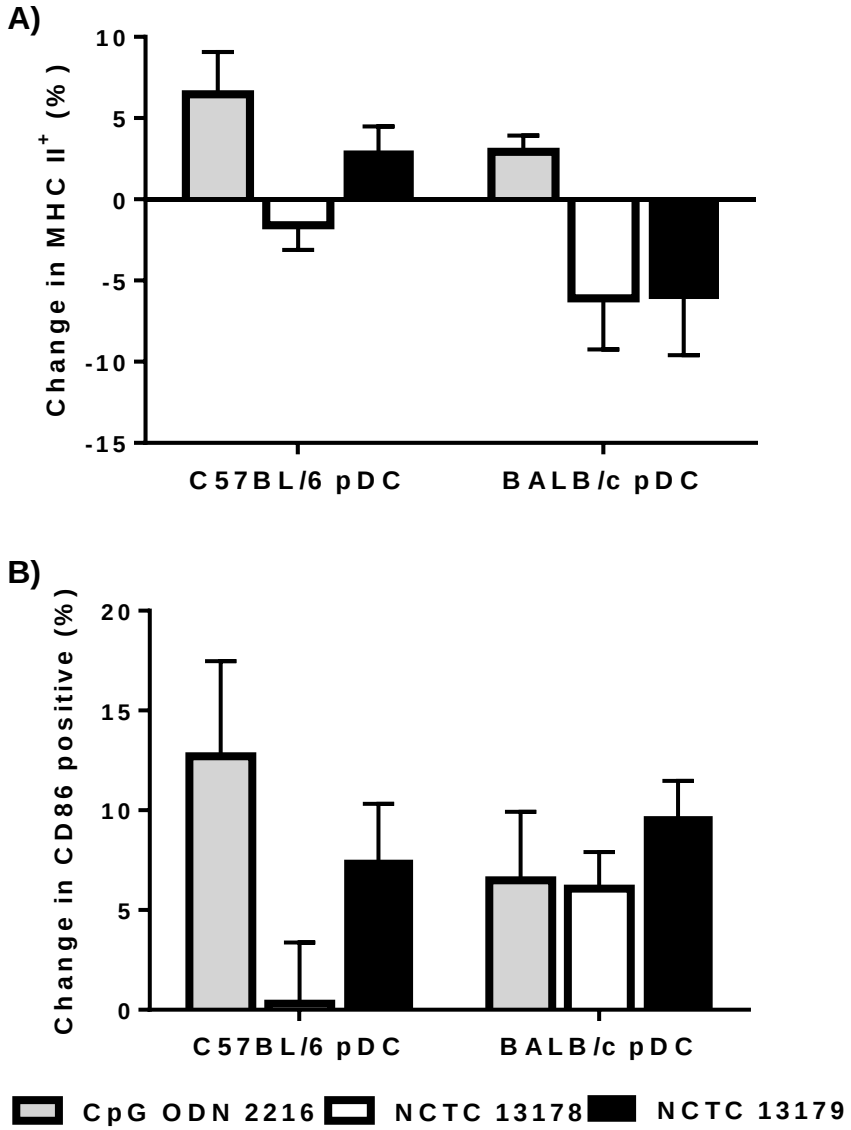

Figure 4.15 Expression of MHC class II and CD86 on murine pDC infected with *B. pseudomallei*

Murine pDC generated from C57BL/6 and BALB/c mice were infected *in vitro* with *B. pseudomallei* isolates NCTC 13178 (high virulence) and NCTC 13179 (low virulence) or stimulated with and CpG ODN 2216. After 24 hr of culture, the level of MHC class II (A) and CD86 (B) expression on murine pDC was determined and expressed as the change in expression compared to uninfected cells. A) Overall, a decrease in MHC class II expression was observed on *B. pseudomallei*-infected murine pDC from C57BL/6 and BALB/c mice. B) In response to *B. pseudomallei,* an increase in CD86 expression was observed on murine pDC from C57BL/6 and BALB/c mice. Bars depict the mean ± SEM of three experiments. No significant differences were observed when data was compared using a 2way ANOVA with multiple comparison tests.

4.4 Discussion

Plasmacytoid DC were first described as potent IFN-producing cells that were effector cells against viruses. More recently, investigations of the functional responses of pDC during bacterial infections indicate that pDC are also capable of responding to bacteria and that their antibacterial mechanisms have been underappreciated (Villadangos and Young, 2008). Defining the role of pDC in response to bacterial infections is complicated due to limited literature and conflicting findings demonstrating both beneficial and detrimental roles. Although pDC develop a mature phenotype similar to cDC following stimulation, pDC are often considered immunomodulating cells that are poor antigen presenting cells in comparison to cDC due to conflicting evidence of pDC capacity to efficiently internalise and present exogenous antigen (Villadangos and Young, 2008). Therefore, the current study investigated the functional responses of pDC toward *B. pseudomallei* by assessing the ability of pDC to internalise and kill *B. pseudomallei*, produce type I IFN and to undergo phenotypic maturation in response to *B. pseudomallei* infection. The current study is the first to demonstrate internalisation and intracellular killing of *B. pseudomallei* by both human and murine pDC. Murine pDC derived from *B. pseudomallei*-susceptible hosts demonstrated reduced bactericidal activity and significantly increased IFN-α responses to *B. pseudomallei* compared to murine pDC from *B. pseudomallei*-resistant hosts. Importantly, murine pDC were as efficient as murine cDC at internalising and killing intracellular *B. pseudomallei,* providing the first evidence for the potential role of pDC in processing *B. pseudomallei* antigens for presentation during infection.

Conventional DC are considered professional antigen presenting cells due to their ability to internalise pathogens, process antigens and develop a mature phenotype for efficient antigen presentation to naïve T cells. Despite accumulating evidence that pDC possess attributes and processes of professional antigen presenting cells, the role of pDC as professional antigen presenting cells is unclear. Observation of pDC phagocytosis of fluorescent micro-particles confirmed that pDC are capable of internalising exogenous antigen (Tel *et al.*, 2010). Furthermore, pDC are efficient at internalising particulate

antigen and uptake of bacteria, such as *S. aureus* (Tel *et al.*, 2010; Michea *et al.*, 2013). The ability of pDC to internalise bacteria appears to vary for different bacterial species. Michea *et al*. (2013) reported 8% *S. aureus* internalisation by human pDC (MOI 10:1, 1 hr), while Jounai *et al*. (2012) found murine pDC internalised spherical lactic acid bacteria (*Lactococcous lactis)* but not bacilli-shaped *Lactobacillus rhamnousus.* In the current study, both human and murine pDC were capable of internalising *B. pseudomallei.* Importantly, the *B. pseudomallei* internalisation efficiency of murine pDC was comparable to murine cDC.

Degradation and processing of internalised bacteria for presentation of bacterial antigen on the cell surface is another requirement of professional antigen presenting cells. Although there is limited evidence of antigen processing by pDC, a study by Tel *et al*. (2010) used micro-particles containing the self-quenched model protein DQ-BSA (BSA protein labelled with fluorescent BODIPY dye) to determine the ability of pDC to process internalised antigen. In this particular assay, degradation of the micro-particles released the DQ-BSA into the cell where subsequent processing by cellular proteases restores the fluorescence of the dye. The increased fluorescence of pDC over time indicated that pDC are capable of processing particulate antigen (Tel *et al.*, 2010). In the current study, the bactericidal activity of pDC against intracellular *B. pseudomallei* was investigated. Murine pDC were as efficient and possibly superior at killing intracellular *B. pseudomallei* compared to murine cDC. No significant differences in intracellular survival were observed between *B. pseudomallei* isolates of high and low virulence. There was also a trend for higher intracellular *B. pseudomallei* survival in pDC derived from the *B. pseudomallei*-susceptible BALB/c mice compared to *B. pseudomallei*-resistant C57BL/6 pDC. Importantly, human and murine pDC were capable of internalising and killing *B. pseudomallei*. These findings demonstrate the ability of pDC to kill *B. pseudomallei*, potentially producing antigen for loading onto MHC class II or I molecules for antigen presentation, similar to cDC, during *B. pseudomallei* infection.

Plasmacytoid DC are described as professional IFN producing cells due to the speed and magnitude of type I IFN produced in response to stimulation (Liu, 2005). However, the type I IFN response of pDC varies for different infections and can be detrimental to the host by driving excessive cytokine signalling (Decker *et al.*, 2005). Excessive production of pro-inflammatory cytokines, IFN-γ, IL-12, IL-15 and IL-18, has been demonstrated in the plasma of patients with sepsis caused by melioidosis (Lauw *et al.*, 1999; Lauw *et al.*, 2000). Assessment of gene expression profiles of whole blood from patients with melioidosis sepsis demonstrated that monocytes from patients have increased mRNA expression of IL-1β, IL-1RA, MIP-1α, NF-κB1, NF-κB1A, and TNF receptor 1, which also corresponded with increased mortality (Wiersinga *et al.*, 2007a). In another study, gene expression profiling of whole blood from patients with acute melioidosis, demonstrated increased type I IFN expression profiles, similar to type II IFN (Koh *et al.*, 2013a). It is possible that pDC and type I IFN signalling are involved in the signalling cascades that lead to excessive pro-inflammatory cytokine release in patients with melioidosis sepsis. In the current study, human and murine pDC were functionally capable of producing type I IFN, demonstrated by strong IFN-α and IFN-β production in response to CpG ODN 2216. This particular oligodeoxynucleotide sequence, containing unmethylated CpG motifs, was previously proven to induce high type I IFN production in pDC (Krug *et al.*, 2001). However, the production of IFN-α/β by human and murine pDC infected with *B. pseudomallei* was low in comparison to cytokine levels observed in CpG ODN 2216 stimulated cultures. Interestingly, *B. pseudomallei* isolates of high and low virulence induced comparable type I IFN responses by pDC. Contrasting type I IFN responses by pDC toward bacteria have been reported. Bacteria such as *S. aureus, Haemophilus influenzae* and *Neisseria meningitidis,* are reported to stimulate high type I IFN responses by pDC (Michea *et al.*, 2013). Other bacteria such as *Streptococcus pyogenes* stimulate low type I IFN responses in human pDC, similar to that observed for *B. pseudomallei*-infected pDC. CpG DNA, like bacterial and viral DNA, is recognised by the pattern recognition receptor (PRR), TLR-9. The expression of TLR-7 and TLR-9 on pDC for recognising RNA and DNA viruses respectively, is well described (Bao and Liu, 2013). Stimulation of pDC via TLR-7 or TLR-9 induces strong type I IFN production (Decker *et al.*, 2005).

However, these are not the only PRR expressed by pDC. Furthermore, triggering of other PRR such as Siglec-H, BDCA2 or DCIR, causes inhibition of type I IFN production despite evidence that DCIR-activated pDC still undergo maturation, present antigen and activate adaptive immune responses (Meyer-Wentrup *et al.*, 2008; Bao and Liu, 2013). Furthermore, pDC have been shown to be effective at combating bacterial infections such as *L. pneumophila* in mice without producing type I IFN (Ang *et al.*, 2010). In light of these reports and the findings of the current study, it is possible that pDC may contribute to the early control of *B. pseudomallei* by internalising and killing the bacteria despite the fact that *B. pseudomallei*-infected pDC do not produce large quantities of type I IFN like CpG ODN 2216-stimulated pDC however; further studies are required to investigate this.

BALB/c mice are known to be poor IFN-α/β producers compared to C57BL/6 mice. This innate difference in IFN-α/β production between BALB/c and C57BL/c mice occurs because BALB/c mice carry the *If-1*^{l} low expression allele for IFN-α/β, while C57BL/6 mice carry the *If-1*^{h} high expression allele for IFN-α/β (Shankar *et al.*, 1996). In the current study, uninfected BALB/c pDC produced less IFN-α than uninfected C57BL/6 pDC. However, *B. pseudomallei*-infected BALB/c pDC demonstrated a 44-fold increase in IFN-α production, significantly higher than the 5-fold increase observed for *B. pseudomallei*-infected C57BL/6 pDC. BALB/c mice are highly susceptible to *B. pseudomallei* infection and develop disease that parallels the acute form of human melioidosis (Leakey *et al.*, 1998). pDC from *B. pseudomallei*-susceptible mice demonstrated a significant change in IFN-α production compared to pDC from *B. pseudomallei*-resistant mice, which suggests that the increased IFN-α may be detrimental to the host during *B. pseudomallei* infection. Other studies have found that hyper-inflammatory responses and impaired bacterial killing by innate cells contributes to the increased bacterial burden and disease severity in *B. pseudomallei*-susceptible BALB/c hosts (Leakey *et al.*, 1998; Ulett *et al.*, 2000a; Liu *et al.*, 2002). It is possible that increased type I IFN production in BALB/c mice infected with *B. pseudomallei*, contributes to signalling pathways driving the hyper-production of pro-inflammatory cytokines and enhances the adverse effects of early innate immune responses, similar to

L. monocytogenes infection (Auerbuch *et al.*, 2004; Stockinger *et al.*, 2004). Given pDC from susceptible hosts significantly increased IFN-α production in response to *B. pseudomallei* in the current study and that expression of type I IFN genes in the whole blood of patients with acute melioidosis is increased (Koh *et al.*, 2013a), future studies are warranted to investigate the ability of pDC and type I IFN to modulate immune responses to *B. pseudomallei.* Important areas for further investigation include whether pDC are recruited to the site of *B. pseudomallei* infection in a murine model of melioidosis, the level of type I IFN they produce during infection, immune cell modulation and cytokine production mediated by pDC and type I IFN and how this differs between resistant and susceptible hosts.

The phenotypic maturation of pDC, like cDC, is important for activation of naive T cells (Mitchell *et al.*, 2011). Following activation, pDC lose their plasmacytoid morphology and develop a morphology similar to cDC, which includes increased cell surface expression of antigen presenting (MHC class II) and T cell co-stimulatory (CD86) molecules. Whilst pDC develop a mature phenotype, it is still debated whether pDC are also professional antigen presenting cells. There is also evidence that pDC maturation responses can be exploited to develop an inappropriate immune response that benefits persistence of the bacteria within the host. For example, *S. aureus* uses pDC activation to develop B cell-mediated immune tolerance (Parcina *et al.*, 2013). Activation of pDC by *S. aureus* appears to occur via TLR9, which subsequently leads to the maturation of pDC. Mature *S. aureus*-activated pDC were shown to support the proliferation of IL-10-producing B cells that suppress the immune response to *S. aureus* (Parcina et al., 2013). In the current study, the cell surface expression of MHC class II and CD86 was altered on murine pDC following exposure to *B. pseudomallei.* Different trends were observed for C57BL/6 pDC and BALB/c pDC following infection with *B. pseudomallei*. Notably, a decrease in MHC class II expression was observed on BALB/c pDC. However, the changes observed were masked by high baseline expression of MHC class II and CD86 on uninfected murine pDC. The expression levels of MHC class II and CD86 on unstimulated cDC is known to increase in culture over time and in addition from manipulation during harvesting (Grauer et al 2002). FLT-3L supplementation for 10

days to improve pDC yield and manipulation of cells to positively select the PDCA-1^+ pDC are likely to have contributed to the elevated baseline expression of MHC class II and CD86 (Grauer *et al.*, 2002). Therefore, the maturation response of *B. pseudomallei*-infected pDC is inconclusive. Additional studies using techniques that reduce the manipulation of pDC, such as enrichment of pDC using negative selection, are required to determine whether *B. pseudomallei* infection stimulates the maturation of pDC.

In summary, this is the first description of the functional responses of human and murine pDC toward *B. pseudomallei*. The ability of pDC to internalise and kill *B. pseudomallei* was demonstrated. No differences in *B. pseudomallei* killing or type I IFN production was observed between pDC exposed to *B. pseudomallei* isolates of high (NCTC 13178) or low (NCTC 13179) virulence. In resistant hosts (C57BL/6 mice), it appears that *B. pseudomallei* activation of pDC stimulates low type I IFN production. Differences in bactericidal activity and IFN-α production were observed for murine pDC generated from *B. pseudomallei*-susceptible BALB/c mice compared to *B. pseudomallei*-resistant C57BL/6 mice. Our findings implicate pDC as an additional cell type contributing to the altered immune responses that underlie the susceptibility of BALB/c mice toward *B. pseudomallei*. As *B. pseudomallei*-infected BALB/c mice exhibit excessive pro-inflammatory cytokine production, similar to that observed in patients with melioidosis sepsis, further investigation is warranted to determine if IFN−α production by pDC contributes to the signalling cascades that cause excessive production of pro-inflammatory cytokines in susceptible hosts. Further studies on the downstream responses of pDC and type I IFN signalling are required to elucidate their ability to modulate other innate immune cells and to activate *B. pseudomallei*-specific T cell responses during the early phases of *B. pseudomallei* infection.

CHAPTER 5
MATURATION AND MIGRATION CAPACITY OF BONE MARROW-DERIVED DENDRITIC CELLS IN RESPONSE TO *BURKHOLDERIA PSEUDOMALLEI IN VITRO*

5.1 Introduction

Dendritic cells (DC) provide innate immune responses and function as professional antigen presenting cells to activate T cell-mediated adaptive immune responses (Banchereau *et al.*, 2000). As part of the innate immune response, highly phagocytic, immature DC internalise and kill pathogens at the site of infection (Shortman and Naik, 2007). DC-pathogen interactions trigger DC maturation, a process whereby DC up-regulate expression of antigen presenting molecules (MHC class I and II), T cell co-stimulatory molecules (e.g. CD80, CD86) and receptors for migration to secondary lymphoid organs (e.g. CC-chemokine receptor 7 [CCR7]; Banchereau *et al.*, 2000; Shortman and Liu, 2002). Mature DC also demonstrate increased production of cytokines: interleukin (IL) 6, IL-12 and tumour necrosis factor alpha (TNF-α). Migration of mature, antigen-loaded DC from the site of infection to secondary lymphoid tissues is largely co-ordinated by CCR7 and its ligands, CCL19 and CCL21 (Comerford *et al.*, 2013). At the site of infection, CCL21 expression on high endothelial venules and lymphatic endothelial cells initiates the intravasation and migration of DC toward secondary lymphoid tissues (Saeki *et al.*, 1999; Ricart *et al.*, 2011). The process of inward migration to T cell rich zones, within the paracortex of lymph nodes or periarteriolar lymphoid sheaths (PALS) of the spleen, also occurs in a CCR7/CCL21/CCL19 dependent manner. DC follow CCL21 expression on the surface of fibroblastic reticular cells, while soluble CCL19 influences the direction of DC migration to interact with T cells (Ricart *et al.*, 2011; Comerford *et al.*, 2013). Upon reaching the T cell rich zones of secondary lymphoid tissues, mature DC expressing high levels of antigen presenting and T cell co-stimulatory molecules (MHC class II, CD80 and CD86) efficiently activate pathogen-specific T cell responses (Savina and Amigorena, 2007; Villadangos and Schnorrer, 2007).

To date, functional studies on DC-*B. pseudomallei* interactions have confirmed that DC internalise and kill intracellular *B. pseudomallei in vitro* (Charoensap *et al.*, 2008; Williams *et al.*, 2008; Horton *et al.*, 2012). *B. pseudomallei* infection also triggers DC maturation, demonstrated by increased expression of molecules for antigen presentation and T cell co-stimulation, along with increased cytokine production (Williams *et al.*, 2008; Horton *et al.*, 2012). Furthermore, host susceptibility to *B. pseudomallei* infection is associated with altered DC functional responses (Williams *et al.*, 2008). Despite significant maturation and IL-12 production in bone marrow (BM)-derived DC (BMDC) from *B. pseudomallei*-susceptible BALB/c, their bactericidal activity against intracellular *B. pseudomallei* was lower than observed for BMDC from *B. pseudomallei*-resistant C57BL/6 mice (Williams *et al.*, 2008). *In vitro* studies have also demonstrated that DC stimulated with heat-killed *B. pseudomallei* can present antigen to *B. pseudomallei*-specific memory T cells (Healey *et al.*, 2005; Elvin *et al.*, 2006; Charoensap *et al.*, 2008; Charoensap *et al.*, 2009; Tippayawat *et al.*, 2011). Furthermore, when used as a vaccine vector, DC-loaded with *B. pseudomallei* antigens initiated the activation of T cells and provided partial protection in the highly susceptible BALB/c murine model of melioidosis (Healey *et al.*, 2005; Elvin *et al.*, 2006).

Migration of DC from the site of infection to secondary lymphoid tissues is important for the development of pathogen-specific immune responses. Modulation or interference at any step in this process has the potential to affect the ability of the host to mount a protective immune response. Virulence of the intracellular pathogen *Yersinia pestis* for example, is in part, attributed to the bacterium's ability to impair DC migration preventing infected DC from presenting antigen and activating T cells in draining lymph nodes. DC infected with *Y. pestis* had impaired ability to rearrange their cytoskeleton, were unable to adhere to surfaces and failed to migrate toward CCL19 (Velan *et al.*, 2006). Currently, the effect of *B. pseudomallei* infection on DC migration capacity is unknown. Therefore, the work presented in this Chapter focused on elucidating the *in vitro* migration response of DC following exposure to *B. pseudomallei*. Subsequent studies on the role of DC migration as a vehicle for *B. pseudomallei* dissemination are discussed in Chapter 6.

The specific aims of the studies outlined in this Chapter were to:

i) Demonstrate the maturation of BMDC in response to *B. pseudomallei* exposure *in vitro*
ii) Assess the bactericidal capacity of BMDC against intracellular *B. pseudomallei in vitro*
iii) Determine if *B. pseudomallei* affects the *in vitro* migration capacity of BMDC
iv) Determine whether the migration of BMDC facilitates *in vitro* trafficking of *B. pseudomallei*

5.2 Methods and Materials

5.2.1 Generation of BMDC and co-culture with *B. pseudomallei*

Murine BMDC were cultured from the BM of C57BL/6 mice as described previously (Section 3.4.1). BMDC were harvested on day 10 and seeded into 6 well plates (Nunc, Thermo Fisher Scientific Pty Ltd, Scoresby, Australia) at 10^6 cells/ml in BMDC culture media (RPMI-1640 with 10 % HI-FBS, 1.5 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-Mercaptoethanol; Appendix 1). BMDC were infected with log phase *B. pseudomallei* (NCTC 13179, low virulence), prepared as described in Section 3.3.4, at a multiplicity of infection (MOI) 1:1 or stimulated with *Escherichia coli* lipopolysaccharide (LPS; 50 ng/ml; Sigma-Aldrich, Sydney, Australia; Appendix 1) and incubated in 5 % CO_2 at 37 ºC according to previously published co-culture conditions (Williams *et al.*, 2008; Williams *et al.*, 2011). *E. coli* LPS was used for comparison to show positive stimulation of DC maturation and migration (Martìn-Fontecha *et al.*, 2003). The concentration of *E. coli* LPS used to stimulate BMDC was determined from previously published literature (Martìn-Fontecha *et al.*, 2003; Williams *et al.*, 2008). To determine if *B. pseudomallei* isolates of high and low virulence differentially influenced the migration of BMDC *in vitro*, where specified, BMDC were also infected with NCTC 13178, a highly virulent clinical *B. pseudomallei* isolate at a MOI 1:1 (Barnes and Ketheesan, 2005). Antibiotic protection of BMDC was required to enable enumeration of intracellular bacteria only and to prevent uncontrolled

extracellular bacterial replication from affecting BMDC viability, as mentioned in Section 4.2.3 (Feterl *et al.*, 2006; Williams *et al.*, 2008). Therefore, kanamycin (250 μg/ml; Sigma-Aldrich, Sydney, Australia; Appendix 1) was added after 4 hr of stimulation to kill extracellular bacteria. At designated time points, BMDC were harvested to assess *in vitro* migration in response to stimulation.

5.2.2 Analysis of BMDC maturation and *B. pseudomallei* killing

5.2.2.1 Influence of *B. pseudomallei* on BMDC maturation

The effect of *B. pseudomallei* on BMDC maturation was investigated by measuring the expression of surface markers CD11c, CD86, MHC class II and CCR7 by flow cytometry. BMDC infected with *B. pseudomallei* isolate NCTC 13179 (low virulence) for 6, 12, 18 and 24 hr, as previously described (Section 5.2.1), were transferred to FACS tubes (10^6 BMDC/tube; BD Biosciences, North Ryde, Australia) and centrifuged (400 G, 5 min). The cells were washed with 2 ml sodium azide buffer (SAB; Appendix 1), resuspended in 20 μl of diluted antibody (Table 5.1) and incubated on ice for 30 min. Stained cells were washed twice with 2 ml SAB then fixed in 100 μl 4 % paraformaldehyde (ProSciTech, Kirwan, Australia) and incubated at 4 °C for 15 min. Fixed cells were then washed with 2 ml phosphate buffered saline (PBS; pH 7.2: Appendix 1) and resuspended in 300 μl PBS and analysed by Flow cytometry as previously described (Section 3.5). Data was expressed as the mean percentage of cells expressing CD11c, MHC class II, CD86 or CCR7 (% ± SEM).

Table 5.1 Antibodies (anti-mouse) used for flow cytometric analysis of BMDC maturation

Antibody	Clone	Conjugate	Isotype	μg/10^6 cells
CD11c	HL3	FITC	Armenian Hamster IgG_1	0.05
I-A/I-E (MHC class II)	M5/114.15.2	PE	Rat IgG_{2b}	0.04
CD86	GL1	PE	Rat IgG_{2a}	0.04
CCR7	4B12	PerCP-Cy5.5	Rat IgG_{2a}	0.04

Note: Anti-mouse CD11c, I-A/I-E and CD86 antibodies were purchased from BD Biosciences, North Ryde, Australia. Anti-mouse CCR7 antibodies were purchased from eBioscience, Jomar Bioscience Pty Ltd, Stepney, Australia. The optimal amount of antibody required for staining 10^6 BMDC in a final volume of 20 μl was determined by titration of the antibodies in preliminary experiments (data not shown).

5.2.2.2 Fluorescence microscopy evaluation of BMDC internalisation of *B. pseudomallei* and maturation

Fluorescence microscopy was also used to observed *B. pseudomallei* internalisation and evaluate the maturation of BMDC using fluorescently labelled antibodies against CD11c and MHC class II. In addition, intracellular *B. pseudomallei* were fluorescently labelled using a *B. pseudomallei* OmpA antibody, an anti-serum against the *B. pseudomallei* outer membrane protein A (OmpA) raised in rabbits, which was kindly provided by Professor Ifor Beacham, Institute for Glycomics, Griffith University Gold Coast Campus, Qld, Australia (Allwood *et al.*, 2008). After harvesting BMDC, the cells were seeded into 16 well chamber slides (Nunc Lab-Tek, Thermo Fisher Scientific Pty Ltd, Scoresby, Australia) at a concentration of 10^5 cells/well and infected with *B. pseudomallei* (NCTC 13179, MOI 1:1) for 24 hr (with kanamycin 250 μg/ml after 4 hr of culture. Uninfected and *B. pseudomallei*-infected BMDC were fixed in 100 μl 4 % paraformaldehyde for 15 min on ice then wash with cold PBS (pH 7.2) for 5 min. To quench endogenous peroxidase, cells were incubated with 3 % H_2O_2 (Sigma-Aldrich, Sydney, Australia) for 10 min at room temperature, then washed with PBS (pH 7.2) for 5 min. Endogenous avidin and biotin were blocked by incubating the cells in avidin and

biotin solutions (10 min at RT each; Vectastain®, Abacus ALS, East Brisbane, Australia) with PBS (pH 7.2) wash steps. The cells were then incubated in 50 µl 1 % blocking reagent (TSA Kit, Life Technologies Australia Pty Ltd, Mulgrave, Australia) with 0.1 % saponin (Sigma-Aldrich, Sydney, Australia) for 15 min then washed with TNT buffer (Appendix 1). Tyramide signal amplification was used to fluorescently stain intracellular *B. pseudomallei* using *B. pseudomallei* OmpA antibody (30 min at RT) followed by HRP anti-rabbit IgG (30 min at RT; Vector Laboratories, Abacus ALS, East Brisbane, Australia) then Tyramide-Alexa Fluor (AF) 594 (10 min at RT; TSA Kit, Life Technologies Australia Pty Ltd, Mulgrave, Australia) according to manufacturer's protocol. To confirm the tyramide signal amplification was specific for the *B. pseudomallei*, negative control samples were included where the *B. pseudomallei* OmpA antibody (primary antibody) was omitted prior to applying the HRP anti-rabbit IgG and Tyramide-AF594. Cells were then stained for either CD11c (Biotinylated anti-CD11c, 30 min at RT; BD Biosciences, North Ryde, Australia) or MHC class II (Biotinylated anti-MHC class II, 30 min at RT; BD Biosciences, North Ryde, Australia) followed by streptavidin-AF488 (30 min at RT; Life Technologies Australia Pty Ltd, Mulgrave, Australia). Isotype control staining for CD11c and MHC class II was performed on parallel samples by substituting anti-CD11c and anti-MHC class II for biotinylated Hamster IgG1 and Rat IgG2b, respectively (BD Biosciences, North Ryde, Australia). After performing the final washes, slides were dried then coverslips were set using mounting medium with DAPI (Vectashield®, Abacus ALS, East Brisbane, Australia). The cells were then imaged using an Axio Imager 2 Light microscope with Axiovision 4.8 software (Carl Zeiss Pty Ltd, North Ryde, Australia). Cell nuclei = blue (DAPI); *B. pseudomallei* = red (AF594); CD11c or MHC class II = green (AF488).

5.2.2.3 Assessment of *B. pseudomallei* killing by BMDC

The number of viable intracellular bacteria within BMDC infected with *B. pseudomallei* for 6, 12, 18 and 24 hr was assessed in parallel with flow cytometric analysis of BMDC phenotype. To determine the number of intracellular bacteria within 10^6 BMDC, cells were washed twice with 2 ml PBS then lysed with 0.1 % Triton-X in sterile deionised

water (Appendix 1) to release the intracellular *B. pseudomallei.* Serial 10-fold dilutions in PBS were performed and 10 µl samples of each dilution plated in triplicate on Ashdown Agar (Appendix 1). Agar plates were incubated for 48 hr at 37 °C then colony forming units (CFU) were enumerated. Data was expressed as the mean $\log_{10}$ CFU/10^6 BMDC (± SD; minimum limit of detection was 100 CFU/10^6 BMDC).

5.2.3 Assessment of BMDC migration *in vitro*

5.2.3.1 Transwell® *in vitro* migration assay

The *in vitro* migration of BMDC was assessed by determining their ability to migrate from the upper chamber of a 24 well Transwell® plate with a 5 µm pore polycarbonate membrane insert (Corning, Sigma-Aldrich, Sydney, Australia) toward a chemokine in the lower chamber. Prior to conducting the assay, the Transwell® plates were equilibrated by filling the upper (100 µl) and lower (600 µl) chambers with RPMI 1640 (Invitrogen, Life Technologies Australia Pty Ltd, Mulgrave, Australia) containing 1 % heat inactivated foetal bovine serum (HI-FBS; Invitrogen, Life Technologies Australia Pty Ltd, Mulgrave, Australia; Appendix 1). Plates were incubated for 1 hr at 37 °C in 5 % CO_2. Uninfected, *B. pseudomallei*-infected and *E. coli* LPS-stimulated BMDC (Section 5.2.1) were harvested, centrifuged (400 G, 5 min) then resuspended at 10^7 cells/ml in RPMI 1640 with 1 % HI-FBS. The media from the upper chamber was removed and 100 µl of BMDC (10^6 cells/well) was added. Chemokines, CCL19 (100 ng/ml; Peprotech, Abacus ALS, East Brisbane, Australia) and/or CCL21 (100 ng/ml; Peprotech, Abacus ALS, East Brisbane, Australia) were added to the lower chambers where appropriate and plates incubated at 37 °C in 5 % CO_2 for 2 hr (Figure 5.1). The concentration of chemokines and assay incubation time were determined from previously published studies (Bar-Haim *et al.*, 2008; Ricart *et al.*, 2011). Optimisation of the *in vitro* migration was performed to determine the effect of stimulation time on migration by comparing BMDC stimulated with *E. coli* LPS for 2, 4, 6, 12, 18 and 24 hr (Section 5.3.2.1) or exposed to *B. pseudomallei* for 6, 12, 18 and 24 hr (Section 5.3.2.2), prior to seeding the BMDC into the Transwell® plate. After the Transwell® plates were

seeded with BMDC, the culture plates were incubated for 2 hr then migrated BMDC in the lower chambers were removed and fixed with 4 % paraformaldehyde (ProSciTech, Kirwan, Australia) for 15 mins at 4 °C. The cells were centrifuged (400 G, 5min), washed with 2 ml PBS, resuspended in 300 µl PBS then enumerated by flow cytometry (unstained using forward and side scatter FACS plots) as previously described (Section 3.5). For the optimisation experiments determining the effect of stimulation time on migration, data was expressed as the mean cell migration (% ± SEM). For all other *in vitro* migration experiments, data was expressed as the mean cell migration toward a chemokine (% ± SEM) above the baseline (mean cell migration toward media alone).

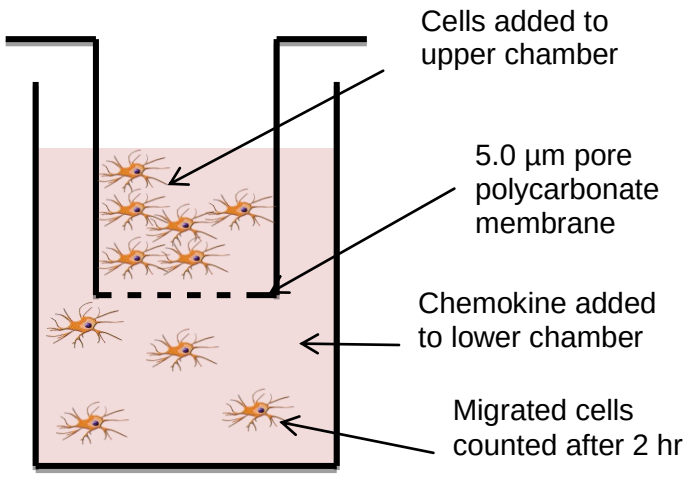

Figure 5.1 Schematic diagram of an individual chamber in a Transwell® migration plate

5.2.3.2 Enumeration of *B. pseudomallei* persisting within migratory BMDC

Parallel wells of the Transwell® *in vitro* migration assays were used to determine the bacterial burden of migrated BMDC. To enumerate the number of viable intracellular bacteria, migrated BMDC in the lower chambers were removed, washed twice with sterile PBS, and resuspended in 0.1 % Triton-X to disrupt cells and release intracellular *B. pseudomallei.* Serial 10-fold dilutions in PBS were performed and 10 µl samples of each dilution were plated in triplicate on Ashdown Agar. Agar plates were incubated for

48 hr at 37 °C then colonies were enumerated and expressed as the mean total CFU/lower chamber (± SD; minimum limit of detection was 70 CFU/lower chamber).

5.2.4 Statistical Analysis

Statistical analysis of data was performed using GraphPad Prism 6 Software and reported in Appendix 2. For characterisation of the *in vitro* interactions of *B. pseudomallei* with BMDC, the changes in MHC class II, CD86 and CCR7 expression were analysed for statistical significance using a 2way ANOVA, with recommended Tukey's posthoc multiple comparisons test to compare *in vitro* stimulation of BMDC time points (6, 12, 18 and 24 hr) and stimulant groups (uninfected, NCTC 13179 and *E. coli* LPS). The decrease in intracellular survival of *B. pseudomallei* within BMDC over time (6, 12, 18 and 24 hr) was analysed using a 1way ANOVA, with recommended Tukey's posthoc multiple comparisons test. For *in vitro* migration of BMDC, statistical significance was determined using 2way ANOVA, with recommended Tukey's or Sidak's posthoc multiple comparisons test to enable comparison between *in vitro* stimulation of BMDC at different time points, stimulation groups (uninfected, NCTC 13178, NCTC 13179 and *E. coli* LPS) and chemokine (CCL19 and CCL21). Differences between tested groups were considered significant if the P value ≤ 0.05 and displayed on figures as * = P values ≤ 0.05 and ** = P values ≤ 0.01.

5.3 Results

5.3.1 Maturation of BMDC exposed to *B. pseudomallei*

The effect of *B. pseudomallei* infection on BMDC expression of CD11c, CD86, MHC class II and CCR7 at 6, 12, 18 and 24 hr following *B. pseudomallei* exposure was analysed by flow cytometry (Figure 5.2). BMDC generated from C57BL/6 mice demonstrated high CD11c expression that was maintained over 24 hr on uninfected and infected cells (Figure 5.2A). The levels of MHC class II and CD86 on BMDC are indicators for DC maturation and capacity for antigen presentation. In response to *B. pseudomallei* and *E. coli* LPS (positive control), expression of MHC class II and CD86 on BMDC was significantly increased compared to uninfected BMDC (Figure 5.2B and C). The time in culture also caused an increase in expression of MHC class II and CD86 on uninfected BMDC. The level of CCR7 expression on BMDC, an indicator for migration capacity, was also determined (Figure 5.2D). In response to *B. pseudomallei* and *E. coli* LPS, BMDC significantly increased CCR7 expression in comparison to uninfected BMDC.

Fluorescence microscopy was used, in parallel to flow cytometry, to visualise the expression of CD11c and MHC class II on uninfected and *B. pseudomallei*-infected BMDC. Fluorescence microscopy confirmed the high level of CD11c expression on BMDC (Figure 5.3). Uninfected BMDC were observed to have a distinctive immature DC morphology that is more spherical with many small dendrites extending from the cell. Following exposure to *B. pseudomallei*, an increase in MHC class II was also observed on *B. pseudomallei*-infected BMDC in comparison to uninfected BMDC (Figure 5.4A and B). In addition to increased expression of MHC class II, mature *B. pseudomallei*-infected BMDC exhibited characteristic extension of large membrane processes from the main cell body (Figure 5.4B). Individual *B. pseudomallei* were identified by fluorescently labelling the bacteria with *B. pseudomallei* OmpA antibody (red; Figure 5.4B, E and F). Intracellular *B. pseudomallei* were also observed within BMDC expressing high levels of MHC class II (Figure 5.4B).

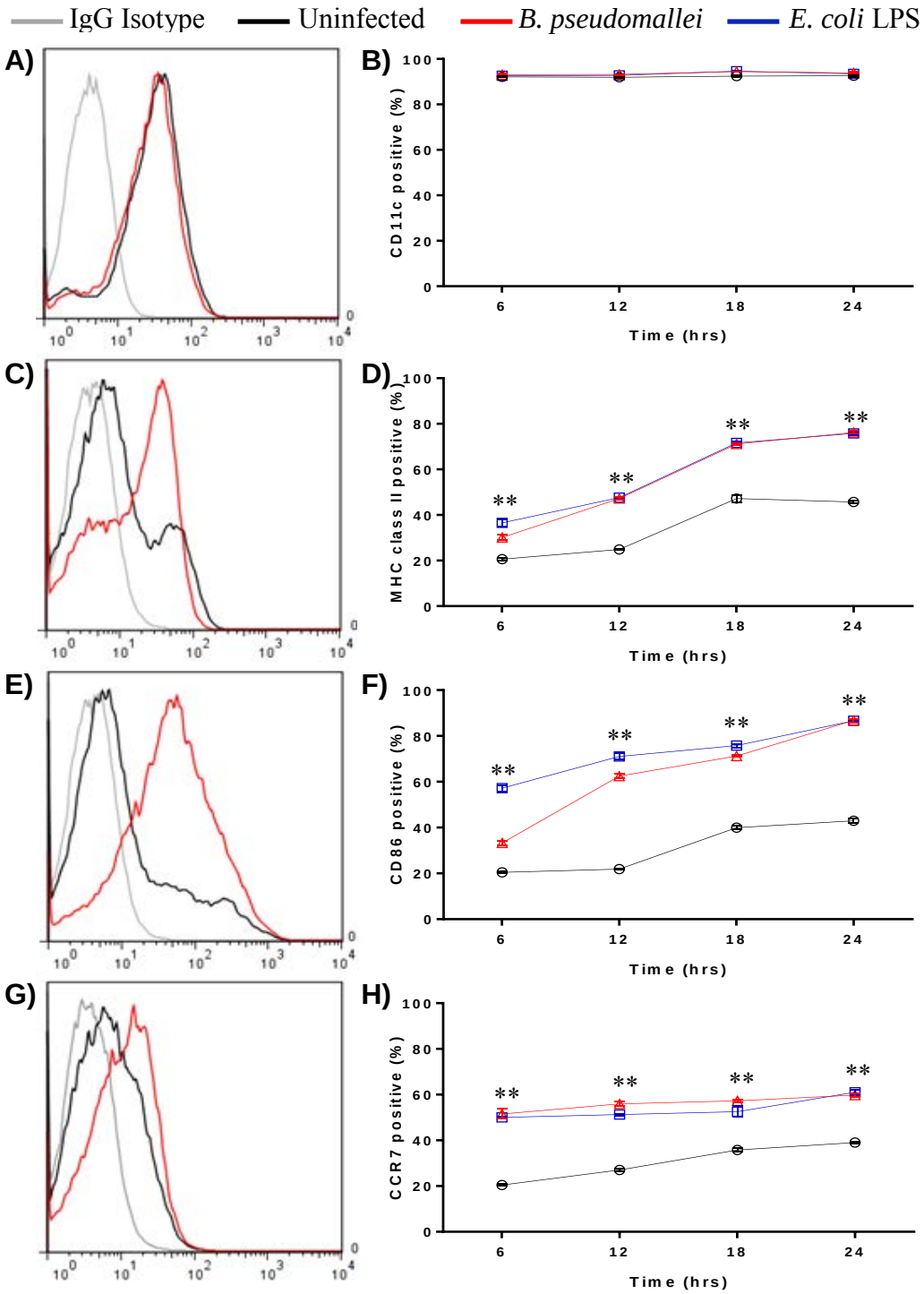

Figure 5.2 Phenotype of BMDC infected with *B. pseudomallei*

The expression of CD11c, MHC class II, CD11c and CCR7 on uninfected, *B. pseudomallei*-infected and *E. coli* LPS-stimulated BMDC at 6, 12, 18 and 24 hr was analysed by flow cytometry. Example histograms of the expression of A) CD11c, C) MHC class II, E) CD86 and G) CCR7 on uninfected and *B. pseudomallei*-infected BMDC after 12 hr. High expression of B) CD11c was observed on uninfected, *B. pseudomallei*-infected and *E. coli* LPS-stimulated BMDC over time. The percentage of cells expressing D) MHC class II, F) CD86 and H) CCR7 significantly increased in response to *B. pseudomallei* or *E. coli* LPS. Data points depict the mean ± SEM of two experiments. ** = P≤ 0.01 determined using a 2way ANOVA with multiple comparison tests.

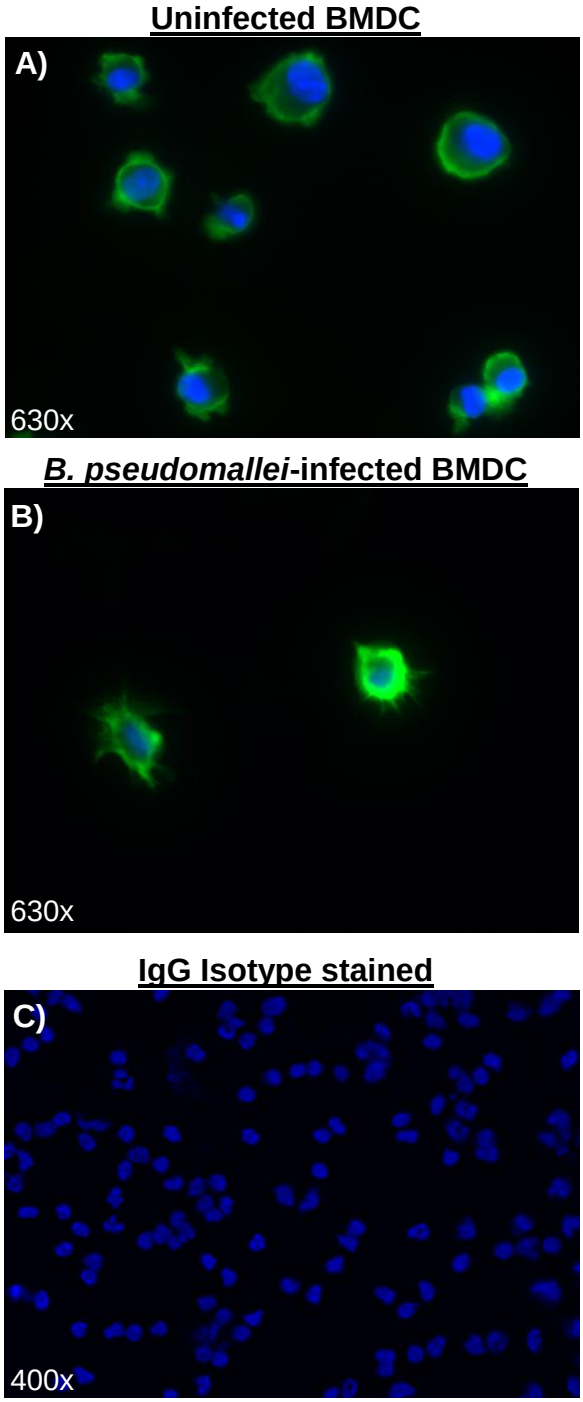

Figure 5.3 CD11c expression on BMDC infected with *B. pseudomallei*
Fluorescence microscopy was used to visualise and compare the expression of CD11c on BMDC. A) Example image of CD11c (green) expression on uninfected BMDC magnified 630x. B) Example image of CD11c expression on *B. pseudomallei*-infected BMDC magnified 630x. High levels of CD11c expression was observed on both uninfected and *B. pseudomallei*-infected BMDC. Note: fluorescent staining of *B. pseudomallei* was not performed. C) Example image of isotype staining on uninfected BMDC magnified 400x. CD11c = green; Cell nuclei = blue.

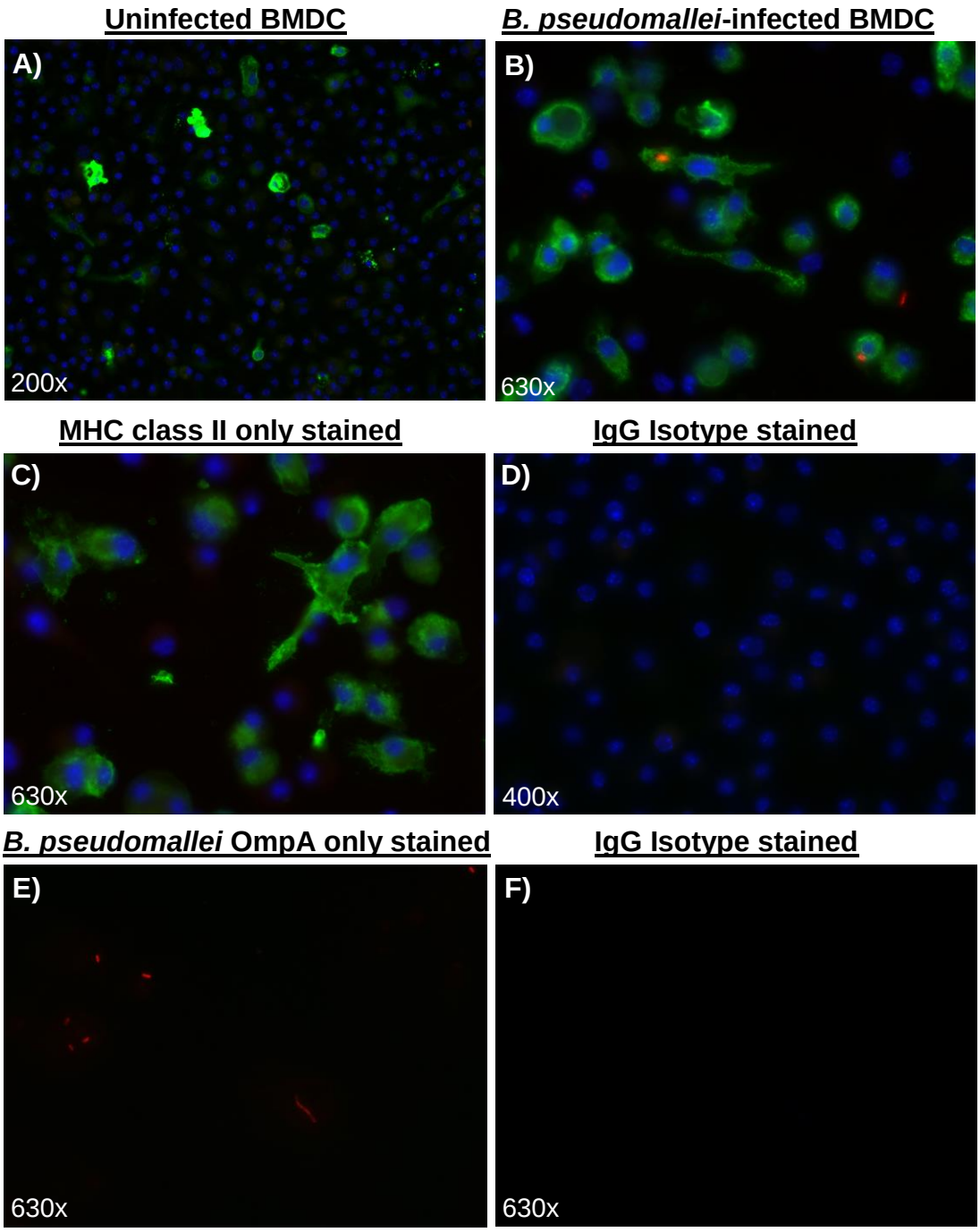

Figure 5.4 MHC class II expression on BMDC infected with *B. pseudomallei*
Fluorescence microscopy was used to visualise and compare the expression of MHC class II on uninfected *B. pseudomallei*-infected BMDC. Example image of A) uninfected and B) *B. pseudomallei*-infected BMDC expressing MHC class II (green); and intracellular *B. pseudomallei* (red); Cell nuclei = blue. Intracellular *B. pseudomallei* were observed within BMDC expressing high MHC class II. MHC class II control staining images are examples of C) single colour staining with MHC class II and D) isotype staining using *B. pseudomallei*-infected BMDC. *B. pseudomallei* OmpA control staining images are examples of E) single colour staining with anti-*B. pseudomallei* OMP and F) no primary antibody control using *B. pseudomallei* alone.

5.3.2 Ability of BMDC to kill intracellular *B. pseudomallei*

Intracellular killing of *B. pseudomallei* by BMDC was also assessed at 6, 12, 18 and 24 hr, in parallel to assessing BMDC maturation. BMDC were efficient at killing intracellular *B. pseudomallei*. This was demonstrated by the ability of BMDC to significantly decrease the number of viable intracellular *B. pseudomallei* over a 24 hr period (Figure 5.5).

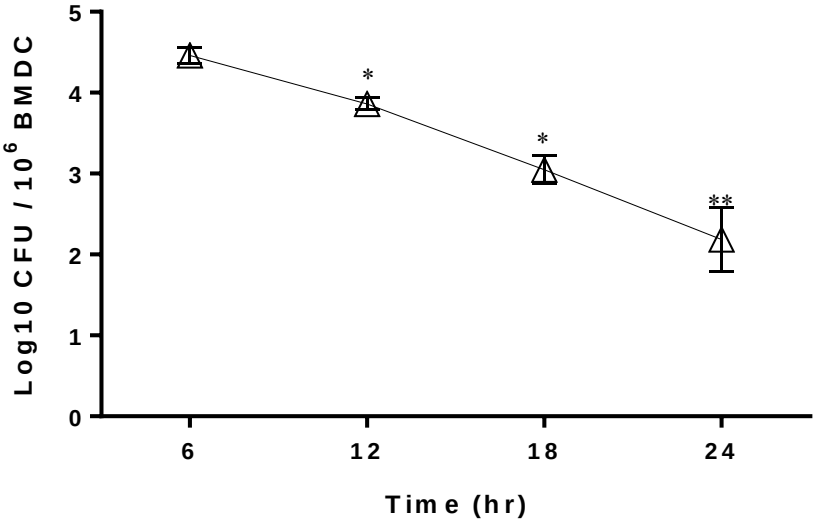

Figure 5.5 Intracellular killing of *B. pseudomallei* by BMDC
The intracellular killing of *B. pseudomallei* by BMDC was assessed at 6, 12, 18 and 24 hr, in parallel to assessing the phenotype of BMDC. At each time point, the number of intracellular bacteria was enumerated and expressed as $\log_{10}$ CFU. BMDC significantly reduced the number of intracellular *B. pseudomallei* over 24 hr. Bars depict the mean ± SD of one experiment. * = P≤ 0.05, ** = P≤ 0.01 determined using a 1way ANOVA with multiple comparison tests.

5.3.3 *In vitro* migration of BMDC

5.3.3.1 Optimisation of *in vitro* migration assay using *E. coli* LPS-stimulated BMDC

Prior to performing experiments to determine the effect of *B. pseudomallei* exposure on BMDC migration, the *in vitro* migration assay was optimised using *E. coli* LPS-stimulated BMDC. BMDC were stimulated with *E. coli* LPS (50 ng/ml) for 2, 4, 6, 12, 18 and 24 hr to determine the effect of the length of stimulation on the migratory

response of BMDC (Figure 5.6). Unstimulated and stimulated BMDC (10^6 cells) were seeded into a Transwell® migration assay plate and the number of cells that migrated *in vitro* from the upper chamber through a 5 μm pore membrane toward the chemokine CCL19 in the lower chamber was assessed after 2 hr. In comparison to media alone, significant migration toward CCL19 was observed for BMDC stimulated with *E. coli* LPS for 4 hr or longer. BMDC stimulated with *E. coli* LPS for 18 or 24 hr demonstrated significantly increased migration in comparison to unstimulated BMDC (Figure 5.6).

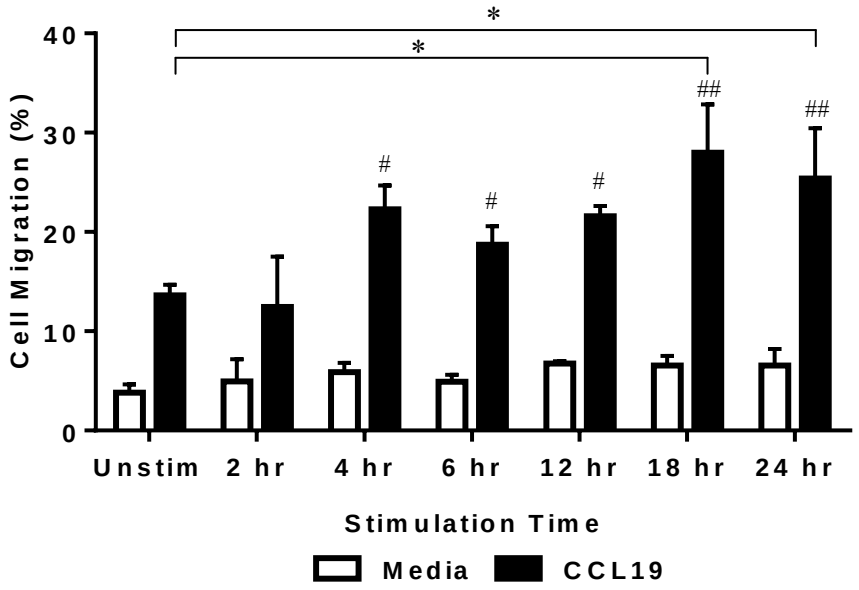

Figure 5.6 *In vitro* migration of BMDC following stimulation with *E. coli* LPS
The *in vitro* migration of BMDC stimulated with *E. coli* LPS (50 ng/ml) for 2, 4, 6, 12, 18 and 24 hr was compared. The number of BMDC that migrated toward CCL19 in the lower chamber was enumerated and expressed as a percentage of the total cells added to the upper chamber. BMDC stimulated with *E. coli* LPS for 18 or 24 hr demonstrated significantly increased migration in comparison to unstimulated BMDC. Bars depict the mean ± SEM of four experiments. # = P≤ 0.05 and ## = P≤ 0.01 denote significant comparisons between media and CCL19, * = P≤ 0.05 denote significant comparisons between unstimulated and stimulated (time), determined using a 2way ANOVA with multiple comparison tests.

5.3.3.2 The migration response of BMDC following exposure to *B. pseudomallei* for different time periods

Increasing the length of time of *in vitro* stimulation with *E. coli* LPS significantly increased the migratory response of BMDC. In addition, BMDC were also shown to

increase the expression of maturation markers when exposed to *B. pseudomallei* for increasing lengths of time (6, 12, 18 and 24 hr). Therefore, the *in vitro* migration of BMDC exposed to *B. pseudomallei* (NCTC 13179) for 6, 12, 18 and 24 hr was compared to uninfected BMDC (Figure 5.7). In comparison to media alone, significant migration toward CCL19 was observed for all uninfected and *B. pseudomallei*-infected BMDC. Similar to *E. coli* LPS-stimulated BMDC, BMDC exposed to *B. pseudomallei* for 18 or 24 hr demonstrated significantly increased migration compared to uninfected BMDC. Together with the BMDC maturation studies, these findings show that, *B. pseudomallei* stimulates BMDC maturation and does not impair the migration capacity of BMDC.

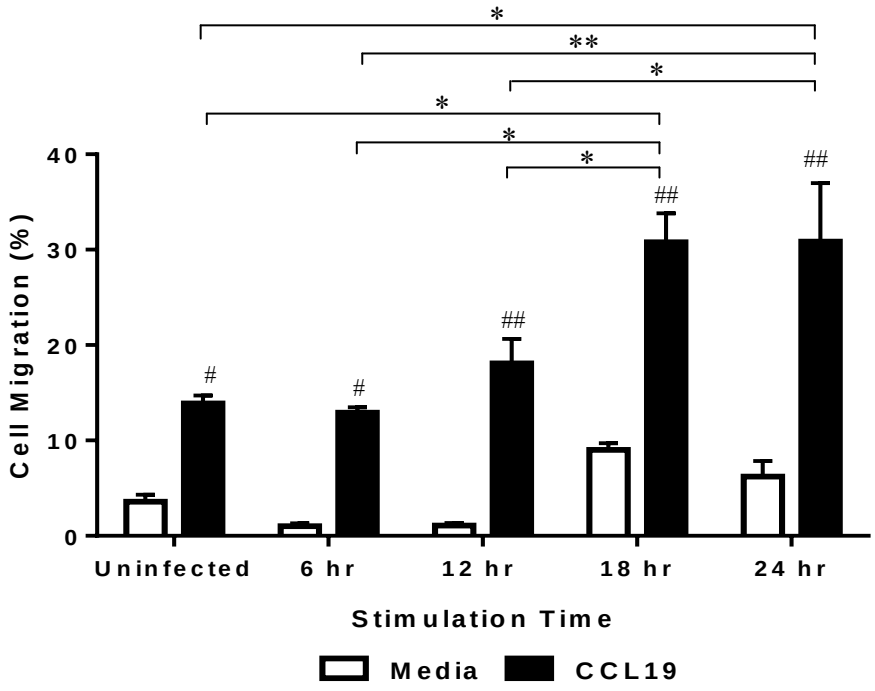

Figure 5.7 *In vitro* migration of BMDC following exposure to *B. pseudomallei*
The *in vitro* migration of BMDC following exposure to *B. pseudomallei* (NCTC 13179, low virulence) for 6, 12, 18 and 24 hr was compared. The number of BMDC that migrated toward CCL19 in the lower chamber was enumerated and expressed as a percentage of the total cells added to the upper chamber. BMDC exposed to *B. pseudomallei* for 18 or 24 hr demonstrated significantly increased migration compared to uninfected BMDC. Bars depict the mean ± SEM of two experiments. # = P≤ 0.05 and ## = P≤ 0.01 denote significant comparisons between media and CCL19, * = P≤ 0.05 and ** = P≤ 0.01 denote significant comparisons as indicated by brackets, determined using a 2way ANOVA with multiple comparison tests.

5.3.3.3 Effect of kanamycin treatment on BMDC migration *in vitro*

To protect BMDC co-cultured with *B. pseudomallei* from the effects of excessive extracellular bacterial growth, kanamycin (250 μg/ml) was added to uninfected and infected BMDC after 4 hr of culture to kill extracellular *B. pseudomallei*. To confirm that the *in vitro* migration of BMDC was not influenced by kanamycin treatment, uninfected and *B. pseudomallei*-infected BMDC were cultured in the presence or absence of kanamycin in culture media (Figure 5.8). Due to the rapid extracellular growth of *B. pseudomallei* in BMDC cultures without kanamycin treatment, BMDC were harvested after a short exposure to *B. pseudomallei* (12 hr) then seeded into the Transwell® migration assays to compare the *in vitro* migration of cells with and without kanamycin treatment. For this study and all future *in vitro* migration studies, the migration of BMDC toward media alone was used as a baseline to determine the percentage of migrated cells. Overall, the addition of kanamycin (250 μg/ml) to BMDC cultures did not have a significant effect on the *in vitro* migration of uninfected or *B. pseudomallei*-infected BMDC. Therefore, kanamycin (250 μg/ml) was added to uninfected and infected BMDC at 4 hr of culture for all future experiments (Figure 5.8).

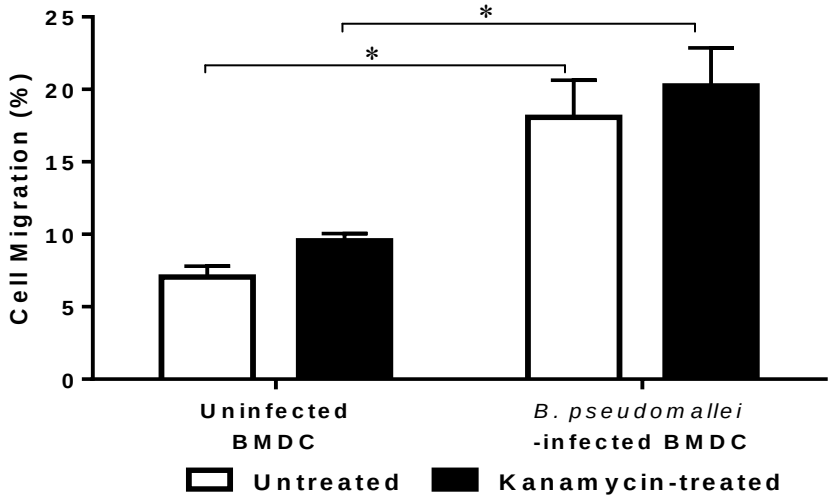

Figure 5.8 Effect of kanamycin treatment on BMDC migration
The *in vitro* migration of BMDC following exposure to *B. pseudomallei* (NCTC 13179, low virulence) with or without kanamycin treatment was compared. The number of BMDC that migrated toward chemokines in the lower chamber was enumerated and expressed as a percentage of the total cells added to the upper chamber. Kanamycin at a concentration of 250 μg/ml in culture media did not have an effect on BMDC migration.

Bars depict the mean ± SEM of three experiments. * = P≤ 0.05, ** = P≤ 0.01 determined using a 2way ANOVA with multiple comparison tests.

5.3.3.4 Migration of BMDC toward the CCR7 ligands, CCL19 and CCL21

Significant *in vitro* migration of BMDC toward the CCR7 ligand CCL19 was observed in response to *B. pseudomallei* infection. However, two CC-chemokine ligands CCL19 and CCL21, are reported to co-ordinate the migration of DC to the lymph nodes via the chemokine receptor CCR7. Therefore, the ability of CCL19 and/or CCL21 to attract *B. pseudomallei*-infected BMDC was investigated. In most instances, the chemokines CCL19, CCL21 and in combination were equally effective at attracting BMDC (Figure 5.9). However, *B. pseudomallei*-infected BMDC demonstrated significantly increased migration toward CCL19 compared to CCL21. No significant differences were observed between CCL19 and CCL19+CCL21 or CCL21 and CCL19+CCL21, for *B. pseudomallei*-infected BMDC. *In vitro* migration of *B. pseudomallei*-infected BMDC was comparable to *E. coli* LPS-stimulated BMDC and both were significantly increased in comparison to uninfected BMDC (Figure 5.9).

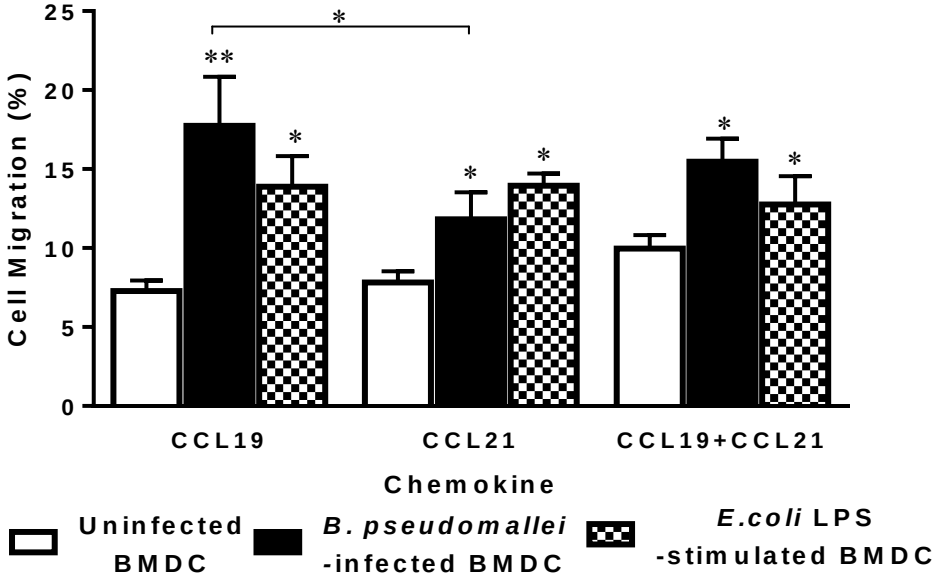

Figure 5.9 *In vitro* migration of BMDC toward CCR7 ligands CCL19 and/or CCL21

The *in vitro* migration of uninfected, *B. pseudomallei*-infected (NCTC 13179, low virulence) and *E. coli* LPS-stimulated BMDC toward the CCR7 ligands, CCL19 and/or CCL21, was compared. The number of BMDC that migrated toward chemokines in the

lower chamber was enumerated after 2 hr and expressed as a percentage of the total cells added to the upper chamber. Migration of BMDC toward the CCR7 ligands, CCL19 and/or CCL21 was observed. Bars depict the mean ± SEM of three experiments. * = P≤ 0.05, ** = P≤ 0.01 denote significant comparisons between unstimulated and stimulated, or as indicated by bracket, determined using a 2way ANOVA with multiple comparison tests.

5.3.3.5 Effect of *B. pseudomallei* virulence on BMDC migration

To determine if *B. pseudomallei* virulence affects the *in vitro* migration of BMDC, two *B. pseudomallei* isolates of high (NCTC 13178) and low (NCTC 13179) virulence were compared (Figure 5.10). BMDC infected with the highly virulent *B. pseudomallei* isolate (NCTC 13178) demonstrated significantly increased *in vitro* migration in comparison to uninfected BMDC. BMDC migration following infection with a *B. pseudomallei* isolate of high virulence was equivalent to that observed for BMDC infected with a *B. pseudomallei* isolate of low virulence (NCTC 13179). *B. pseudomallei* isolates of high virulence do not appear to possess virulence mechanisms that enable them to affect DC migration differently to that of *B. pseudomallei* isolates of low virulence.

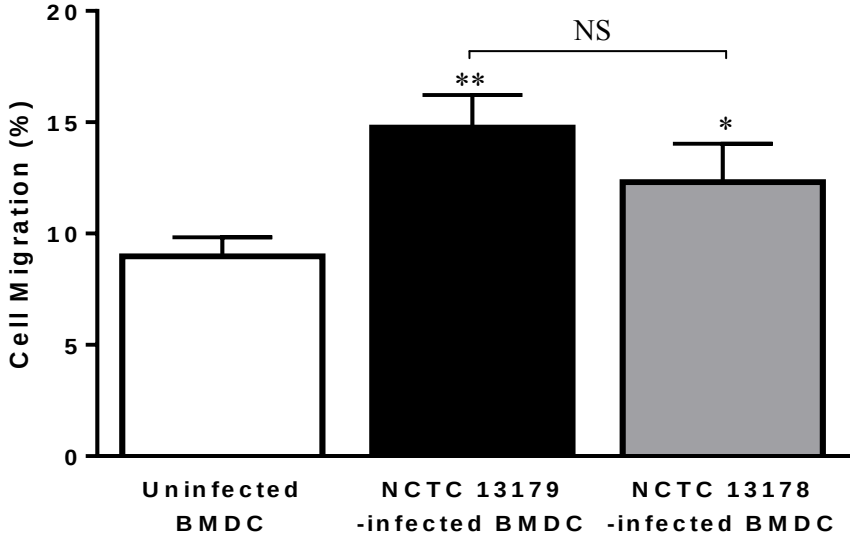

Figure 5.10 *In vitro* migration of BMDC infected with *B. pseudomallei* isolates of high and low virulence for 24 hr
The *in vitro* migration of BMDC exposed to *B. pseudomallei* isolates NCTC 13179 (low virulence) or NCTC 13178 (high virulence) was compared to uninfected BMDC. The number of BMDC that migrated toward CCL19+CCL21 in the lower chamber was

enumerated after 2 hr and expressed as a percentage of the total cells added to the upper chamber. *B. pseudomallei* isolates of high and low virulence stimulated comparable BMDC migration. Bars depict the mean ± SEM of four experiments. * = P≤ 0.05, ** = P≤ 0.01 denote significant comparisons between unstimulated and stimulated, or NS = not significant as indicated by bracket, determined using a 1way ANOVA with multiple comparison tests.

5.3.3.6 Persistence of *B. pseudomallei* within migratory BMDC

The results of the previous sections demonstrate that *B. pseudomallei* stimulates BMDC maturation and migration *in vitro.* As *B. pseudomallei* did not impair DC migration *in vitro*, it was hypothesised that migrating BMDC may harbour live *B. pseudomallei*. To assess this, parallel wells of an *in vitro* migration assay were seeded with *B. pseudomallei* (NCTC 13179) alone or *B. pseudomallei*-infected BMDC (NCTC 13179, 24 hr). Viable *B. pseudomallei* within the lower chambers after 2 hr were enumerated. Persistence of intracellular *B. pseudomallei* within migrated DC suggests that DC-associated transport may potentially facilitate *B. pseudomallei* dissemination *in vivo* (Figure 5.11). This was investigated further with subsequent studies described in Chapter 6.

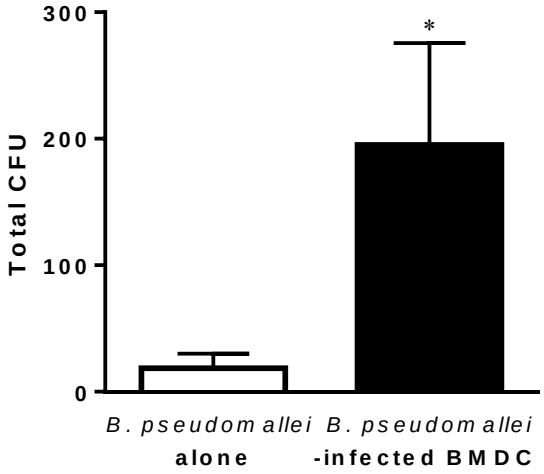

Figure 5.11 Migrated BMDC harbour live *B. pseudomallei*
The movement of *B. pseudomallei* from the upper chamber to the lower chamber of an *in vitro* migration assay in a cell-dependent (*B. pseudomallei*-infected BMDC) compared to independent (*B. pseudomallei* alone) manner was determined by enumerating the bacteria (total CFU) in the lower chamber after 2 hr. The presence of viable intracellular *B. pseudomallei* within BMDC that migrate *in vitro*, demonstrate the propensity of DC to traffic *B. pseudomallei*. Bars depict the mean ± SD of one experiment. * = P≤ 0.05, determined using a Mann Whitney test.

5.4 Discussion

Migration of DC from the site of infection to secondary lymphoid tissues is essential to enable antigen presentation by DC to lymphocytes, thereby driving activation of adaptive immune responses. This is the first study to investigate the effect of *B. pseudomallei* on the migratory capacity of DC *in vitro*. In response to *B. pseudomallei*, DC generated *in vitro* from the BM of C57BL/6 mice developed a mature phenotype with up-regulated expression of MHC class II, CD86 and CCR7, and elicited bactericidal activity against intracellular *B. pseudomallei*. Furthermore, *B. pseudomallei* significantly increased BMDC migration *in vitro* toward the CCR7 ligands, CCL19 and CCL21, in comparison to uninfected BMDC. However, *B. pseudomallei* virulence was not found to differentially influence the migration capacity of BMDC *in vitro*. Importantly, the findings of the work described in this Chapter indicate that *in vitro* trafficking of *B. pseudomallei* is facilitated by BMDC migration.

Dendritic cell maturation in response to infection precedes migration of DC to secondary lymphoid tissue for antigen presentation. Therefore, the first aspect of this study was to determine the effect of *B. pseudomallei* exposure on BMDC maturation and intracellular killing of *B. pseudomallei*. Over 24 hr, BMDC exposed to *B. pseudomallei* developed a mature phenotype, indicated by the increased expression of MHC class II and CD86. BMDC were also bactericidal against intracellular *B. pseudomallei*. These observations are in accordance with previously published studies on DC-*B. pseudomallei* interactions (Williams *et al.*, 2008; Horton *et al.*, 2012). In addition, the expression of the CCR7 on BMDC as an indicator for migration capacity was determined. Mature DC are known to up-regulate CCR7 expression which recognises two CC-chemokine ligands, CCL19 and CCL21. Together, CCL19 and CCL21, co-ordinate the migration of $CCR7^+$ DC to lymph nodes and spleen then direct DC homing to T cell rich regions (Comerford *et al.*, 2013). The increased level of CCR7 expression on *B. pseudomallei*-infected and *E. coli* LPS-stimulated BMDC compared to uninfected BMDC suggests that BMDC exposed to

B. pseudomallei are capable of migrating to secondary lymphoid tissue via CCL19 and CCL21 signalling.

Certain intracellular bacteria such as *Brucella suis* can modulate DC migration by inhibiting CCR7 expression, whilst other pathogens utilise alternative mechanisms to inhibit DC migration. For example, Velan *et al.* (2006) observed that while DC infected with *Y. pestis* underwent maturation and up-regulated CCR7 expression, they failed to migrate. This indicates that *Y. pestis* can use an alternative mechanism to impair DC migration, one that is independent of the CCR7/CCL19/CCL21 axis. Further investigation of *Y. pestis*-infected DC revealed that certain *Y. pestis* strains possess a virulence plasmid pCD1, that enables the bacteria to impair DC attachment and cytoskeleton rearrangement (Velan *et al.*, 2006). Therefore, after determining that *B. pseudomallei* stimulated CCR7 expression on BMDC, the ability of *B. pseudomallei*-infected and uninfected BMDC to migrate toward the CCR7 ligands, CCL19 and/or CCL21 was assessed using an *in vitro* Transwell® migration assay. BMDC infected with *B. pseudomallei* for 18 hr or longer demonstrated significant *in vitro* migration toward CCL19 in comparison to uninfected BMDC. The increased migratory response of BMDC corresponded with increased BMDC maturation over 24 hr. In addition, migration of BMDC infected with *B. pseudomallei* isolates of high (NCTC 13178) and low (NCTC 13179) virulence was comparable. These studies demonstrate that *B. pseudomallei* stimulates both maturation and migration of BMDC *in vitro*. Furthermore unlike *Y. pestis*, *B. pseudomallei* virulence does not appear to be related to contrasting effects on DC migration.

Although both CCL19 and CCL21 are ligands for CCR7 and bind with similar affinities to co-ordinate DC migration to lymphoid organs, they do not mediate overlapping nor redundant functions (Comerford *et al.*, 2013). CCL19 is expressed by stromal cells in lymphoid organs where it is readily available in soluble form to promote chemotaxis. CCL21 differs to CCL19 as it is expressed not only by cells within lymphoid tissue but also by epithelial cells in high endothelial venules and by lymphatic endothelial cells in peripheral tissues (Comerford *et al.*, 2013). The different patterns of CCL19 and CCL21

expression in lymphoid and peripheral tissues indicate that CCL21 is a key migratory cue present in peripheral tissues for initiating DC migration. Studies also suggest that CCL19 provides directional migration of DC within lymphoid tissue to the T cell rich zones and that mature DC are more sensitive to CCL19 than CCL21 (Saeki *et al.*, 1999; Ricart *et al.*, 2011). In addition, migration of mature DC is also influence by the CXC-chemokine receptor 4 (CXCR4) and its ligand CXCL12 (Kabashima *et al.*, 2007). Other studies suggest that CCCR7/CCL19/CCL21-mediated migration of DC is more potent than CXCR4/CXCL12-mediated migration (Ricart *et al.*, 2011). As *B. pseudomallei* did not impair CCR7 expression on mature DC, the role of CXCR4/CXCL12 was not investigated in this *in vitro* study. However, CXCR4/CXCL12 may mediate different DC homing within lymphoid tissue *in vivo* compared to CCCR7/CCL19/CCL21, which may be an avenue for future *in vivo* studies (Ohl *et al.*, 2003; Kabashima *et al.*, 2007). In the current study, *in vitro* migration of *B. pseudomallei*-infected BMDC toward both CCL19 and/or CCL21 was observed and demonstrates the potential for *B. pseudomallei*-infected BMDC to respond to migration signals at the site of infection and within secondary lymphoid tissue.

As *B. pseudomallei* infection of BMDC stimulated maturation and activated migration of BMDC *in vitro*, the potential for BMDC to transport *B. pseudomallei* during migration was investigated. Importantly, migratory DC were found to harbour live *B. pseudomallei*, demonstrating the propensity of DC to traffic *B. pseudomallei in vitro*. As these mature *B. pseudomallei*-infected BMDC have up-regulated expression of CCR7 and migrate toward CCL19 and CCL21 *in vitro*, it is tempting to speculate that the migration of these DC to secondary lymphoid tissues is a mechanism that facilitates the rapid dissemination of *B. pseudomallei* from sites of infection. Therefore, studies described in the following Chapter investigated whether the *in vitro* DC-associated transport of *B. pseudomallei* is indeed relevant *in vivo* (Chapter 6).

In summary, the findings of this study demonstrate that BMDC infected with *B. pseudomallei* develop a mature DC phenotype and exhibit antibacterial activity against intracellular *B. pseudomallei*. *B. pseudomallei*-infected BMDC significantly

increase their expression of CCR7 and demonstrate significantly increased migration toward the CCR7 ligands, CCL19 and CCL21, in comparison to uninfected BMDC. Overall, *B. pseudomallei* did not impair the migration capacity of BMDC rather; migration of BMDC facilitated cell-associated movement of *B. pseudomallei* in the *in vitro* migration assay.

CHAPTER 6
BURKHOLDERIA PSEUDOMALLEI DISSEMINATION VIA DENDRITIC CELL MIGRATION

6.1 Introduction

Pathogen-activated dendritic cells (DC) differentiate into mature cells that utilise lymphatic or haematogenous routes to migrate from the site of infection to secondary lymphoid tissues (Martin-Fontecha *et al.*, 2009). Mature DC can rapidly reach tissue draining lymph nodes by migrating through tissue, across lymphatic vessel walls then traverse the lymphatics with lymph flow and enter lymph nodes via the afferent lymphatic vessel in order to home to T cell rich regions in the paracortex (Martin-Fontecha *et al.*, 2009; Comerford *et al.*, 2013). Mature, antigen-loaded DC can also utilise haematogenous routes, by leaving lymph nodes via efferent lymphatic vessels with lymph fluid re-entering the blood system or directly entering vascular capillaries at the site of infection, to reach to distal secondary lymphoid tissues (Randolph *et al.*, 2008). A negative consequence of lymphatic or haematogenous DC migration is the potential to inadvertently facilitate the systemic spread of a pathogen (Randolph *et al.*, 2008).

Studies investigating the *in vitro* migratory response of DC infected with *B. pseudomallei* were described in the previous chapter (Chapter 5). Mature *B. pseudomallei*-infected DC were shown to express CCR7 and migrate toward the CCR7 ligands, CCL19 and CCL21, *in vitro.* Although intracellular bacteria such as *Yersinia pestis* are known to directly impair DC migration (Velan et al., 2006), *B. pseudomallei* infection of DC did not impair migration *in vitro.* Rather, the *in vitro* studies suggest that trafficking of *B. pseudomallei* may be facilitated by DC migration. Certain intracellular pathogens are reported to use DC migration as a 'Trojan horse' to facilitate dissemination (Pron *et al.*, 2001; Bar-Haim *et al.*, 2008; Rosendahl *et al.*, 2013). DC were visualised as vectors for transporting *Listeria monocytogenes* during the early stages of infection (Pron *et al.*, 2001). Similarly, *Francisella tularensis* hijacks

DC migration to facilitate dissemination from the site of infection in the respiratory tract to the draining mediastinal lymph node. By blocking DC migration *in vivo,* disease progression by *F. tularensis* was prolonged (Bar-Haim *et al.*, 2008). *Streptococcus pneumoniae* also uses DC as a vehicle for dissemination. Ablation of DC in mice infected with *S. pneumoniae,* correlated with enhanced capacity to restrict systemic dissemination, significantly reduced bacterial loads and resistance to *S. pneumoniae* infection (Rosendahl *et al.*, 2013).

While blocking migration of infected DC during the early phases of infection may decrease dissemination of some bacterial pathogens, DC migration to secondary lymphoid tissues is essential for activating protective adaptive immune responses (Muraille *et al.*, 2005; Bar-Haim *et al.*, 2008). The development of $CD8^+$ T cell-mediated immunity during *L. monocytogenes* infection relies on antigen presentation by DC exposed to live bacteria. Following exposure to live *L. monocytogenes*, DC migrated to the T cell rich zones in the spleen where they activated $CD8^+$ T cells. In comparison, DC stimulated with heat-killed *L. monocytogenes* migrated to the marginal zone and red pulp regions of the spleen which corresponded with suboptimal $CD8^+$ T cell priming (Muraille *et al.*, 2005). DC migration has also been shown to facilitate the rapid dissemination of *Bacillus anthracis* spores from the lungs to the thoracic lymph nodes (using transgenic mice developed to specifically express green fluorescent protein [GFP] in DC only [$CX_3CR1^{+/GFP}$]; Brittingham *et al.*, 2005; Cleret *et al.*, 2007). In mice infected with *Salmonella typhimurium*, systemic spread to extra-intestinal tissues, primarily to the mesenteric lymph node, is facilitated by the migration of infected DC (Voedisch *et al.*, 2009). Within the mesenteric lymph node, *S. typhimurium*-infected DC activated protective adaptive immune responses preventing further systemic spread of the pathogen. Although secondary lymphoid tissues are a centralised site for antigen presentation and activation of protective cell-mediated immune (CMI) responses, complications can arise if the pathogen is able to subvert host mechanisms and develop a niche for intracellular persistence and replication. Migration of *F. tularensis*-infected DC from the lung to the mediastinal lymph node in mice, results in rapid and excessive bacterial colonisation of the lymph node characterised by large bacterial foci formation

(Bar-Haim *et al.*, 2008). Consequently, colonisation and progressively increasing bacterial numbers in lymphoid tissues can lead to bacteraemia and systemic spread to multiple organs. Therefore, the migration of infected DC to secondary lymphoid organs can be a crucial tipping point in the development of protection as opposed to disease progression.

The migration of DC from peripheral tissues to secondary lymphoid tissues during steady state or under conditions of inflammation or infection has been well studied. Consequently, numerous techniques have been developed to observe DC migration (Bonasio and von Andrian, 2006; Alvarez *et al.*, 2008). Fluorescent tracer dyes are a popular tool used to visualise the trafficking of DC, as they are readily incorporated into cells where they become cell-impermanent resulting in stable fluorescence of the cells of interest. Common fluorescent tracer dyes include carboxyfluorescein diacetate succinimidyl ester (CFSE) or chloromethyl benzoylamino-tetramethylrhodamine (CMTMR), which are typically used in one of two applications. The tracer dye can be 'painted' onto tissue, such as the skin, where cells within the 'painted' tissue incorporate the fluorescent dye (Bonasio and von Andrian, 2006). Migration of fluorescent cells to draining secondary lymphoid organs can then be detected by fluorescence microscopy or flow cytometry. Alternatively, the tracer dye can be used to fluorescently stain a population of isolated or *in vitro* cultured cells of interest. The fluorescently labelled cells are then injected into an animal to assess their ability to migrate (Weston and Parish, 1990). Weston and Parish (1990) were the first to employ this technique to trace the migration of CFSE-labelled lymphocytes and demonstrated that CFSE-labelled lymphocytes were stable and detectable *in vivo,* 8 weeks post-injection into mice. The versatility of this technique makes it applicable for tracing the trafficking of a particular migratory cell, including DC.

Rodents, such as mice, are a useful animal model for investigating *in vivo* trafficking of DC. Two murine models of models of melioidosis have been previously characterised and published (Leakey *et al.*, 1998). The BALB/c mouse is highly susceptible to *B. pseudomallei* infection and develops a rapidly progressing disease similar to the acute

form of human melioidosis. In comparison, the C57BL/6 mouse is partially resistant to *B. pseudomallei* infection and develops disease similar to the chronic form of human melioidosis (Ulett *et al.*, 2000a; Koo and Gan, 2006). DC derived from the BM of BALB/c mice demonstrate significantly increased maturation response and IL-12 production but have impaired bactericidal activity against *B. pseudomallei* compared to DC from C57BL/6 mice (Williams *et al.*, 2008). Although comparing C57BL/6 and BALB/c mice can provide valuable information on different aspects of the immune system against *B. pseudomallei*, BALB/c mice are not always a suitable model due to their high susceptibility to infection with this pathogen. The LD_{50} for BALB/c mice infected subcutaneously with *B. pseudomallei* isolates NCTC 13178 (high virulence) and NCTC 13179 (low virulence) is 10^3 and $9x10^2$ CFU, respectively (Barnes and Ketheesan, 2005). In comparison, the LD_{50} dose for C57BL/6 mice subcutaneously infected with NCTC 13178 and NCTC 13179 is $9x10^5$ and $>10^8$ CFU, respectively (Barnes and Ketheesan, 2005). Therefore, the feasibility of using both murine models of melioidosis for investigating the effect of *B. pseudomallei* infection on the migratory capacity of DC *in vivo* requires consideration.

Currently, the mechanisms that enable melioidosis to rapidly progress from a localised infection to bacteraemia and infection of multiple organs are largely unknown. The findings presented in the previous chapter indicate that the trafficking of *B. pseudomallei* may be facilitated by DC migration (Chapter 5). Recently, colonisation of the brain in a murine model of neurological melioidosis was found to occur via the transmigration of *B. pseudomallei*-infected $CD11b^+/CD62L^+$ leukocytes across endothelial cells (Liu *et al.*, 2013). Therefore, a mechanism where *B. pseudomallei* uses DC as a 'Trojan horse' to facilitate systemic spread is plausible. To investigate this hypothesis, the current study utilised CFSE-labelled BM-derived DC (BMDC) to determine whether *in vitro* exposure to *B. pseudomallei* affects the *in vivo* migratory capacity of BMDC. These findings were then extended upon by investigating the role of tissue-resident DC in uptake of *B. pseudomallei* at the site of infection and the dissemination of the bacteria. In addition, the current study also investigated the potential implications of DC

migration on the dissemination of *B. pseudomallei* and the development of *B. pseudomallei*-specific T cell responses in the C57BL/6 murine model of melioidosis.

The specific aims of this study were to:

i) Determine if *B. pseudomallei* affects the migration capacity of BMDC *in vivo*
ii) Determine if migration of *B. pseudomallei*-infected BMDC *in vivo* facilitates dissemination of *B. pseudomallei*
iii) Determine if tissue-resident DC at the site of skin infection internalise *B. pseudomallei*
iv) Determine if disseminated *B. pseudomallei* are associated with tissue-resident DC *in vivo*
v) Assess the development of *B. pseudomallei*-specific T cell responses stimulated by *in vivo* migration of *B. pseudomallei*-infected BMDC

6.2 Materials and Methods

6.2.1 Culture of murine BMDC and infection with *B. pseudomallei*

Murine BMDC were cultured from the BM of C57BL/6 mice as previously described (Section 3.4.1). BMDC were harvested on day 10 and seeded into 6 well plates (Nunc, Thermo Fisher Scientific Pty Ltd, Scoresby, Australia) at 10^6 cells/ml in BMDC culture media (Appendix 1). BMDC were infected with log phase *B. pseudomallei* (NCTC 13179, low virulence; Section 3.3.4) at a multiplicity of infection (MOI) 1:1 or stimulated with *Escherichia coli* lipopolysaccharide (LPS, 50 ng/ml; Sigma-Aldrich, Sydney, Australia; Appendix 1) and incubated in 5 % CO_2 at 37 ºC according to previously published co-culture conditions (Williams *et al.*, 2008; Williams *et al.*, 2011). Kanamycin (250 µg/ml; Sigma-Aldrich, Sydney, Australia; Appendix 1) was added at 4 hr of co-culture to kill extracellular bacteria and protect BMDC as previously described (Section 4.2.3; Feterl *et al*., 2006; Williams *et al.*, 2008). BMDC were harvested at designated time points post-exposure to *B. pseudomallei*. BMDC were also stimulated with heat-killed (Hk)-*B. pseudomallei* (Section 3.3.5) at a ratio of Hk-

bacteria:BMDC of 1:1 for 24 hr and used to optimise the kinetics of *B. pseudomallei*-specific T cell development (Section 6.3.5).

6.2.2 Assessment of BMDC migration in C57BL/6 mice

6.2.2.1 CFSE labelling of BMDC and injection into mice

The *in vivo* migration of BMDC was assessed by injecting 10^6 CFSE-labelled BMDC into the left hind footpad of C57BL/6 mice then enumerating CFSE-labelled BMDC present in the draining popliteal lymph node (pLN) at various time points after injection. Uninfected, *B. pseudomallei*-infected and *E. coli* LPS-stimulated BMDC, prepared as described above (Section 6.2.1), were centrifuged (400 G, 5 min), washed with Hanks buffered saline solution (HBSS; Invitrogen, Life Technologies Australia Pty Ltd, Mulgrave, Australia) then resuspended at 10^7 cells/ml in HBSS. BMDC were fluorescently labelled with CFSE (8 μM; Molecular Probes®, Life Technologies Australia Pty Ltd, Mulgrave, Australia) and incubated at 37 °C for 10 min with gentle inversion. The reaction was stopped by adding excess RPMI 1640 (Invitrogen, Life Technologies Australia Pty Ltd, Mulgrave, Australia) with 10 % heat inactivated foetal bovine serum (HI-FBS; Appendix 1). CFSE-labelled BMDC were centrifuged (300 G, 5 min), resuspended in 5 ml RPMI 1640 with 10 % HI-FBS and allowed to rest for 1 hr at 37 °C. Uninfected, *B. pseudomallei*-infected and *E. coli* LPS-stimulated BMDC, with or without CFSE labelling, were centrifuged (300 G, 5 min) and the cell pellets washed with phosphate buffered saline (PBS, pH 7.2; Appendix 1). Finally, the cells were resuspended in PBS at a concentration of 2.5×10^7 BMDC/ml. Mice were injected with 40 μl (10^6 BMDC) into the left footpad as per experimental group design (Table 6.1).

Table 6.1 Experimental group design for assessing the migration of CFSE-labelled BMDC in C57BL/6 mice

Treatment of BMDC	CFSE labelling	Number of mice in group
Uninfected	Unlabelled	3
	CFSE-labelled	3
B. pseudomallei-infected	Unlabelled	3
	CFSE-labelled	3
E. coli LPS-stimulated	Unlabelled	3
	CFSE-labelled	3

6.2.2.2 Detection of migrated CFSE-labelled BMDC within the draining pLN

Detection and enumeration of migrated CFSE-labelled BMDC in the draining pLN was achieved by digesting the pLN with collagenase to release the DC, then analysing the cell suspensions for CFSE-labelled BMDC by flow cytometry. At designated time points (18, 24 or 36 hr) post-injection of CFSE-labelled BMDC, mice were killed by CO_2 asphyxiation and the draining pLN in the left hind limb was harvested. Individual pLN were macerated through 63 μm mesh with 500 μl collagenase type IV (400 U/ml according to published methods (Martìn-Fontecha *et al.*, 2003; Inaba *et al.*, 2009); Gibco®, Life Technologies Australia Pty Ltd, Mulgrave, Australia) in a 6 well plate. Cell suspensions were incubated (37 °C, 30 min) then pipetted vigorously to break up any remaining tissue fragments and filtered through a fresh piece of 63 μm mesh into a new tube. The mesh and well used for digesting the pLN was rinsed twice with 500 μl collagenase (100 U/ml) to collect residual cells. The pLN cells were then centrifuged (280 G, 10 min) and washed with RPMI 1640. Lymph node cells were transferred to FACS tubes (10^6 cells/tube; BD Biosciences, North Ryde, Australia), centrifuged (400 G, 5 min) and washed with 2 ml sodium azide buffer (SAB; Appendix 1). Cell pellets were incubated with 20 μl of diluted anti-mouse CD11c biotin (0.05 μg/10^6 cells; clone HL3; BD Biosciences, North Ryde, Australia) on ice for 30 min. After washing cells with 2 ml SAB, cell pellets were incubated in 20 μl of diluted streptavidin-PE

(0.02 μg/10^6 cells; BD Biosciences, North Ryde, Australia) on ice for 30 min. Stained cells were washed twice with 2 ml SAB then fixed in 100 μl 4 % paraformaldehyde (ProSciTech, Kirwan, Australia) at 4 °C for 15 min. Fixed cells were washed with 2 ml PBS and resuspended in 500 μl PBS. Flow cytometric analysis was performed as previously described (Chapter 3.5). The presence of $CFSE^+/CD11c^+$ cells in the draining pLN identified BMDC that had migrated from the infection site within the footpad injection site. Data was expressed as the mean percentage (% ± SEM) of injected BMDC ($CFSE^+/CD11c^+$) that migrated to the pLN.

6.2.2.3 BALB/c and C57BL/6 murine models of melioidosis

Using both C57BL/6 and BALB/c mice could provide valuable information on how *B. pseudomallei* effects the migration capacity of DC in resistant as opposed to susceptible hosts. Previous data indicates that BMDC internalise between 1-10 % *B. pseudomallei* when infected at a MOI 1:1 (Williams *et al.*, 2008). Over 24 hr, BMDC exhibit killing of intracellular *B. pseudomallei*. Consequently the number of intracellular bacteria within 10^6 BMDC infected with *B. pseudomallei* at a MOI 1:1 can range from 10^2 to 10^4 CFU (Section 5.3.1, Williams *et al.*, 2008). As the LD_{50} for NCTC 13178 (10^3 CFU) and NCTC 13179 ($9x10^2$ CFU) is so low for BALB/c mice (Barnes and Ketheesan, 2005), it was predicted that they would rapidly succumb to infection during an *in vivo* migration assay due to the number of intracellular *B. pseudomallei* within 10^6 BMDC injected into the footpad of a mouse; thus, making it difficult to examine the kinetics of DC migration. Therefore, the feasibility of using both the C57BL/6 and BALB/c and murine models of melioidosis was investigated. C57BL/6 and BALB/c mice were injected with 10^6 BMDC infected with *B. pseudomallei* (with predicted 10^4 CFU intracellular bacteria, NCTC 13178) or 10^4 CFU *B. pseudomallei* (NCTC 13178) alone into the left hind footpad as previously described (Section 6.2.1 and 6.2.2.1). Survival was monitored for 14 days and expressed as a rate of mortality (%). The bacterial loads in the draining pLN and spleen were determined in surviving mice and expressed as the mean $\log_{10}$ CFU/organ (minimum limit of detection was 50 CFU/pLN and 100 CFU/spleen).

6.2.3 Assessment of *B. pseudomallei* dissemination by BMDC in C57BL/6 mice

To compare the cell-dependent and independent dissemination of *B. pseudomallei*, the left hind footpad of C57BL/6 mice was injected with 10^6 *B. pseudomallei*-infected BMDC (with predicted 10^4 CFU intracellular bacteria, NCTC 13179) or 10^4 CFU *B. pseudomallei* (NCTC 13179). The actual number of intracellular *B. pseudomallei* within 10^6 BMDC injected into mice was subsequently determined to be $2x10^2 \pm 1$ CFU/10^6 BMDC. On day 1 post-footpad injection, mice were killed and blood collected into a heparinised tube (BD Biosciences, North Ryde, Australia). Draining pLN, inguinal LN (iLN), spleen and lung were harvested into a 6 well plate on a piece of 63 μm mesh with PBS (500 μl/LN and 1ml/lungs or spleen). Organs were macerated in PBS. Serial 10-fold dilutions of tissue homogenates or heparinised blood were performed and aliquots plated in triplicate on Ashdown Agar (Appendix 1). Agar plates were incubated for 48 hr at 37 °C prior to enumerating colonies. Data was expressed as the mean $\log_{10}$ CFU (± SEM)/organ (minimum limit of detection was 50 CFU/pLN, 50 CFU/iLN, 100 CFU/spleen and 100 CFU/lung).

6.2.4 Assessment of *B. pseudomallei* uptake and dissemination by tissue-resident DC in a murine model of melioidosis

6.2.4.1 Determination of intracellular *B. pseudomallei* loads within tissue-resident DC and non-DC

The internalisation of *B. pseudomallei* by tissue-resident DC in the skin infection site and the presence of disseminated *B. pseudomallei* within tissue-resident DC in the draining pLN, spleen and lung of C57BL/6 mice was determined. Log phase *B. pseudomallei* (10^6 CFU; Section 3.3.4) were injected into the left hind footpad of C57BL/6 mice. At 2 and 4 hr post-infection, tissue-resident DC and non-DC were isolated from the skin of the footpad as described in Section 6.2.4.2 and 6.2.4.3 then intracellular *B. pseudomallei* were enumerated (n=3 mice per time point). At 24 and 48 hr post-infection, tissue-resident DC and non-DC were isolated from the draining

pLN, spleen and lung as described below (Section 6.2.4.2 and 6.2.4.3) and intracellular *B. pseudomallei* enumerated (n=3 mice per time point). Quantification of the number of intracellular *B. pseudomallei* was determined by treating cell suspensions with 0.1 % Triton-X (total 500 µl volume; Appendix 1) to release intracellular bacteria. Lysates were serially diluted in PBS and plated onto Ashdown agar. The intracellular bacteria present within tissue-resident DC and non-DC fractions for each organ were then enumerated on plates that had been cultured at 37 °C for 48 hr. Data was then expressed as the mean ratio of *B. pseudomallei*:10^3 cells (± SEM) for DC and non-DC fractions (minimum limit of detection was 50 CFU/organ DC and non-DC fraction).

6.2.4.2 Collagenase digestion of organs

To determine the number of intracellular *B. pseudomallei* within tissue-resident DC, collagenase digestion is necessary to release the DC from tissues. At 2 and 4 hr post-footpad infection, mice were killed by CO_2 asphyxiation and the skin of the left hind footpad was harvested. The draining pLN, spleen and lung were harvested from mice killed at 24 and 48 hr post-infection. Individual organs were macerated through 63 µm mesh with collagenase type IV and DNase-1 (Sigma-Aldrich, Sydney, Australia) in a 6 well plate using volumes and concentrations as shown in Table 6.3. Cell suspensions were incubated for 30 min at 37 °C to release the DC. Cell suspensions were pipetted vigorously to break up any remaining tissue fragments, then filtered through a fresh piece of 63 µm mesh into a new tube. The mesh and well used for digesting the organ were rinsed twice with HBSS to collect residual cells. The cells were centrifuged (280 G, 10 min) then washed once with RPMI 1640 and once with SAB before resuspending the cells to $2x10^7$ cells/ml in SAB.

Table 6.2 Collagenase and DNase concentrations for digesting organs

Organ	Digestion volume (ml)	Collagenase IV (U/ml)	DNase-1 (U/ml)
Footpad	0.5	400	100
pLN	0.5	400	100
Spleen	1	200	100
Lung	1	200	100

The optimal volume and concentrations for digesting each organ was determined in a prior study (data not shown in this thesis).

6.2.4.3 Magnetic selection of tissue-resident DC from organs

Tissue-resident DC in the skin, pLN, spleen and lung were isolated by magnetically separating $CD11c^+$ cells from the non-DC. Cell suspensions from the collagenase-digested organs as described above (Section 6.2.4.2) were incubated with anti-mouse CD11c Biotin (0.05 μg/10^6 cells; clone HL3; BD Biosciences, North Ryde, Australia) on ice for 15 min. Cells were washed with 1ml IMag Buffer (1x; BD Biosciences, North Ryde, Australia) then all of the supernatant was aspirated. Cell pellets were incubated with streptavidin-magnetic nanoparticles (1 μl/10^6 cells; BD Biosciences, North Ryde, Australia) at 4°C for 30 mins. The cells were resuspended in 500 μl IMag Buffer. To separate the $CD11c^+$ tissue-resident DC from the non-DC, cells were exposed to a strong magnetic force by standing the tubes in an IMag magnet (BD Biosciences, North Ryde, Australia) for 8 mins. With the tubes still in the magnet, the supernatant containing the $CD11c^-$ non-DC cells was transferred to a fresh tube. The magnetic separation twice step was repeated twice by resuspending the remaining $CD11c^+$ tissue-resident DC were resuspended in 500 μl IMag Buffer. Finally, the tissue-resident DC and non-DC fractions (pooled) were washed, resuspended in 110 μl PBS and 100 μl of each fraction was used to enumerate the intracellular *B. pseudomallei* (Section 6.2.4.1). The remaining 10 μl of each cell fraction was reserved to assess the purity of the isolated tissue-resident DC.

6.2.4.4 Assessment of tissue-resident DC purity

Following isolation of tissue-resident DC and non-DC cells, 10 µl of each cell fraction were transferred to FACS tubes (10^6 cells/tube) and centrifuged (400 G, 5 min). The cells were washed with 2 ml SAB, resuspended in 20 µl of diluted anti-mouse CD11c FITC (0.05 µg/10^6 cells; clone HL3; BD Biosciences, North Ryde, Australia) and anti-mouse CD45 PerCP (0.05 µg/10^6 cells; clone 30-F11; BD Biosciences, North Ryde, Australia), then incubated on ice for 30 min. Stained cells were washed twice with 2 ml SAB then fixed in 100 µl 4 % paraformaldehyde (ProSciTech, Kirwan, Australia) and incubated at 4 °C for 15 min. Fixed cells were then washed with 2 ml PBS and resuspended in 300 µl PBS for analysis by flow cytometry as previously described (Chapter 3.5). Tissue-resident DC were identified as $CD45^+$/ $CD11c^+$ cells and the percentage within tissue-resident DC fractions was calculated (Appendix 3) then expressed as the mean purity (% ± SEM).

6.2.5 Assessment of *B. pseudomallei*-specific T cell responses in C57BL/6 mice following injection of *B. pseudomallei*-infected BMDC

6.2.5.1 Detection of *B. pseudomallei*-specific T cell proliferation in mononuclear cell cultures stimulated *in vitro* with *B. pseudomallei* lysate

Development of *B. pseudomallei*-specific T cell responses in C57BL/6 mice, following injection of BMDC that had been exposed to *B. pseudomallei in vitro*, was determined by assessing ^{3}H-thymidine (0.25 µCi/well; Appendix 1) incorporation by proliferating T cells stimulated with *B. pseudomallei* lysate *in vitro*. Uninfected BMDC (10^6 cells; Section 6.2.1), *B. pseudomallei*-infected BMDC (10^6 cells; Section 6.2.1), Hk-*B. pseudomallei*-stimulated BMDC (10^6 cells; Section 6.2.1) or *B. pseudomallei* alone (10^4 CFU; Chapter 3.3.4) were injected into the left hind footpad of C57BL/6 mice. The survival of the mice was monitored until they were killed at 7, 14 or 21 days post-injection by CO_2 asphyxiation to assess the *B. pseudomallei*-specific T cell responses in the draining pLN and spleen. Mononuclear cells isolated from spleen and pLN as

described below (Section 6.5.2 and 6.5.3) were seeded (100 µl; 10^5 cells/well) in triplicate into U-bottom 96 well plates (Nunc, Thermo Fisher Scientific Pty Ltd, Scoresby, Australia) containing 100 µl double strength culture media (Appendix 1). Cultures were stimulated with either *B. pseudomallei* lysate (NCTC 13179, 1 µg/ml; Section 3.3.6) to activate recall response proliferation of *B. pseudomallei*-specific T cells or concanavalin A (2 µg/ml; Appendix 1) as a positive proliferation control. Proliferation was quantified by assessing the incorporation of H^3-Thymidine at 48, 72, 96 and 120 hr of culture. For each time point, cells were cultured in the presence of H^3-thymidine for 18 hr then fixed with 4 % paraformaldehyde (4 °C, 40 min) and harvested using a FilterMate cell harvester (Perkin Elmer, Melbourne, Australia) onto glass fibre filter paper. Incorporation of H^3-thymidine was then analysed using a Beta scintillation counter (Perkin Elmer, Melbourne, Australia). From the data generated (counts per minute [CPM]), stimulation indices (SI) were calculated (Appendix 3) and expressed as the mean maximum SI (± SEM) recorded over the four time points.

6.2.5.2 Isolation of mononuclear cells from the spleen

Individual spleens were macerated through 63 µm mesh with 2 ml RPMI 1640 containing kanamycin (500 µg/ml) in a 6 well plate. Cell suspensions were transferred to a conical 15 ml tube (BD Biosciences, North Ryde, Australia). Residual cells on the mesh and well were collected by washing twice with 2 ml RPMI 1640 containing kanamycin (500 µg/ml). Kanamycin was added to the isolation media to kill any *B. pseudomallei* surviving in the harvested tissue. The use of kanamycin in the isolation media instead of cell culture media was optimised as described in the following results Section 6.3.5.2. Tissue fragments were allowed to settle out before the supernatant was carefully layered over 3 ml Ficoll-paque PLUS (GE Healthcare Australia Pty Ltd, Rydalmere, Australia) to enable isolation of mononuclear cells by density centrifugation (500 G, 20 min). After centrifugation, the mononuclear cells were collected from the interface between Ficoll-paque PLUS and RPMI 1640, washed twice with RPMI 1640 (kanamycin free; 500 G, 10 min) then resuspended to 10^6 cells/ml in RPMI 1640 (kanamycin free) for subsequent assessment of T cell proliferation (Section 6.2.5.1).

6.2.5.3 Isolation of mononuclear cells from the pLN

Popliteal lymph nodes were pooled according to treatment group and macerated through 63 μm mesh with 2 ml RPMI 1640 containing kanamycin (500 μg/ml) in a 6 well plate. Cell suspensions were filtered through a fresh piece of 63 μm mesh into a 10 ml tube (BD Biosciences, North Ryde, Australia). Residual cells on the mesh and well were collected by washing with 1 ml RPMI 1640 containing kanamycin (500 μg/ml). Mononuclear cells isolated from the pLN were then washed with RPMI 1640 (kanamycin free; 500 G, 10 min) and resuspended to 10^6 cells/ml in RPMI 1640 (kanamycin free) for subsequent assessment of T cell proliferation (Section 6.2.5.1).

6.2.6 Statistical analysis

Statistical analysis of data was performed using GraphPad Prism 6 Software and details are reported in Appendix 2. To optimise the *in vivo* migration assay, differences between *B. pseudomallei* infection time on BMDC migration (18 and 24 hr) and harvest time of the draining pLN post-injection of DC (18, 24 and 36 hr) were tested for statistical significance using a 2way ANOVA, with recommended Sidak's and Tukey's posthoc multiple comparisons. For the *in vivo* migration studies, differences between BMDC stimulant groups (uninfected, *B. pseudomallei*-infected or *E. coli* LPS-stimulated) and BMDC infected with *B. pseudomallei* isolates of high and low virulence (NCTC 13178 and NCTC 13179) were tested for statistical significance using a 1way ANOVA, with recommended Tukey's multiple comparison test, or a 2way ANOVA with recommended Sidak's and Tukey's posthoc multiple comparisons. The effect of kanamycin on lymphocyte proliferation was tested for statistical significance using an unpaired t test with recommended Welch's correction. The proliferation of *B. pseudomallei*-specific T cells from mice was tested for statistical significance using a 2way ANOVA, with recommended Sidak's posthoc multiple comparisons. The survival of mice in different treatment groups were compared for statistical differences using Log Rank (Mantel Cox) tests on survival curves. Differences in organ loads of mice (*B. pseudomallei*-infected BMDC, 10^4 or 10^6 CFU *B. pseudomallei* alone) and

intracellular *B. pseudomallei* within organ cell fractions ($CD11c^+$ or $CD11c^-$) were tested for statistical significance using a 2way ANOVA, with recommended Sidak's posthoc multiple comparisons. Differences between tested groups were considered significant if the P value ≤ 0.05. Significant differences were displayed on figures where * = $P \leq 0.05$ and ** = $P \leq 0.01$.

6.3 Results

6.3.1 Optimisation of the murine model for assessing *in vivo* migration of BMDC following *in vitro* exposure to *B. pseudomallei*

6.3.1.1 Flow cytometric analysis of CFSE-labelled BMDC

The use of CFSE to fluorescently label and track the migration of cells is a well-established technique (Weston and Parish, 1990). In this study, CFSE labelling of BMDC was used to track the *in vivo* migration of BMDC from the left hind footpad of C57BL/6 mice to the draining pLN. This technique was used to investigate whether the *in vivo* migration capacity of BMDC is affected following exposure to *B. pseudomallei*. Successful labelling of uninfected BMDC with CFSE was demonstrated by flow cytometry (Figure 6.1) prior to performing the *in vivo* migration assays. The unlabelled and CFSE labelled BMDC, gated as shown in the FSC against SSC plot (Figure 6.1A), were compared for CFSE expression (Figure 6.1B). Compared to unlabelled BMDC, fluorescence of CFSE-labelled BMDC was apparent in the FL1 channel. The process of CFSE labelling of BMDC did not affect the phenotype of uninfected BMDC, demonstrated by similar patterns of CD11c and CD86 expression on unlabelled and CFSE-labelled BMDC (Figure 6.1C and D).

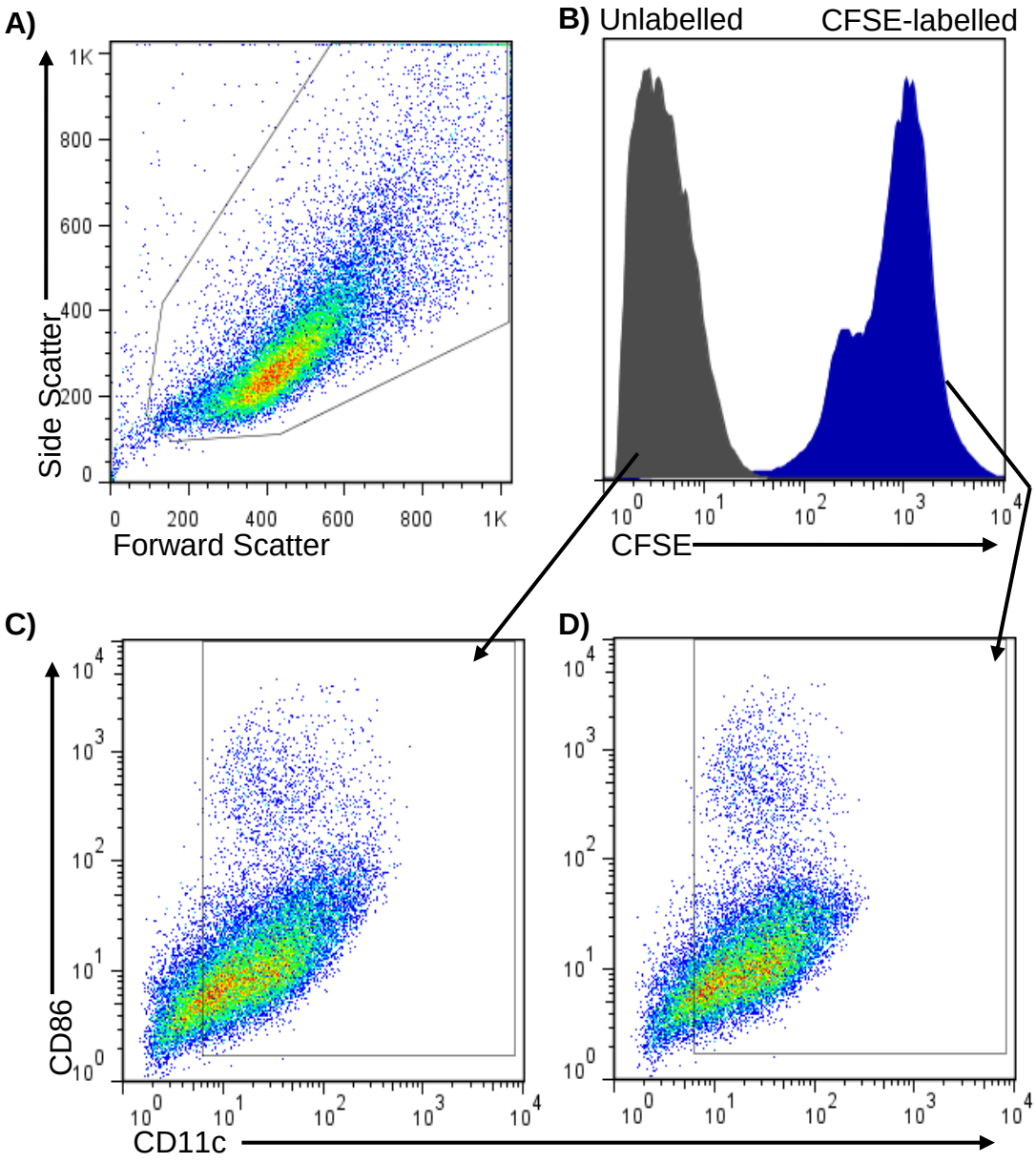

Figure 6.1 Demonstration of CFSE-labelled BMDC
The fluorescence of uninfected BMDC (10^6) with or without CFSE labelling was compared. Representative FACS plot and histogram demonstrate the A) cell scatter of unlabelled BMDC and B) comparison of the fluorescence between unlabelled and CFSE-labelled BMDC. Fluorescence of CFSE-labelled BMDC was detected in the FL1 channel (B). Comparison of CD11c and CD86 expression on C) unlabelled and D) CFSE-labelled BMDC demonstrated that CFSE labelling of BMDC did not affect phenotype.

6.3.1.2 Feasibility of the C57BL/6 murine model of melioidosis for assessing the *in vivo* migration of BMDC

Infection of BALB/c and C57BL/6 mice with *B. pseudomallei* causes disease similar to the acute and chronic forms of human melioidosis, respectively. As BALB/c mice are highly susceptible to *B. pseudomallei* infection, it was predicted that BALB/c mice would succumb to infection during an *in vivo* migration assay due to the number of intracellular *B. pseudomallei* surviving within 10^6 BMDC (10^2 to 10^4 CFU; Section 5.3.1; Williams *et al*., 2008). Therefore, the feasibility of using both the C57BL/6 and BALB/c and murine models of melioidosis was investigated. Injection of 10^6 BMDC infected with *B. pseudomallei* (NCTC 13178) or 10^4 CFU *B. pseudomallei* (NCTC 13178) alone into the left hind footpad was lethal for 100 % of BALB/c mice and caused 33 % mortality in C57BL/6 mice within 4 days post-injection. Although comparison of BMDC migration in both murine models of melioidosis would have been beneficial for determining how *B. pseudomallei* effects the migration capacity of DC in resistant compared to susceptible hosts, it was confirmed that BALB/c mice were not a suitable model for investigating the kinetics of *in vivo* migration of BMDC.

Organ bacterial loads were also determined for the C57BL/6 mice that survived for 14 days post-footpad injection of *B. pseudomallei*-infected BMDC or *B. pseudomallei* alone. These surviving mice were heavily infected with *B. pseudomallei*, demonstrated by enlarged spleens and pLN that had visible abscesses and high bacterial burden (Figure 6.2). As the *B. pseudomallei* isolate NCTC 13178 (high virulence) caused heavy systemic infection in C57BL/6 mice, the *B. pseudomallei* isolate NCTC 13179 (low virulence) was selected for further studies. The use of the *B. pseudomallei* isolate NCTC 13179 is supported by the findings of the previous Chapter 5 where it was determined that *B. pseudomallei* isolates of high (NCTC 13178) and low virulence (NCTC 13179) elicited similar effects on the *in vitro* migration of BMDC. Therefore, *B. pseudomallei*-resistant C57BL/6 mice and the low virulence *B. pseudomallei* isolate (NCTC 13179) were used to investigate *in vivo* migration of BMDC, dissemination of

B. pseudomallei and generation of *B. pseudomallei*-specific T cell responses by migrated *B. pseudomallei*-infected BMDC.

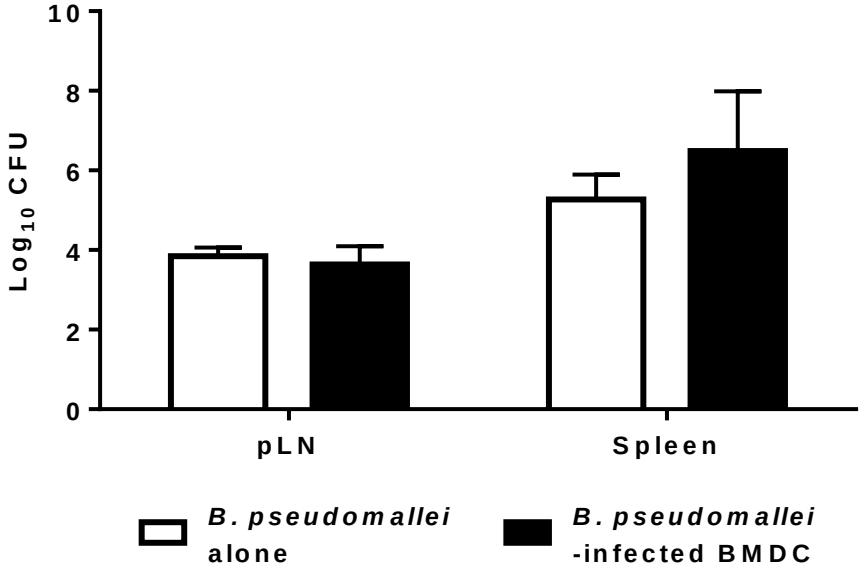

Figure 6.2 Bacterial loads in the pLN and spleen of C57BL/6 mice following footpad injections with *B. pseudomallei*-infected BMDC or *B. pseudomallei* alone
The organ loads of surviving C57BL/6 mice (n=9, 3 mice per group) was determined on day 14 post-footpad injection with either live *B. pseudomallei* (NCTC 13178, 10^4 CFU), *B. pseudomallei*-infected BMDC (10^6 BMDC) or uninfected BMDC (10^6 BMDC). *B. pseudomallei* was not recovered from any tissues of mice that received 10^6 uninfected BMDC (data not shown). Bars depict the mean bacterial load (± SD) per organ of one experiment expressed as Log_{10} CFU.

6.3.1.3 Identification of migrated BMDC in the draining pLN

Having confirmed the methods for labelling BMDC with CFSE, the suitable *B. pseudomallei* isolate and mouse strain, we next investigated the migration of CFSE-labelled BMDC from the footpad to the draining pLN of C57BL/6 mice. Uninfected, *B. pseudomallei*-infected and *E. coli* LPS-stimulated BMDC, with or without CFSE labelling were injected into the left hind footpad of C57BL/6 mice (Table 6.1). The draining pLN was removed 24 hr post-footpad injection. The baseline for detection of $CFSE^+/CD11c^+$ cells was based on the background fluorescence in the draining pLN of mice injected with unlabelled BMDC. The presence of $CFSE^+/CD11c^+$ cells in the

draining pLN, identified by flow cytometry, indicated migration of BMDC from the skin injection site (Figure 6.3).

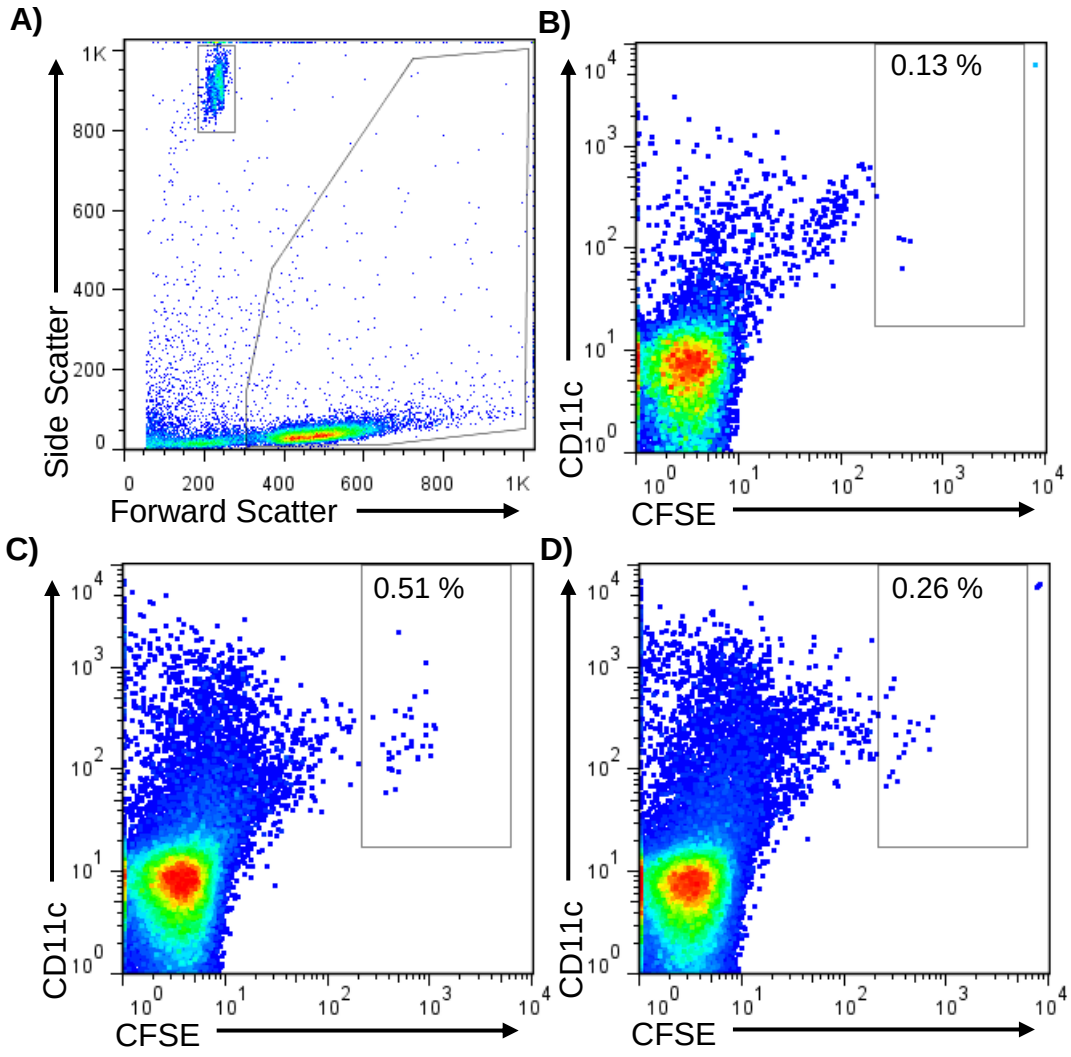

Figure 6.3 Quantification of $CFSE^+/CD11c^+$ cells that have migrated to the draining pLN

Example FACS plots from three experiments demonstrate the identification of migrated BMDC ($CFSE^+/CD11c^+$) in the draining pLN 24 hr after mice were injected with BMDC. A) FSC against SSC plot of pLN from mice injected with uninfected. Cells gated in the FSC against SSC plot were then compared for CFSE and CD11c expression to identify migrated BMDC ($CFSE^+/CD11c^+$) in the pLN of mice injected with B) uninfected BMDC, C) *B. pseudomallei*-infected (NCTC 13179) BMDC and D) *E. coli* LPS stimulated BMDC, stimulated for 24 hr *in vitro* and labelled with CFSE. The fluorescence of cells in the pLN of mice injected with unlabelled BMDC were used as a baseline for detecting $CFSE^+$ cells. Countbright^{TM} counting beads gated in the upper left corner of forward scatter against side scatter plots enabled enumeration of migrated BMDC.

6.3.1.4 Optimisation of the *in vivo* migration assay using *E. coli* LPS-stimulated BMDC

In the previous Chapter (Chapter 5) it was demonstrated that *B. pseudomallei* and *E. coli* LPS stimulate BMDC to develop a mature phenotype with increased expression of MHC class II, CD86 and CCR7. Furthermore, increased stimulation time with *B. pseudomallei* and *E. coli* LPS corresponded with increased maturation and *in vitro* migration response of BMDC (Section 5.3.1 and 5.3.2). Therefore, to determine if *in vitro* stimulation time also affected the migration response of BMDC *in vivo,* the *in vivo* migration assay was optimised using BMDC stimulated *in vitro* with *E. coli* LPS for 6, 12, 18 and 24 hr prior to footpad injection (Figure 6.4). Following injection into the left hind footpad, *E. coli* LPS-stimulated BMDC demonstrated increased migration to the draining pLN compared to unstimulated BMDC. Consistent with the previous *in vitro* migration findings (Chapter 5), BMDC stimulated *in vitro* with *E. coli* LPS for 18 hr demonstrated the highest migration responses *in vivo*, with an average of 8569 $CFSE^+/CD11c^+$ cells (equals 0.82% of the injected $CFSE^+/CD11c^+$ BMDC) detected in the pLN.

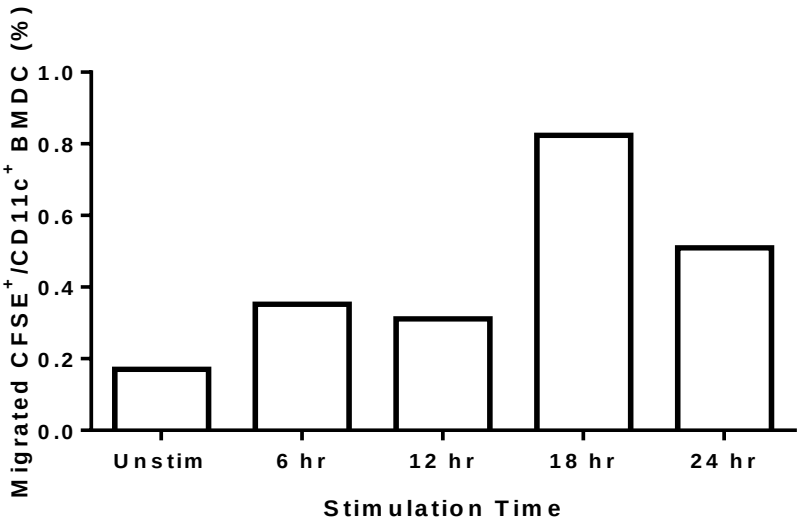

Figure 6.4 *In vivo* migration of BMDC stimulated with *E. coli* LPS for 6, 12, 18 and 24 hr prior to footpad injection
The *in vivo* migration of BMDC stimulated *in vitro* with *E. coli* LPS (50 ng/ml) for 6, 12, 18 and 24 h prior to foot-pad injection of C57BL/6 mice (n=3 mice per group) was compared. At 24 hr post-foot-pad injection, pLN were harvested and pooled according to group then migrated BMDC, defined as $CFSE^+/CD11c^+$, were identified by flow cytometry. BMDC stimulated with *E. coli* LPS for 18 and 24 hr demonstrated the highest *in vivo* migration response. Bars depict the percentage migrated BMDC (averaged for 3 mice) from one experiment.

6.3.2 Migratory response of *B. pseudomallei*-infected BMDC in C57BL/6 mice

6.3.2.1 Effect of *in vitro* exposure time with *B. pseudomallei* on the *in vivo* migration of BMDC

Having found that BMDC stimulated *in vitro* with *E. coli* LPS for 18 hr demonstrated the highest migration responses *in vivo*, we next sought to determine the optimal exposure time for *B. pseudomallei*-infected BMDC. Therefore, the *in vivo* migration of BMDC infected *in vitro* with *B. pseudomallei* for 18 and 24 hr prior to footpad injection was compared. Both, 18 and 24 hr *B. pseudomallei*-infected BMDC demonstrated significant *in vivo* migration compared to uninfected BMDC (Figure 6.5). All subsequent *in vivo* migration experiments were conducted using BMDC that were exposed to *B. pseudomallei* for 24 hr *in vitro* prior to footpad injection.

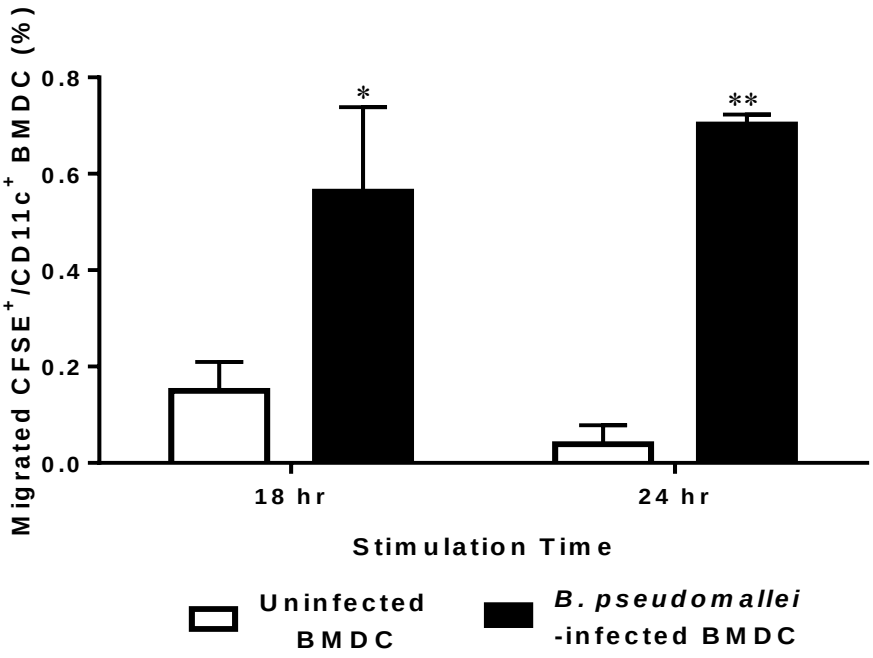

Figure 6.5 *In vivo* migration of BMDC infected with *B. pseudomallei* (NCTC 13179) for 18 and 24 hr prior
The *in vivo* migration of BMDC infected *in vitro* with *B. pseudomallei* for 18 and 24 hr prior to injection into the footpad of C57BL/6 mice (n=3 mice per group) was compared. BMDC demonstrated significantly increased *in vivo* migration following *B. pseudomallei* infection for 18 and 24 hr compare to uninfected BMDC. Bars depict the mean ± SEM of two experiments. * = P≤ 0.05, ** = P≤ 0.01 determined using a 2way ANOVA with multiple comparisons tests.

6.3.2.2 *In vivo* migration kinetics of *B. pseudomallei*-infected BMDC

To better understand the kinetics of BMDC migration *in vivo* and to identify the optimal time for assessing the peak BMDC migration to the draining pLN, different harvest times post-footpad injection of BMDC were compared. Hind footpads of mice were injected with unlabelled or CFSE-labelled BMDC that had been infected *in vitro* with *B. pseudomallei* for 24 hr. At 18, 24 and 36 hr post-footpad injection, mice were killed and the draining pLN harvested then digested to enable enumeration of migrated BMDC by flow cytometry (Figure 6.6). Peak *in vivo* migration of *B. pseudomallei*-infected BMDC from the footpad to the pLN was observed 24 hr post-injection. *In vivo* migration of BMDC was therefore assessed at 24 hr post-footpad injection for all subsequent *in vivo* migration assays.

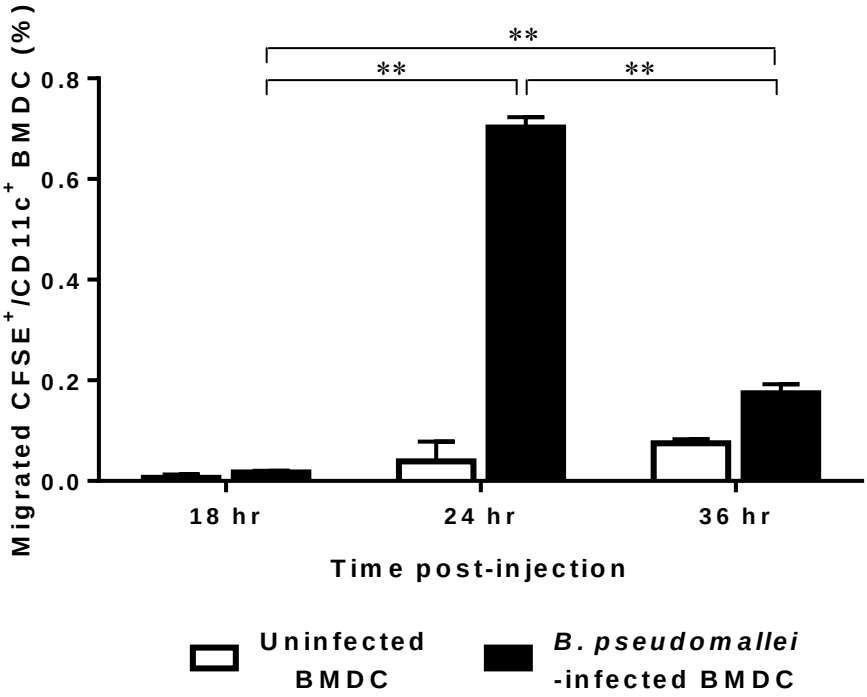

Figure 6.6 Effect of time post-footpad injection (18, 24 and 36 hr) on assessing *in vivo* migration of BMDC infected with *B. pseudomallei* (NCTC 13179) for 24 hr
The *in vivo* migration of BMDC infected *in vitro* with *B. pseudomallei* (NCTC 13179, 24 hr) was assessed at 18, 24 and 36 hr post-footpad injection of C57BL/6 mice (n= 3 mice per group). Peak *in vivo* migration of *B. pseudomallei*-infected BMDC from the footpad to the pLN was observed 24 hr post-injection. Bars depict the mean ± SEM of one experiment. * = P≤ 0.05, ** = P≤ 0.01 denote significant differences between uninfected infected BMDC (unless indicated by brackets), determined using a 2way ANOVA with multiple comparisons tests.

6.3.2.3 *In vivo* migration of BMDC following *in vitro* exposure to *B. pseudomallei*

Using the optimal *in vitro* exposure time (24 hr) and *in vivo* migration time (24 hr post-footpad injection) determined in the previous sections, the *in vivo* migration of *B. pseudomallei*-infected and *E. coli* LPS-stimulated BMDC within C57BL/6 mice was determined. BMDC infected with *B. pseudomallei* (NCTC 13179) or stimulated with *E. coli* LPS (positive control) elicited similar migration responses *in vivo*, which were significantly higher than that observed for uninfected BMDC (Figure 6.7). Although *B. pseudomallei* exposure caused significantly increased migration of BMDC to the pLN compared to uninfected BMDC, the percentage of migrated BMDC detected in the draining pLN after 24 hr was low compared to previously published studies (Martìn-Fontecha *et al.*, 2003). Nevertheless, these studies demonstrate that BMDC exposed to *B. pseudomallei* migrate to secondary lymphoid tissue.

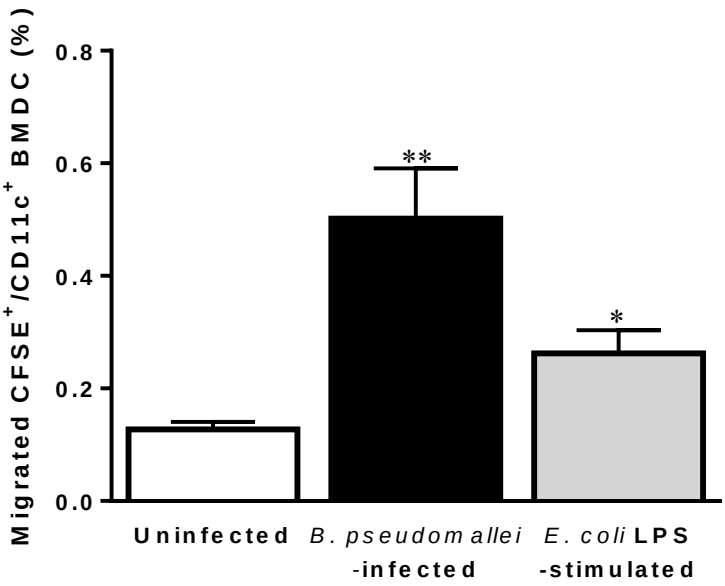

Figure 6.7 Assessment of BMDC migration *in vivo*
The *in vivo* migration of uninfected, *B. pseudomallei*-infected (NCTC 13179, 24 hr) or *E. coli* LPS-stimulated BMDC was assessed 24 hr post-footpad injection of C57BL/6 mice (n=3 mice per group). Stimulation of BMDC with *B. pseudomallei* and *E. coli* LPS elicited similar BMDC migration responses *in vivo.* Bars depict the mean ± SEM of three experiments. * = P≤ 0.05, ** = P≤ 0.01 denote significant differences when compared to uninfected BMDC, determined using a 1way ANOVA with multiple comparisons tests.

6.3.3 Effect of BMDC migration on the dissemination of *B. pseudomallei* in C57BL/6 mice

To determine if the *in vivo* migration of *B. pseudomallei*-infected BMDC facilitate host cell-dependent dissemination of the pathogen, the bacterial burden within draining pLN, iLN, spleen, lung and blood, 24 hr after mice were injected with *B. pseudomallei*-infected BMDC (10^6 BMDC) or *B. pseudomallei* alone (10^4 CFU) was determined. Although the number of intracellular bacteria within 10^6 *B. pseudomallei*-infected BMDC can range from 10^2 to 10^4 CFU, the actual number of intracellular bacteria within the 10^6 BMDC injected into mice was retrospectively determined to be $2x10^2 \pm 1$ CFU/10^6 BMDC (Section 5.3.1). Mice injected with *B. pseudomallei* alone received 10^4 CFU, 50 times the number of CFU associated with 10^6 *B. pseudomallei*-infected BMDC. However, the bacterial burden of the spleen and lung was significantly higher in C57BL/6 mice that received *B. pseudomallei*-infected BMDC compared to *B. pseudomallei* alone (Figure 6.8). This demonstrates that *B. pseudomallei* utilise migration of infected BMDC to facilitate cell-dependent dissemination to multiple organs.

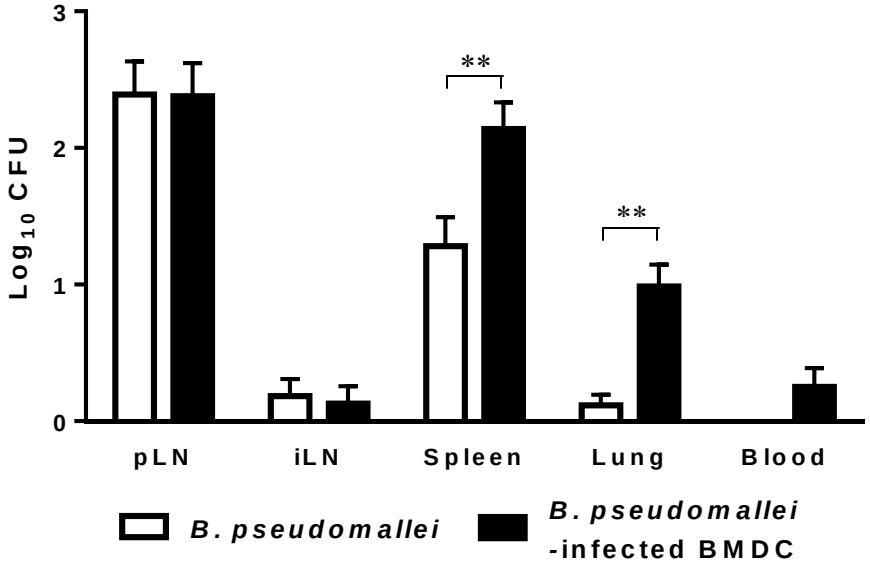

Figure 6.8 Bacterial burden following footpad injection with *B. pseudomallei*-infected BMDC
The organ bacterial load in the draining pLN, iLN, spleen, lung and blood of C57BL/6 mice injected with *B. pseudomallei*-infected BMDC (10^6 BMDC, n=5 mice) or *B. pseudomallei* only (10^4 CFU, n=5 mice) was determined on day 1 post-footpad

injection. Cell-associated *B. pseudomallei* used DC migration to facilitate significantly increased dissemination to the spleen and lung of C57BL/6 mice. Bacteria were not detected in the blood of mice infected with *B. pseudomallei* alone (limit of detection = 2 CFU/ml blood). Bars depict the mean $\log_{10}$ CFU ± SEM of three experiments. ** = P≤ 0.01 determined using a 2way ANOVA with multiple comparisons tests.

6.3.4 Role of tissue-resident DC in the dissemination of *B. pseudomallei* in C57BL/6 mice

6.3.4.1 Effect of collagenase digestion on *B. pseudomallei* viability

In the previous sections, it was determined that BMDC migration facilitated the dissemination of *B. pseudomallei.* However, the contribution of tissue-resident DC at the site of *B. pseudomallei* infection is unknown. Therefore, internalisation of *B. pseudomallei* by skin DC at the site of infection and dissemination of *B. pseudomallei* to secondary lymphoid tissues was subsequently investigated. Collagenase digestion of organs is required to release tissue-resident DC prior to enriching DC and determining the number of intracellular *B. pseudomallei.* Preliminary experiments were conducted to confirm that collagenase digestion of tissue had no effect on *B. pseudomallei* viability (Figure 6.9).

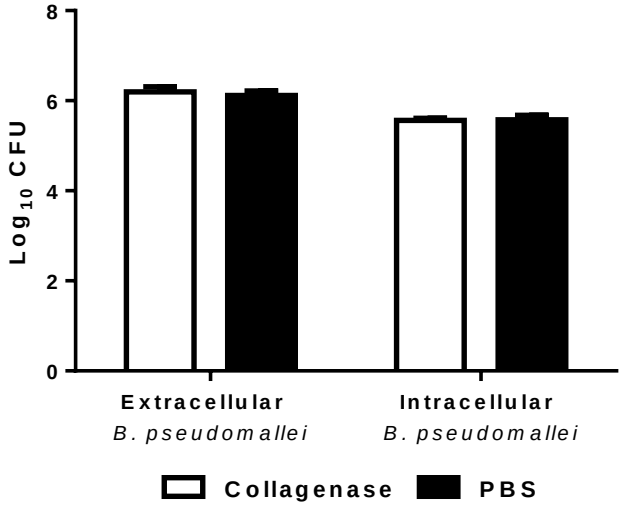

Figure 6.9 Effect of collagenase digestion on *B. pseudomallei* viability
The effect of collagenase digestion (400 U/ml, 30 min at 37 °C) on the viability of extracellular *B. pseudomallei* and intracellular *B. pseudomallei* within BMDC was determined by comparing bacterial numbers in groups with or without exposure to collagenase. Collagenase digestion had no effect on the viability of *B. pseudomallei.* Bars depict the mean ± SD of one experiment.

6.3.4.2 Isolation and purity of tissue-resident DC

Having optimised tissue digestion protocols, tissue-resident DC within the footpad, draining pLN, spleen and lung were subsequently isolated by magnetically selecting the $CD11c^+$ (DC) cells from the $CD11c^-$ (non-DC) cells. Tissue-resident DC fractions were analysed by flow cytometry and defined as $CD45^+/CD11c^+$ cells using the gating strategy depicted in Figure 6.10A and 6.10B. From the FACS data, both the quantity and cell purity of the tissue-resident DC enriched from each organ was determined (Figure 6.10C).

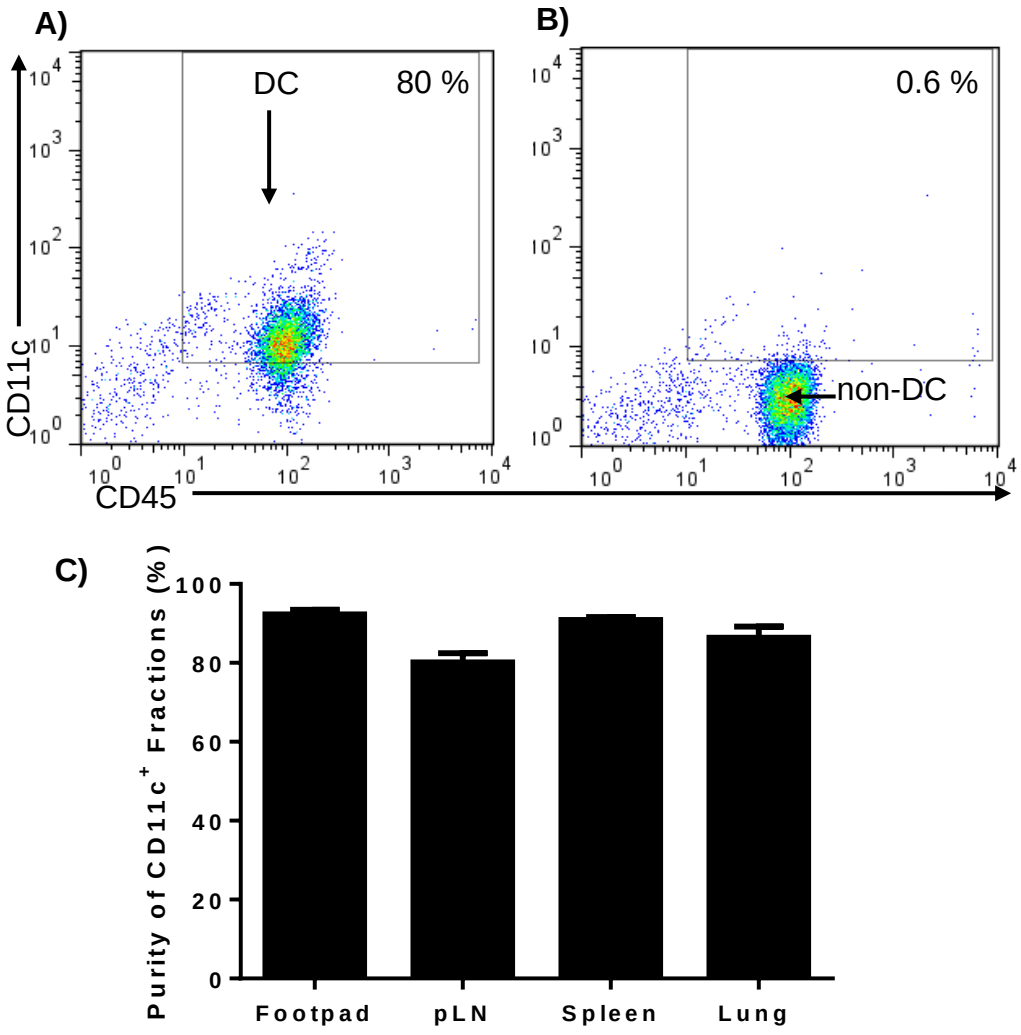

Figure 6.10 Selection of $CD11c^+$ DC
The purity of tissue-resident DC isolated from the footpad, draining pLN, spleen and lungs of C57BL/6 mice was analysed by flow cytometry. Example FACS plots depict how tissue-resident DC were identified as $CD45^+/CD11c^+$ in pLN cells following

A) enrichment of tissue-resident DC ($CD11c^+$ fraction) from B) non-DC ($CD11c^-$ fraction). C) The purity of tissue-resident DC enriched from the footpad, draining popliteal lymph node, spleen and lung was calculated. Bars depict the mean ± SEM of three experiments.

6.3.4.3 *B. pseudomallei* uptake by tissue-resident DC in the footpad

Internalisation of *B. pseudomallei* by tissue-resident DC ($CD11c^+$) and non-DC ($CD11c^-$) at the site of infection was determined. C57BL/6 mice were infected with *B. pseudomallei* (NCTC 13179, 10^6 CFU) via the left hind footpad. At 2 and 4 hr post-injection, the infected footpad was excised and tissue-resident DC separated from non-DC. The intracellular *B. pseudomallei* within tissue-resident DC and non-DC were enumerated and expressed as the ratio of *B. pseudomallei* per 10^3 cells since the percentage of tissue-resident DC within the footpad, pLN and spleen ranges between 0.5-4 % of the nucleated cell population (Duriancik and Hoag, 2009). Internalisation of *B. pseudomallei* by tissue-resident DC in the skin infection site was demonstrated. While non-DC at the site of infection also internalised some *B. pseudomallei*, the ratio of bacteria to non-DC was lower compared to tissue-resident DC. This is highlighted by the fact that the ratio of intracellular *B. pseudomallei* within infection site DC was significantly higher than non-DC at 2 hr post-infection (Figure 6.11).

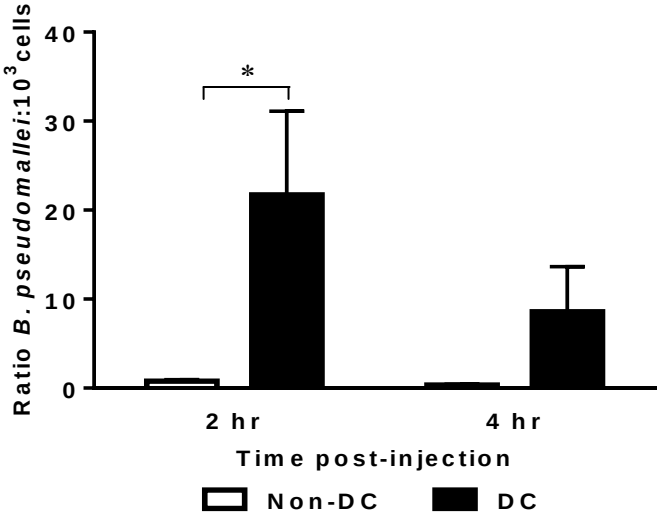

Figure 6.11 Internalisation of *B. pseudomallei* by tissue-resident DC in the footpad
The internalisation of *B. pseudomallei* by tissue-resident DC at the footpad infection site was determined by quantifying the intracellular bacteria within tissue-resident DC ($CD11c^+$) and non-DC ($CD11c^-$) fractions at 2 and 4 hr post-footpad infection of

C57BL/6 mice with *B. pseudomallei* (NCTC 13179, 10^6 CFU, n=3 mice). The intracellular bacteria present within tissue-resident DC ($CD11c^+$) and non-DC ($CD11c^-$) fractions of the footpad were enumerated then expressed as the ratio of intracellular *B. pseudomallei* per 10^3 cells. At 2 hr post-infection, the ratio of *B. pseudomallei* to skin DC was significantly higher than non-DC. Bars depict mean ± SEM of three experiments. * = $P \leq 0.05$, determined using a 2way ANOVA with multiple comparisons tests.

6.3.4.4 *B. pseudomallei* dissemination by tissue-resident DC

Having demonstrated internalisation of *B. pseudomallei* by tissue-resident DC in the skin at the infection site, the dissemination of *B. pseudomallei* within tissue-resident DC to the draining pLN, spleen and lungs was subsequently investigated at 24 and 48 hr post-infection (Figure 6.12). Intracellular *B. pseudomallei* were detected in the draining pLN, within tissue-resident DC and non-DC, at 24 hr post-infection (Figure 6.12A). The ratio of *B. pseudomallei* to tissue-resident DC compared to non-DC in the pLN was increased however, this trend was not statistically significant (p=0.21; Figure 6.12A). At 48 hr post-infection, bacterial loads were undetectable in the draining pLN. However, the ratio of *B. pseudomallei* within spleen DC increased between 24 and 48 hr (Figure 6.12B). No intracellular *B. pseudomallei* were detectable within DC and non-DC in the lungs at 24 and 48 hr post-infection (data not shown). As DC and non-DC fractions for each organ were lysed with 0.1 % Triton-X in a total volume of 500 µl before diluting and plating 10 µl on Ashdown agar, the limit of detection for *B. pseudomallei* in this particular assay was 50 CFU per organ DC or non-DC fraction. Therefore, *B. pseudomallei* may have been present in the lungs or pLN (48 hr) at levels below the detection limit. Overall, DC at the site of infection internalised *B. pseudomallei*. Furthermore, disseminated *B. pseudomallei* were detected within tissue-resident DC in the draining pLN and spleen, which is consistent with the previous *in vivo* migration studies.

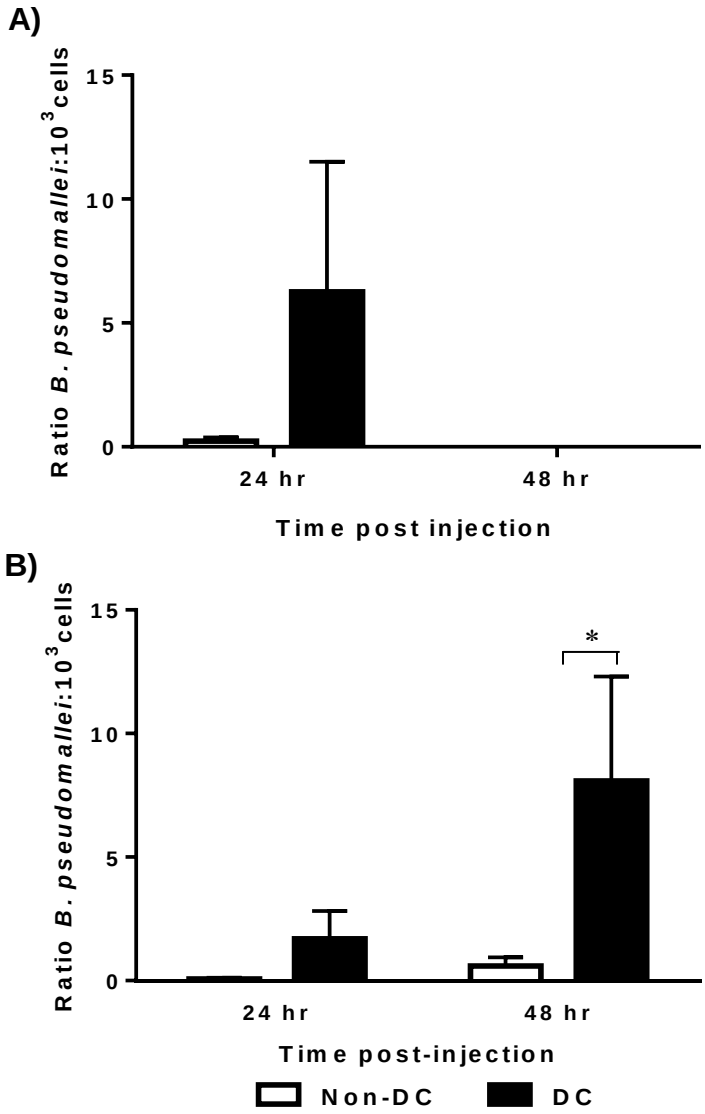

Figure 6.12 Dissemination of *B. pseudomallei* within tissue-resident DC to the draining pLN and spleen

The association of disseminated *B. pseudomallei* with tissue-resident DC in the draining pLN and spleen was determined by quantifying the bacterial burden of tissue-resident DC ($CD11c^+$) and non-DC ($CD11c^-$) fractions at 24 and 48 hr post-footpad infection of C57BL/6 mice with *B. pseudomallei* (NCTC 13179, 10^6 CFU, n=3 mice). The intracellular bacteria present within tissue-resident DC and non-DC fractions of A) pLN and B) spleen were enumerated then expressed as the ratio of intracellular *B. pseudomallei* per 10^3 cells. At 24 hr post-infection, disseminated *B. pseudomallei* were detected within tissue-resident DC in the pLN and spleen. After 48 hr, no intracellular *B. pseudomallei* were detected in the pLN. However, *B. pseudomallei* replication within tissue-resident DC in the spleen was observed, such that at 48 hr post-infection, the ratio of intracellular *B. pseudomallei* within DC (*Bps*:10^3 cells) was significantly higher than non-DC in the spleen. Bars depict mean ± SEM of three experiments. * = $P \leq 0.05$, determined using a 2way ANOVA with multiple comparisons tests.

6.3.5 Ability of *B. pseudomallei*-infected BMDC to activate *B. pseudomallei*-specific T cell responses in C57BL/6 mice

6.3.5.1 Kinetics of *B. pseudomallei*-specific T cell activation

DC migration to secondary lymphoid tissues is important for the development of protective adaptive immune responses. In the previous sections, it was determined that migration of *B. pseudomallei*-infected DC inadvertently facilitated the systemic spread of *B. pseudomallei.* To understand how this affects the development of adaptive immune responses against *B. pseudomallei,* the ability of *in vitro B. pseudomallei*-infected BMDC to activate *B. pseudomallei*-specific T cell responses in C57BL/6 mice was investigated. In preliminary experiments, the kinetics of *B. pseudomallei*-specific T cell activation at 7, 14 and 21 days post-footpad injection of BMDC stimulated with heat-killed (Hk)-*B. pseudomallei* (NCTC 13179) was assessed (Figure 6.13). Significant *B. pseudomallei*-specific T cell responses in the draining pLN and spleen were observed 21 days post-footpad injection of Hk-*B. pseudomallei*-stimulated BMDC (Figure 6.13). *B. pseudomallei*-specific T cell responses were assessed at 21 days post-footpad injection for all subsequent proliferation assays.

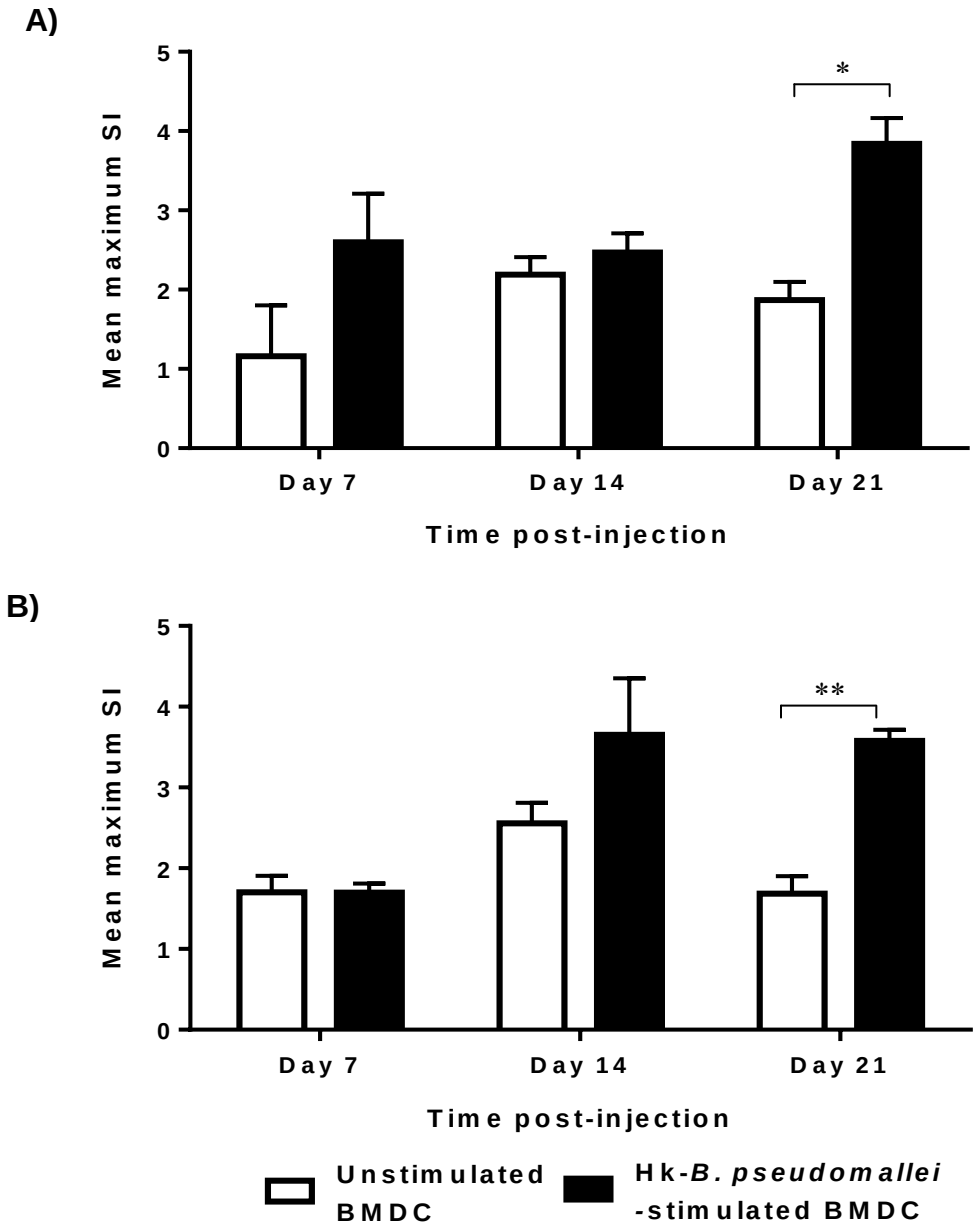

Figure 6.13 Time point for assessing *B. pseudomallei*-specific T cell response
The kinetics of *B. pseudomallei*-specific T cell responses in C57BL/6 mice was determined following footpad injection of BMDC stimulated with Hk-*B. pseudomallei*. On days 7, 14 and 21 post-footpad injection, A) the draining pLN and B) spleen were harvested from mice (n=3 mice per group at each time point) to assess the proliferation response of T cells to *B. pseudomallei* lysate by determining H^3-thymidine incorporation. Significant *B. pseudomallei*-specific T cell recall responses were detected 21 days post-footpad injection. Bars depict the mean maximum stimulation index (SI) to *B. pseudomallei* lysate of three animals. * = P≤ 0.05, ** = P≤ 0.01 determined using a 2way ANOVA with multiple comparisons tests. Raw CPM data reported in Appendix 4.

6.3.5.2 Effect of kanamycin inclusion in mononuclear cell culture media on the *in vitro* proliferation of T cells

Earlier studies investigating the bacterial loads of C57BL/6 mice following footpad injection with *B. pseudomallei*-infected BMDC or *B. pseudomallei* alone indicated that live *B. pseudomallei* is likely to be present within splenocytes at 21 days post-infection (Chapter 6.3.1.2). Intracellular persistence of *B. pseudomallei* within mononuclear cells has the potential to affect the viability and proliferation of T cells in an *in vitro* assay system. Antibiotic protection of cells can be provided by the addition of kanamycin to kill extracellular *B. pseudomallei*. Therefore, the effect of kanamycin (250 μg/ml) in culture media used throughout cell re-stimulation period for T cell proliferation assays was investigated. Kanamycin treatment of mononuclear cell culture media caused significantly increased proliferation of T cells (Figure 6.14).

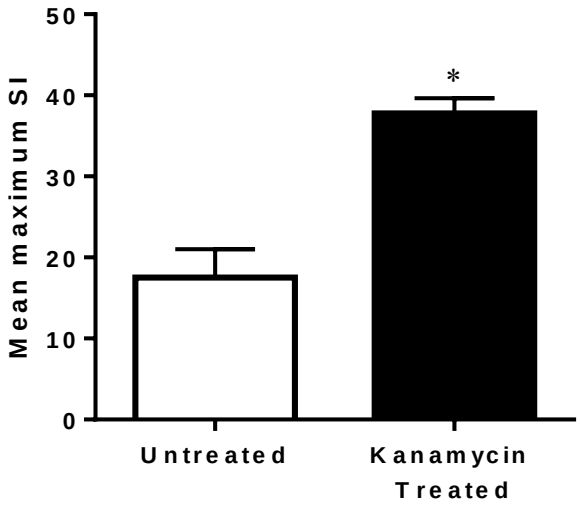

Figure 6.14 Effect of kanamycin in cell culture media on concanavalin A-induced T cell proliferation
The determine if the inclusion of kanamycin in culture media effects mononuclear cell proliferation, cells isolated from the spleens of naïve C57BL/6 mice (n=3, pooled) were culture with or without kanamycin treatment (250 μg/ml). The proliferation response of T cells stimulated with concanavalin A, was assessed by determining H^3-thymidine incorporation. Kanamycin treatment of cell culture media during the lymphocyte proliferation assay caused significantly increased proliferation. Bars depict the mean maximum SI ± SD of one experiment. *=P≤ 0.05 determined using an unpaired t test with Welch's correction. Raw CPM data reported in Appendix 4.

Consequently, culturing mononuclear cells for up to 120 hr in kanamycin treated culture media (250 μg/ml) is unsuitable as it significantly affected the proliferative response of T cells and could potentially affect the reliability and interpretation of results obtained under such conditions. Therefore, the efficacy of using a higher concentration of kanamycin (500 μg/ml) in the media used only during the isolation of mononuclear cells and the subsequent effect on T cell proliferation was investigated (Figure 6.15). The use of a high concentration of kanamycin during the cell isolation period only was effective at reducing the bacterial burden of isolated mononuclear cells. Importantly, a high concentration of kanamycin during the mononuclear cell isolation procedure did not affect T cell proliferation (Figure 6. 15). For subsequent studies, kanamycin (500 μg/ml) was only used during the isolation of mononuclear cells.

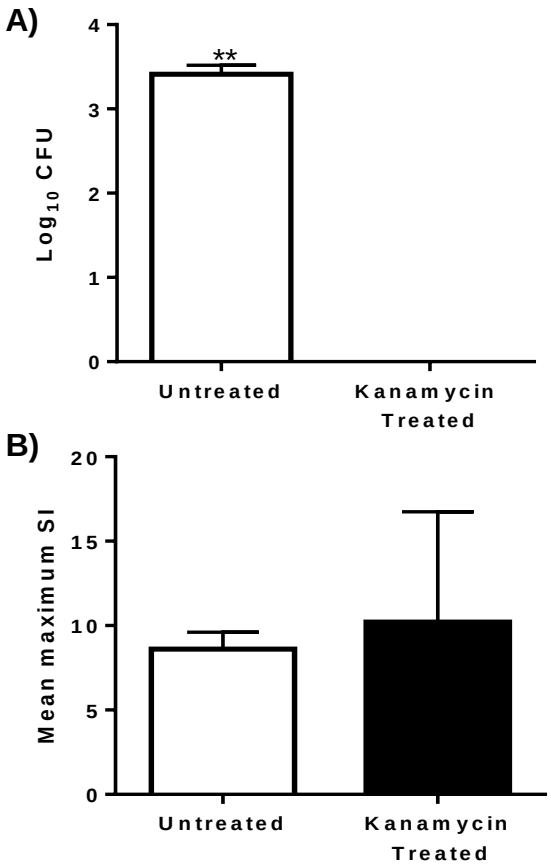

Figure 6.15 Effect of kanamycin on concanavalin A-induced T cell proliferation when included in isolation media only

To determine the efficacy of high concentration kanamycin (500 μg/ml) in mononuclear cell isolation media to kill *B. pseudomallei* and the subsequent effect on T cell proliferation, mononuclear cells were isolated with or without kanamycin treatment from the spleens of *B. pseudomallei*-infected C57BL/6 mice then cultured in media free of

kanamycin and stimulated with concanavalin A. (A) Kanamycin treatment was effective as no bacteria were detectable in mononuclear cells isolated with kanamycin from spleens of *B. pseudomallei*-infected mice. B) Kanamycin treatment during isolation only did not affect T cell proliferation following stimulation with concanavalin A. Bars depict the mean Log_{10} CFU ± SD (A) and the mean maximum SI ± SD (B) of one experiment. ** = $P \leq 0.01$ determined using an unpaired t test with Welch's correction.

6.3.5.3 *B. pseudomallei*-specific T cell responses following migration of *B. pseudomallei*-infected BMDC

After determining the optimal time post-injection to detect *B. pseudomallei*-specific T cell responses (21 days post-injection) and that kanamycin treatment (500 μg/ml) during the isolation of mononuclear cells from the pLN and spleen is effective for killing persistent *B. pseudomallei* within organs, the *B. pseudomallei*-specific T cell responses generated by *B. pseudomallei*-infected BMDC were investigated. Uninfected BMDC, *B. pseudomallei*-infected BMDC and *B. pseudomallei* alone were injected into the left hind footpad of C57BL/6 mice. The survival of mice was monitored for 21 days at which time the mice were killed to determine organ bacterial loads and the development of *B. pseudomallei*-specific T cell responses in mononuclear cells derived from the draining pLN and spleen. The survival of C57BL/6 mice injected with *B. pseudomallei*-infected BMDC or *B. pseudomallei* alone was 90 % for both groups (Figure 6.16A). Organ bacterial loads demonstrated that all surviving mice had cleared the infection (data not shown). However, poor *B. pseudomallei*-specific T cell recall responses were observed in the pLN and spleen of mice injected with *B. pseudomallei*-infected BMDC or *B. pseudomallei* alone compared to mice injected with uninfected BMDC (Figure 6.16B). The antigen load delivered by injecting 10^6 *B. pseudomallei*-infected BMDC or 10^4 CFU *B. pseudomallei* alone was likely to be low considering less than 1 % of *B. pseudomallei*-infected BMDC migrate to the draining pLN and 10^4 CFU *B. pseudomallei* (NCTC 13179) equates to 0.0001 LD_{50} for C57BL/6 mice. Therefore, it is possible that the amount of *B. pseudomallei* antigen available for presentation to naïve T cells in the spleen and pLN was insufficient to stimulate detectable levels of *B. pseudomallei*-specific T cell proliferation in recall assays using techniques employed.

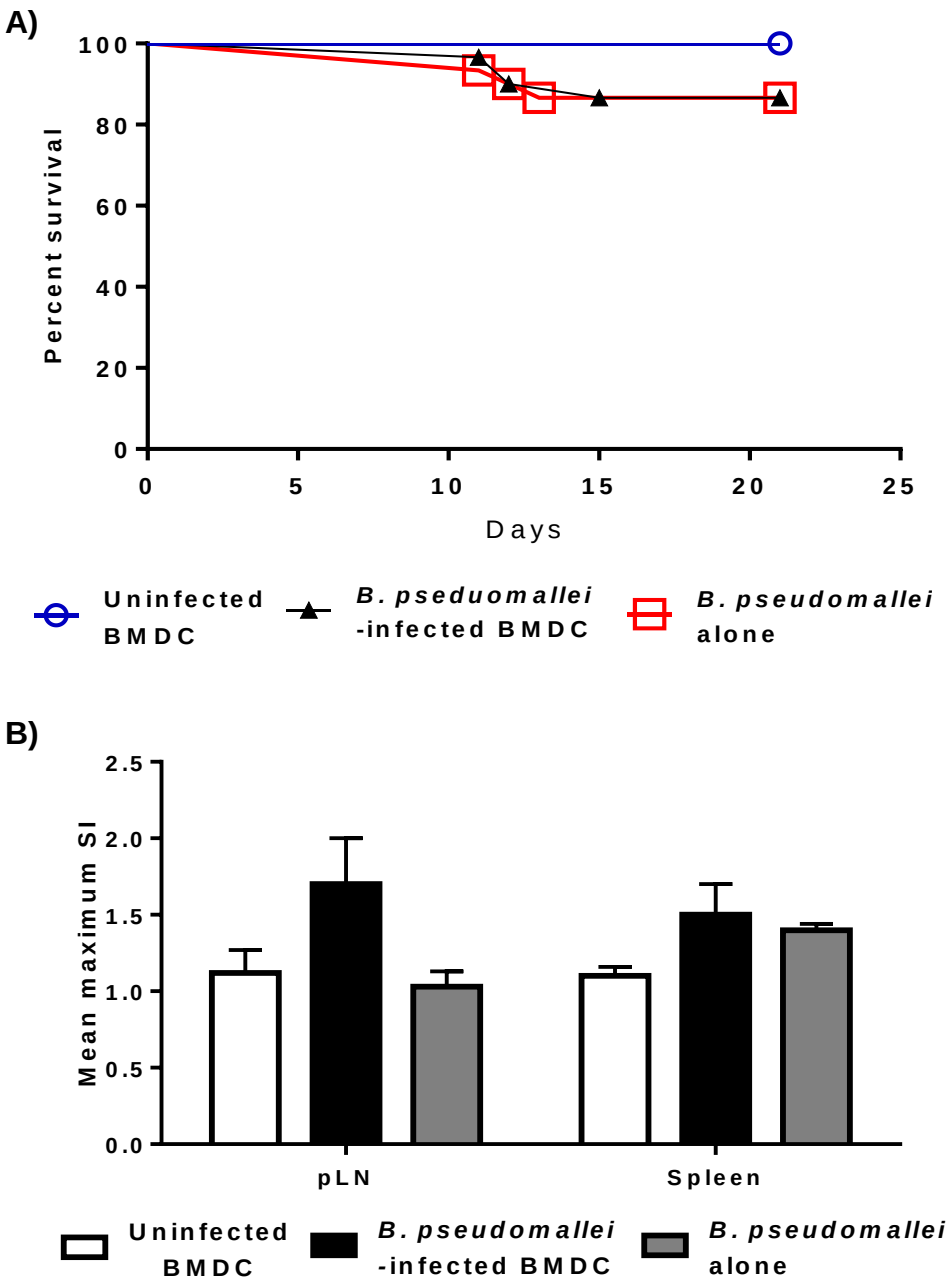

Figure 6.16 Survival of mice and proliferation responses of T cells following footpad injection with *B. pseudomallei*-infected BMDC

The survival of mice and generation of *B. pseudomallei*-specific T cell responses in C57BL/6 mice (n= 5 mice per group) was determined 21 days post-footpad injection with uninfected BMDC (10^6), *B. pseudomallei*-infected BMDC (10^6) or *B. pseudomallei* alone (10^4 CFU). A) The survival of each group was monitored over 21 days. B) The proliferation of mononuclear cells stimulated with *B. pseudomallei* lysate was quantified by determining H^3-thymidine incorporation. Poor *B. pseudomallei*-specific T cell recall responses were observed in mononuclear cell cultures derived from both the pLN and spleen. The graph depicts the combined survival (A) and the bars depict the mean maximum SI ± SEM of (B) of three experiments.

6.3.5.4 Effect of antigen load on the generation of *B. pseudomallei*-specific T cell responses

To determine whether the weak *B. pseudomallei*-specific T cell response observed in the previous studies was an effect of low antigen load, the organ bacterial burden and proliferation response of T cells was assessed in response to a 100 fold higher *B. pseudomallei* dose (10^6 CFU = 0.01 LD_{50}). At 24 hr post-infection, the bacterial burden in the spleen and lung of C57BL/6 mice infected with 10^6 CFU was significantly higher in comparison to mice infected with 10^4 CFU (Figure 6.17A). Furthermore, increased antigen load also corresponded with significant *B. pseudomallei*-specific T cell proliferation in the spleen 21 days post-infection (Figure 6.17B). These findings demonstrate that detection of measurable antigen-specific T cell responses is influenced by the initial antigen load delivered to mice and may explain why mice injected with *B. pseudomallei*-infected BMDC or 10^4 CFU *B. pseudomallei* failed to demonstrated detectable *B. pseudomallei*-specific T cell memory in Section 6.3.5.3.

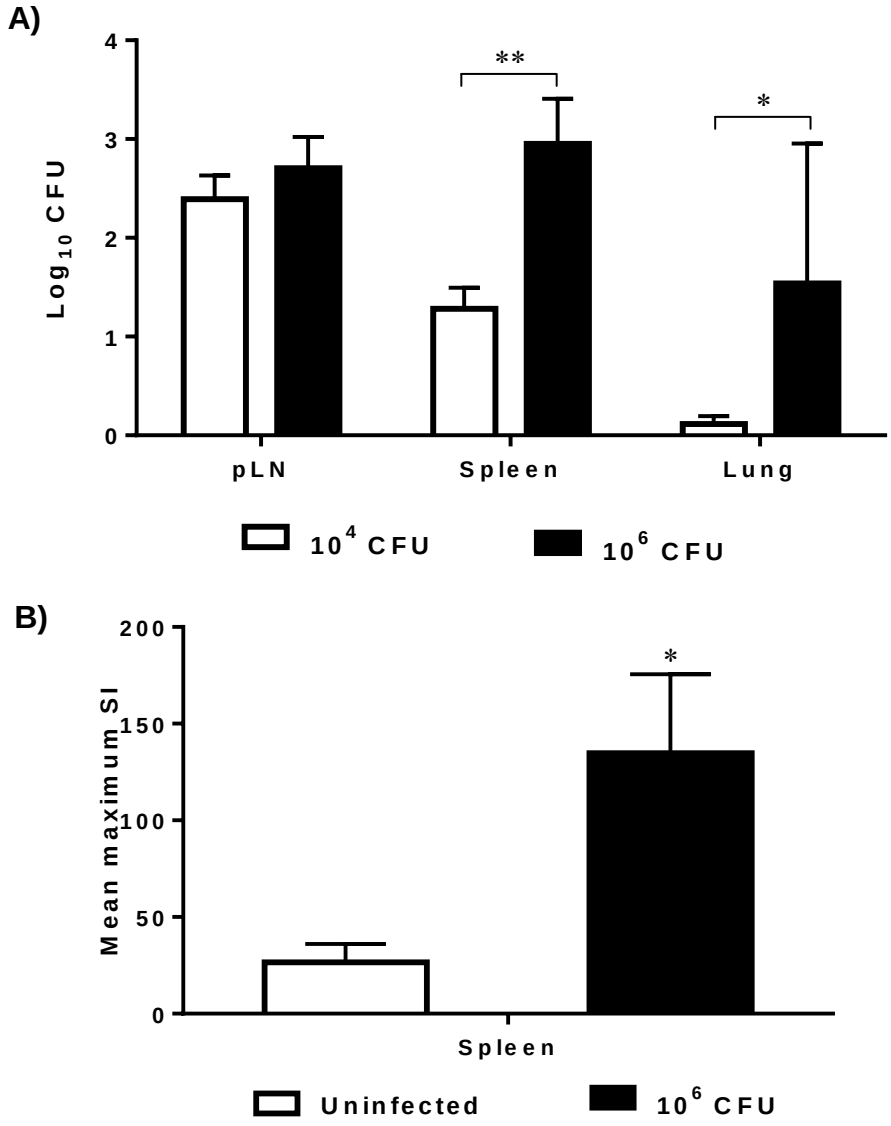

Figure 6.17 Bacterial burden and generation of *B. pseudomallei*-specific T cell responses in C57BL/6 mice following footpad injection with 10^6 CFU *B. pseudomallei*

To determine if a higher dose of *B. pseudomallei* would active the development of *B. pseudomallei*-specific T cell responses, C57BL/6 mice were infected with *B. pseudomallei* only (10^6 CFU; NCTC 13179) or left uninfected (PBS) via the left hind footpad. A) The organ bacterial load of C57BL/6 mice (n=5 mice per group) was assessed 24 hr post-injection. B) At day 21 post-infection, the proliferation of T cells stimulated with *B. pseudomallei* lysate was quantified by determining H^3-thymidine incorporation. An increased dose of *B. pseudomallei* corresponded with significantly increased *B. pseudomallei*-specific proliferation of T cells when challenged *in vitro* with *B. pseudomallei* lysate (NCTC 13179). Bars depict the mean $\log_{10}$ CFU ± SD (A) and the mean maximum SI ± SD (B) of one experiment.* = P≤ 0.05, ** = P≤ 0.01 determined using A) 2way ANOVA with multiple comparisons tests or B) unpaired t test with Welch's correction.

6.4 Discussion

In the previous Chapter (5), it was demonstrated that *B. pseudomallei* could be trafficked within migrating BMDC *in vitro*. The current studies investigated the *in vivo* relevance of these findings. In response to *B. pseudomallei* infection, DC were shown to migrate from the site of infection to secondary lymphoid tissues. This was confirmed by tracking the migration of *B. pseudomallei*-infected BMDC, fluorescently labelled with CFSE, from the footpad to the draining pLN. Furthermore, migration of *B. pseudomallei*-infected BMDC correlated with significantly increased systemic dissemination of *B. pseudomallei*. By isolating tissue-resident DC from different organs of *B. pseudomallei*-infected mice, it was demonstrated that DC at the site of infection internalised *B. pseudomallei* and disseminated *B. pseudomallei* were present within tissue-resident DC in the pLN and spleen. Furthermore, spleen DC provided a niche for *B. pseudomallei* persistence and replication. Although DC migration facilitated the dissemination of *B. pseudomallei* infection, in the current study, migrated *B. pseudomallei*-infected BMDC failed to stimulate the development of detectable levels of *B. pseudomallei*-specific T cell recall responses as assessed by quantifying ^{3}H-thymidine incorporation by proliferating T cells.

To investigate the role of DC migration in the dissemination of *B. pseudomallei,* the *in vivo* migration of *B. pseudomallei*-infected BMDC, from the footpad injection site to the draining pLN was first investigated. BMDC infected with *B. pseudomallei* (NCTC 13179) or stimulated with *E. coli* LPS (positive control) elicited similar migration responses *in vivo*. However, less than 1 % of BMDC were found to migrate to the draining pLN, which is low compared to published studies on DC migration (Martìn-Fontecha *et al.*, 2003; Zhao *et al.*, 2006). Zhao *et al.* (2006) assessed the migration of *S. typhimurium*-infected BMDC to the mesenteric lymph node by injecting 10^{7} *S. typhimurium*-infected BMDC (labelled with CFSE) in the peritoneum of BALB/c mice and found ~750 injected cells/2x10^{6} lymph node cells. Direct comparison with our study is difficult as Zhao *et al.* (2006) used different assay conditions, such as BALB/c mice. However, injection of 10^{7} BMDC into the peritoneum and harvesting of the

mesenteric lymph node 48 hr post injection, resulted in higher migration of *S. typhimurium*-infected BMDC than that observed for *B. pseudomallei*-infected BMDC in our study. Martìn-Fontecha *et al*. (2003) observed that the *in vivo* migration efficiency of LPS-stimulated BMDC reached 3 % when 1-2x10^6 BMDC were injected into the hind footpad of BALB/c mice. For this study, 10^6 BMDC were injected into the hind footpad of C57BL/6 mice. Differences in the *in vivo* migration observed between this study and that by Martìn-Fontecha *et al.* (2003) and Zhao *et al.* (2006) may reflect variation in the migratory capacity of DC isolated from different mouse strains. The *in vivo* migration of DC within BALB/c infected with *Leishmania major* was found to be eight times higher than that observed in C57BL/6 mice (Misslitz *et al.*, 2004). Comparison of the *in vivo* migratory response of *B. pseudomallei*-infected BMDC from C57BL/6 and BALB/c mice was not feasible due the high susceptibility of BALB/c mice to this bacterium. The subcutaneous LD_{50} dose for BALB/c mice infected with the low virulence isolate NCTC 13179 is 9x10^2 CFU compared to >10^8 CFU for *B. pseudomallei*-resistant C57BL/6 mice (Barnes and Ketheesan, 2005). This is further complicated by the impaired *B. pseudomallei*-killing efficiency of BMDC derived from BALB/c mice (Williams *et al.*, 2008). Therefore, BALB/c mice received a lethal higher dose of bacteria when injected with 10^6 BMDC compared to C57BL/6 mice. Consequently, BALB/c mice injected with *B. pseudomallei*-infected BMDC succumbed to infection within 4 days and did not appear to be a suitable model for investigating the *in vivo* migration of BMDC infected with *B. pseudomallei*. Therefore, the *in vivo* migration of BMDC was only investigated within the *B. pseudomallei*-resistant C57BL/6 mice. There is scope for future studies to investigate the dissemination of *B. pseudomallei* by tissue-resident DC in BALB/c mice infected with an appropriate low dose of bacteria. Overall, using the C57BL/6 model, *in vivo* migration of *B. pseudomallei*-infected BMDC was observed, which was similar to *E. coli* LPS-stimulated BMDC and significantly increased compared to unstimulated BMDC.

Despite relatively low numbers of migratory *B. pseudomallei*-infected BMDC, significant numbers of *B. pseudomallei* were isolated from spleen and lung of mice by 24 hr post-injection. Furthermore, the combined bacterial burden within popliteal and

inguinal LN, spleen and lung of mice was higher than the original number of intracellular *B. pseudomallei* surviving within the injected BMDC. Here, the total bacterial loads were determined in organs rather than the intracellular burden of DC within the organ. Therefore, it is not possible to distinguish whether *B. pseudomallei* replication has occurred within BMDC or after escape from the BMDC. However, these findings do demonstrate that DC migration served as a vehicle for dissemination of *B. pseudomallei.*

Bacteria that breach the skin are readily internalised by resident DC or recruited DC responding to inflammation. DC-pathogen interactions and inflammatory cytokines, such as TNF-α and IL-1 produced by keratinocytes, trigger the maturation and migration of DC from the site of infection to skin draining lymphoid organs via the CCR7/CCL19/CCL21 axis (Wang *et al.*, 1999; Comerford *et al.*, 2013). Although cutaneous inoculation is a major route of *B. pseudomallei* infection, the role of tissue-resident DC at the site of active *B. pseudomallei* infection has not been explored. In initial studies, *in vivo* migration assays demonstrated that *in vitro* cultured and infected BMDC migrate and traffic *B. pseudomallei* when injected into the skin of mice. To determine the *in vivo* relevance of these findings, the ability of tissue-resident skin DC at the site of infection to internalise *B. pseudomallei* and the subsequent dissemination of *B. pseudomallei* was investigated. Tissue-resident skin DC at the site of infection internalised *B. pseudomallei* and facilitated dissemination of *B. pseudomallei* to the pLN and spleen but not to the lungs. Migration of DC to secondary lymphoid tissues has been shown to facilitate dissemination of other intracellular bacteria, such as *S. typhimurium*. However, in mice infected with *S. typhimurium*, the draining mesenteric LN is able to restrict the dissemination of *S. typhimurium*-infected DC (Voedisch *et al.*, 2009). In contrast, the draining pLN was unable to contain and prevent the dissemination of *B. pseudomallei* to the spleen.

Subsequently, persistence and replication of *B. pseudomallei* within tissue-resident DC in the spleen was observed. This is consistent with the *in vivo* BMDC migration studies that suggested that *B. pseudomallei* may replicate within DC in the spleen. These

observations are consistent with the mechanisms utilised by *F. tularensis*, where DC migration facilitates dissemination of the bacteria to the draining lymph node leading to subsequent systemic dissemination and colonisation of multiple organs (Bar-Haim *et al.*, 2008). However, persistence and replication of *B. pseudomallei* within spleen DC *in vivo* contradicts our *in vitro* studies whereby murine BMDC were capable of killing intracellular *B. pseudomallei.* Previous *in vitro* studies using human and murine DC have consistently shown that DC grown under *in vitro* culture conditions and protected by antibiotics are capable of killing intracellular *B. pseudomallei* (Williams *et al.*, 2008; Horton *et al.*, 2012). However, in the *in vivo* studies of the current study, organ specific differences in the ability of DC to kill intracellular *B. pseudomallei* were observed *in vivo*, in particular within the spleen where DC were a site for *B. pseudomallei* persistence. This highlights the need for caution when interpreting *in vitro* studies and the requirement for subsequent studies using animal models to determine the true *in vivo* relevance of *in vitro* observations. Collectively, our data is the first to demonstrate internalisation of *B. pseudomallei* by tissue-resident DC in the skin the infection site and dissemination of *B. pseudomallei*-infected DC to secondary lymphoid tissues. Furthermore, DC within the spleen, but not within the skin infection site or draining pLN appear to provide an intracellular niche for *B. pseudomallei* persistence and replication. Further studies are warranted to determine which DC subset/s (migrating skin DC, resident spleen DC or both) facilitate *B. pseudomallei* persistence in the spleen.

Migration of activated DC to secondary lymphoid tissues is important for antigen presentation to naïve T cells and the development of pathogen-specific adaptive immune responses. However, the strength and type of immune responses activated vary depending on the maturation state of the infected DC, the subset of DC, bacterial factors that influence the antigen presentation of DC and whether the DC migrate to the appropriate T cell rich zones within the lymphoid tissue (Baldwin *et al.*, 2004; Muraille *et al.*, 2005; Coutanceau *et al.*, 2007; Aoshi *et al.*, 2008; Igyarto *et al.*, 2011; Horton *et al.*, 2012). Previous studies have also demonstrated that the magnitude of antigen-specific T cell responses is directly proportional to the number of migrated DC (Martìn-Fontecha *et al.*, 2003). In the current study, the percentage of migrated *B. pseudomallei*-

infected BMDC and the low *B. pseudomallei* alone dose (10^4 CFU) were inadequate for activating strong *B. pseudomallei*-specific T cell responses. Previous work comparing *B. pseudomallei*-specific T cell responses generated by 0.1 and 0.01 LD_{50} doses of *B. pseudomallei* also demonstrated that the strength of *B. pseudomallei*-specific T cell responses is directly proportional to the *B. pseudomallei* dose (Barnes, 2004). For this study, 10^4 CFU *B. pseudomallei* (NCTC 13179) were injected into C57BL/6 mice, equivalent to 0.0001 LD_{50}. Increasing the *B. pseudomallei* dose to 10^6 CFU (0.01 LD_{50}) correlated with increased bacterial burden of organs and significantly increased *B. pseudomallei*-specific T cells responses, which supports the previous work by Barnes (2004). Similar observations have been reported for other intracellular bacteria such as *S. typhimurium.* While a low dose of *S. typhimurium* is sufficient to cause infection and colonisation of the spleen, mice fail to develop a *S. typhimurium*-specific $CD4^+$ T cell response which is observed in mice infected with a higher dose of bacteria (Srinivasan *et al.*, 2004). Therefore, it appears that the strength of *B. pseudomallei*-specific T cell recall responses is proportional to the infectious dose of *B. pseudomallei* and the number of migrated *B. pseudomallei*-infected DC.

The inability of *B. pseudomallei*-infected BMDC to activate strong *B. pseudomallei*-specific T cell responses is contradictory to the findings of two previous studies, which used heat-killed *B. pseudomallei*-stimulated BMDC as vaccine vectors to activate protective *B. pseudomallei*-specific T cell responses in mice (Healey *et al.*, 2005; Elvin *et al.*, 2006). The differences in *B. pseudomallei*-specific T cell responses observed between this study and the DC immunisation studies published by Healy *et al.* (2005) and Elvin *et al*. (2006) are perhaps reflections of variations in experimental design. Healy *et al*. (2005) and Elvin *et al*. (2006) evaluated BMDC as vaccine vectors for *B. pseudomallei* and therefore used susceptible BALB/c mice and heat-killed *B. pseudomallei* to stimulate BMDC, with animals given a booster at day 28 after the primary vaccination. Whilst the DC immunisation studies were designed to maximise the use of DC as vaccine vectors for *B. pseudomallei*, the current study investigated the role of DC migration in protection against or facilitation of *B. pseudomallei* infection. Furthermore, the migratory capacity of *B. pseudomallei*-infected BMDC within different

mouse strains is an important consideration. Previous studies demonstrate that the functional responses of DC toward *B. pseudomallei in vitro* differ for *B. pseudomallei*-susceptible BALB/c mice compared to *B. pseudomallei*-resistant C57BL/6 mice (Williams *et al.*, 2008). The migratory capacity of DC infected with *L. major* is significantly different for BALB/c mice compared to C57BL/6 mice (Baldwin *et al.*, 2004; Misslitz *et al.*, 2004). Therefore, it is plausible that differences exist in *B. pseudomallei*-infected DC migration and subsequent T cell activation between BALB/c and C57BL/6 mice and will be an important avenue for further research.

The current study is the first to demonstrate that DC migration inadvertently acts as a mechanism for the systemic dissemination of *B. pseudomallei*. The findings of this study warrant additional investigations that employ different techniques to further define the role of DC in the development of protection as opposed to disease progression during *B. pseudomallei* infection. In the current study, the migration of *B. pseudomallei*-infected DC to secondary lymphoid tissues was confirmed. However, future immuno-histochemical analysis of lymphoid tissue would be beneficial to confirm that *B. pseudomallei*-infected DC migrate to appropriate T cell rich regions for activation of naïve T cells (Aoshi *et al.*, 2008). Ablation of DC or blocking of DC migration in *B. pseudomallei*-infected mice may also be useful for elucidating the contribution of DC to the development of protection compared to disease progression. How different *B. pseudomallei* antigen preparations affect antigen presentation and the type of CMI initiated by DC has not been investigated. Horton *et al.* (2012) demonstrated that live *B. pseudomallei* are not required to activate DC maturation however, it is not known if the viability of *B. pseudomallei* affects antigen presentation by DC (Horton *et al.*, 2012). Furthermore, intracellular bacteria such as *S. typhimurium* attempt to prevent mature DC from priming effector T cells by inducing apoptosis of the DC before it can present antigen (Biedzka-Sarek and El Skurnik, 2006; Fabrik *et al.*, 2013). Additional investigations of *B. pseudomallei* interactions with different DC subsets within the skin and secondary lymphoid tissues could provide important information on the type of adaptive immune responses activated, which would be informative for future vaccine design. In the current study, the role of tissue-resident DC was investigated by

comparing the total DC population to non-DC. Studies on *Candida albicans* skin infections have demonstrated that skin-resident DC subsets, identified by their unique phenotypes, activate different antigen-specific T cell responses by cytotoxic $CD8^+$ T cells or $CD4^+$ T cells and T helper 17 cells (Igyarto *et al.*, 2011). DC subsets also demonstrate differential susceptibility to infection with *L. major* and cytokine production which can lead to polarisation of different T_h1 or T_h2 immune responses (Baldwin *et al.*, 2004).

A limitation of this study is that extensive phenotyping of BMDC and tissue-resident DC was not performed. Although the BMDC culture method used in the current study is well established for generating a non-adherent high $CD11c^+$ cell with DC morphology, it does not achieve 100 % purity. Furthermore, whilst high CD11c expression is distinctive for DC, CD11c is not exclusively expressed on DC (Lutz *et al.*, 1999; Hashimoto *et al.*, 2011). Therefore, additional low frequencies of CD11c-expressing cell types, such as inflammatory macrophages, could potentially have been included within the tissue-resident DC fractions. In addition, without extensive DC phenotyping it is not possible to distinguish migratory DC from tissue resident DC in the current study. Since it is plausible that *B. pseudomallei* spreads from migratory skin DC to resident spleen DC, future studies employing techniques for more extensive phenotyping will enable determination of which DC subset/s (migratory skin DC, resident spleen DC or both) facilitate *B. pseudomallei* persistence in the spleen. Importantly, our findings identify $CD11c^+$ DC migration as a mechanism for *B. pseudomallei* cell-dependent dissemination. These findings warrant further characterisation studies, possibly using transgenic mice with specific green fluorescent protein (GFP) expression in DC or extensive characterisation of the DC phenotype, to confirm the beneficial as opposed to detrimental role of DC subsets and indeed other phagocytic cells involved in the *in vivo* trafficking of *B. pseudomallei.*

In summary, this study investigated the effect of *B. pseudomallei* on the *in vivo* migration of DC and their potential to inadvertently facilitate systemic dissemination of the bacteria. Following *B. pseudomallei* infection, BMDC demonstrated significant *in*

vivo migration compared to uninfected BMDC. Although DC are capable of killing intracellular *B. pseudomallei*, the findings of this study indicate that *B. pseudomallei* that persist within DC are disseminated to other organs via DC migration. Furthermore, *B. pseudomallei* replicated inside spleen DC in mice contradicting our *in vitro* studies, whereby murine BMDC were capable of killing intracellular *B. pseudomallei.* Previous *in vitro* studies using human and murine DC have consistently shown that DC grown under *in vitro* culture conditions and protected by antibiotics are capable of killing intracellular *B. pseudomallei* (Williams *et al.*, 2008; Horton *et al.*, 2012). However, in the current study, DC within the spleen were a site for *B. pseudomallei* persistence. This highlights the need for caution when interpreting *in vitro* studies and the requirement for subsequent studies using animal models to determine the true *in vivo* relevance of *in vitro* observations. Further studies are warranted to determine which DC subset/s facilitate *B. pseudomallei* persistence in the spleen. Overall, DC migration facilitated dissemination of *B. pseudomallei* to secondary lymphoid tissues. The migration of mature *B. pseudomallei*-infected DC to secondary lymphoid tissues suggests that these cells have the potential to present antigen to naïve T cells. However, *B. pseudomallei*-infected BMDC failed to activate a detectable *B. pseudomallei*-specific T cell response using the current experimental design. Given DC migration is central to development of protective adaptive immune responses to infection, further studies to clarify the contribution of DC migration to disease progression as opposed to the development of protection in *B. pseudomallei* infection are paramount and may facilitate identification of potential novel immunomodulatory therapies and preventative strategies.

CHAPTER 7
BURKHOLDERIA PSEUDOMALLEI-SPECIFIC T CELL RESPONSES IN PATIENTS DIAGNOSED WITH MELIOIDOSIS

7.1 Introduction

Adaptive cell-mediated immune (CMI) responses play an important role in protection against intracellular pathogens by providing pathogen-specific responses and the development of long term immunity (Murphy *et al.*, 2011). Antigen presenting cells, such as mature antigen-loaded dendritic cells (DC), activate adaptive immune responses by presenting antigen to naïve $CD4^+$ and $CD8^+$ T cells. Activated $CD8^+$ T cells direct their cytolytic activity against host cells infected with intracellular pathogens and produce pro-inflammatory cytokines, such as interferon (IFN) γ, to induce inflammation and recruit additional effector cells. Following activation, $CD4^+$ T cells differentiate into T helper (T_H) cells that can provide B cell help and $CD8^+$ T cell activation (Muraille and Leo, 1998). T_h cells are distinguished by the type of cytokines secreted, which subsequently results in polarised immune responses to a given pathogen (O'Shea and Paul, 2010). The original paradigm of T_h cells described the role of T_h1 and T_h2 cells in response to infection, where T_h1 cells produce IFN-γ along with other cytokines to activate the effector functions of inflammatory cells such as monocytes. Whilst T_h2 cells produce cytokines, such as interleukin (IL)-4 and IL-13, they also promote antibody production along with the activation of mast cells and eosinophils (O'Shea and Paul, 2010). T_h subsets have now been expanded to include T follicular helper (T_{fh}) cells, which produce IL-12 to support antibody production by B cells in response to T cell dependent antigen. T_h17 cells produce IL-17a and IL-17f during infection by activating neutrophils and macrophages and have been implicated as effector cells driving inflammation in diseases such as rheumatoid arthritis and Crohn's disease (Tesmer *et al.*, 2008; Korn *et al.*, 2009). T regulatory (T_{reg}: producing IL-10, IL-35 and transforming growth factor-β) have been demonstrated to play a role in establishing immune tolerance and regulating immune responses (Muraille and Leo, 1998; O'Shea and Paul, 2010).

Following infection with *B. pseudomallei,* the activation of adaptive CMI responses is important for protection against disease progression (Barnes *et al.*, 2004; Haque *et al.*, 2006b). *B. pseudomallei*-specific T cell responses were first demonstrated in patients who had recovered from culture-confirmed melioidosis by determining recall responses to *B. pseudomallei* antigen, *in vitro* (Ketheesan et al., 2002). Compared to healthy controls, recognition of *B. pseudomallei* antigen by memory T cells in peripheral blood from patients with a history of melioidosis led to increased T cell proliferation and IFN-γ production (Ketheesan *et al.*, 2002). This was the first study to demonstrate the potential role of T cells in protection against melioidosis and to identify the potential use of adaptive CMI assays for monitoring disease progression in patients.

The development of *B. pseudomallei*-specific CMI responses in healthy individuals with a history of *B. pseudomallei* exposure but who have never developed clinical melioidosis has also been investigated (Barnes *et al.*, 2004; Govan and Ketheesan, 2004). Potential exposure to *B. pseudomallei* is increased during severe weather events or activities associated with potentially contaminated soil and water in endemic regions (Howe *et al.*, 1971; Currie *et al.*, 2004). During the Vietnam War, it is highly possible that soldiers were repeatedly exposed to *B. pseudomallei* contaminated soil and water. This is supported by the findings that healthy Vietnam veterans demonstrated strong *B. pseudomallei*-specific T cell responses 30 years post-exposure, despite having no clinical history of melioidosis (Govan and Ketheesan, 2004). Similarly, following an outbreak of culture-confirmed melioidosis in Papua New Guinea, *B. pseudomallei*-specific T cell responses were demonstrated in both patients with melioidosis and also in their healthy relatives (Barnes *et al.,* 2004). Importantly, the relatives who may have been exposed to *B. pseudomallei* but had not history of clinical melioidosis demonstrated significantly stronger *B. pseudomallei*-specific T cell responses compared to the patients with melioidosis (Barnes *et al.*, 2004). Tippayawat *et al.* (2009) provided evidence that both indirect haemagglutination assay (IHA)-positive patients who have recovered from culture confirmed melioidosis and healthy individuals who are IHA-positive but have no history of clinical melioidosis, develop protective *B. pseudomallei*-specific T cell responses which are mediated by terminally differentiated effector-

memory T cells (T_{EMRA}) of mixed $CD4^+$ and $CD8^+$ subsets. When rechallenged *in vitro* with purified *B. pseudomallei* antigen or DC stimulated with *B. pseudomallei* antigen, these T_{EMRA} cells release IFN-γ and the level of IFN-γ released was found to correlate with IHA titre (Tippayawat *et al.*, 2009; Tippayawat *et al.*, 2011). Collectively, these studies indicate that the development of a strong *B. pseudomallei*-specific T cell recall response following exposure to *B. pseudomallei* provides protection against the development of clinical melioidosis (Barnes *et al.*, 2004; Govan and Ketheesan, 2004).

Commercially developed antibody detections assays are routinely used in clinical settings to aid diagnosis of infectious diseases, often for diseases that are difficult to isolate or culture from clinical specimens such as syphilis (Ratnam, 2005). CMI response-based assays provide an alternative, though less common format for diagnosing infectious diseases. The QuantiFERON®-TB Gold is a commercial CMI response-based assay that is used for the diagnosis of tuberculosis. The QuantiFERON®-TB Gold is an enzyme-linked immunosorbent assay (ELISA) that detects the release of IFN-γ in fresh heparinised whole blood following incubation with synthetic proteins simulating *M. tuberculosis* early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10; Mori *et al.*, 2004). As *M. tuberculosis* is an intracellular pathogen, host CMI responses are crucial for control of *M. tuberculosis* infection. Similar to *B. pseudomallei*-infected hosts, individuals with active or latent tuberculosis develop *M. tuberculosis*-specific T cells which produced IFN-γ upon re-stimulation. Therefore, QuantiFERON®-TB Gold assay can be used to identify individuals who have developed *M. tuberculosis*-specific T cells, from active or latent *M. tuberculosis* infection, by high IFN-γ release in blood samples stimulated with *M. tuberculosis* antigens (Figure 7.1).

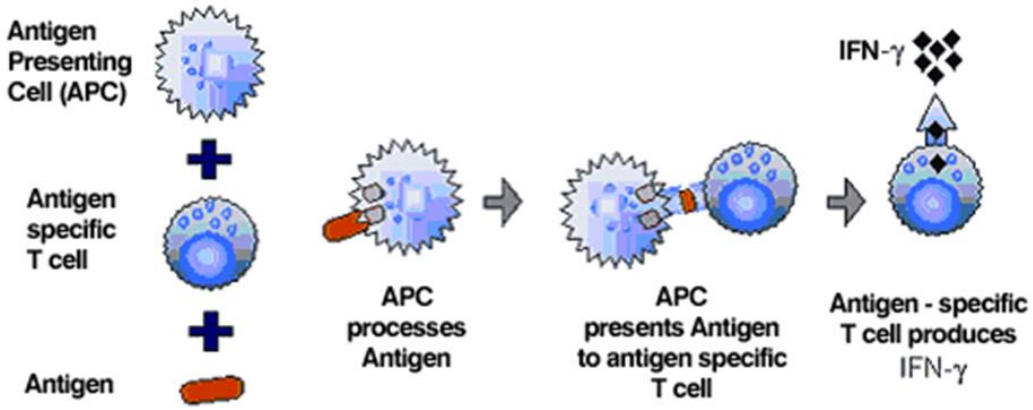

Figure 7.1 Principles of the QuantiFERON®-TB Gold
Heparinised blood samples are mixed with stimulatory antigens (synthetic *M. tuberculosis* proteins). In individuals with active or latent tuberculosis, antigen presenting cells present the antigens to *M. tuberculosis*-specific T cells which stimulates the release of high levels of IFN-γ. High IFN-γ release is indicative of active or latent infection as blood samples from uninfected individuals with no *M. tuberculosis*-specific T cells will produce low (non-specific) levels of IFN-γ (Adapted from Stanford University, 2014).

Currently, diagnosis of melioidosis is confirmed by bacterial culture of clinical specimens (Peacock *et al.*, 2005). The IHA is widely used as a diagnostic aid to detect *B. pseudomallei*-specific antibodies (Cheng *et al.*, 2006a). However, the IHA provides results with variable sensitivity (73 %) and specificity (64 %; Chantratita *et al.*, 2007c). In North Queensland, only 51 % of patients with culture-confirmed melioidosis have positive IHA serology (IHA-positive patients) on admission (Harris *et al.*, 2009). On subsequent testing, the majority of patients with initial negative IHA serology demonstrate IHA-conversion by 1 month. However, approximately 10 % of patients with melioidosis remain IHA negative when retested up to 55 months later. This cohort of patients with melioidosis that have negative IHA serology on admission and on subsequent testing (minimum 1 month post-discharge) are defined as persistently IHA-negative patients. Whilst an initially negative IHA result was correlated with melioidosis bacteraemia, there are no specific patient factors that correlate with persistently negative IHA serology (Harris *et al.*, 2009). Although negative IHA serology at initial diagnosis with melioidosis may be possible, a persistently negative

IHA result during the course of infection and following recovery seems less likely (Harris *et al.*, 2009).

Presumably, persistently negative serology observed for IHA-negative patients is attributed to limitations associated with the IHA, rather than a lack of *B. pseudomallei*-specific immune responses (Harris *et al.*, 2009). Importantly, the IHA is a poor rapid diagnostic aid since it is not indicative of active infection, rather positive results simply reflect evidence of prior exposure to *B. pseudomallei* (Ketheesan *et al.*, 2002). There is also no standardised protocol for conducting the IHA or for the preparation of *B. pseudomallei* antigen for the assay between laboratories (Norton *et al.*, 2012). Furthermore, the level of antibody responses in a patient with melioidosis is not predictive of disease progression, nor outcome following infection (Ho *et al.*, 1997). Despite evidence for the importance of adaptive CMI responses in protection against *B. pseudomallei*, assessment of *B. pseudomallei*-specific T cell responses in patients with melioidosis to demonstrate exposure and monitor disease progression in clinical human melioidosis is underutilised. The adaptive CMI response of persistently IHA-negative patients with melioidosis has not been investigated. It is predicted that the use of adaptive CMI assays would be beneficial to clinicians monitoring IHA-negative patients with melioidosis. Such an assay would also have a place as a rapid diagnostic aid and for monitoring the development of protective adaptive immune responses in patients with melioidosis, similar to the Quanti-FERON®-TB Gold assay used to diagnose *M. tuberculosis* infection. Therefore, this study aimed to compare the *B. pseudomallei*-specific T cell response of IHA-negative patients who have recovered from melioidosis and healthy controls.

The specific aims of this study were to:

i) Demonstrate the development of *B. pseudomallei*-specific T cell responses in IHA-negative patients who have recovered from culture-confirmed melioidosis
ii) Investigate whether the *B. pseudomallei*-specific T cell response of IHA-negative patients is $CD4^+$ or $CD8^+$ T cell dominant

iii) Determine if the *B. pseudomallei*-specific T cells produce IFN-γ upon antigen recall challenge *in vitro*

7.2 Materials and Methods

7.2.1 Isolation and culture of human peripheral blood mononuclear cells

7.2.1.1 Study participants

Between January 1996 and January 2010, a total of 14 persistently IHA-negative patients with culture-confirmed melioidosis were treated at the Townsville Hospital (JCU Human Ethics Approval #H4470 and Queensland Health Townsville Hospital and Health Services Ethical Approval #71/04). Of these, seven IHA-negative patients were contactable and volunteered to participate in the study. One patient was later excluded due to treatment of an unrelated medical condition with an immune-suppressive agent, cyclosporin. The six IHA-negative patients (2 males, 4 females) all had a history of culture-confirmed melioidosis (mean time since discharge, 8.6 ± 4.9 years) but had recovered and no longer demonstrated clinical evidence of melioidosis (Table 7.1). All six IHA-negative patients have demonstrated persistently negative IHA serology for melioidosis (personal communication with Dr Robert Norton, The Townsville Hospital, Townsville, Australia) and were IHA-negative at the time of this study (Table 4.2). The control group consisted of age and sex matched healthy individuals (n=6) with no history of melioidosis and negative IHA serology. Peripheral blood (25 ml) from IHA-negative patients and controls was collected in lithium heparin (4 tubes for peripheral blood mononuclear cell (PBMC) isolation) and clot activator (1 tube for serum separation) vacutainers (BD Biosciences, North Ryde, Australia) by a trained phlebotomist as previously described (Section 3.2).

Table 7.1 IHA-negative patient clinical features

Patient	Age* (Years)	Gender	Admitted (Year)	IHA Titre
1	54	Female	1997	<5
2	47	Male	2008	<5
3	72	Male	2002	<5
4	52	Female	2002	<5
5	52	Female	1996	<5
6	48	Female	2004	<5

Note: Data supplied by Dr Robert Norton, The Townsville Hospital, Townsville, Australia. * Age on admission.

7.2.1.2 Indirect haemagglutination assay

Serum from all IHA-negative patients and controls was tested for *B. pseudomallei* antibodies using the IHA to re-confirm their IHA titre. Assays were performed according to previously published methods (Ashdown, 1987; Lazzaroni *et al.*, 2008; Harris *et al.*, 2009). Red blood cells (RBC) were isolated from 20 ml peripheral blood of sheep (kindly provided by the College of Public Health, Medical and Veterinary Sciences, JCU, Townsville, Australia) freshly collected in sodium citrate vacutainers (BD Biosciences, North Ryde, Australia). RBC were prepared by washing with saline solution (10 ml, Appendix 1) three times (1000 G, 20 min), resuspended in saline to obtain a 10 % RBC suspension. Prepared RBC were diluted (750 µl 10 % RBC added to 9.25 ml saline) then "sensitised" by incubating with IHA antigen (6.7 µl/ml; kindly provided by Dr Robert Norton, The Townsville Hospital, Townsville, Australia) for 1 hr at 37 °C. Non-sensitised RBC were incubated in saline without IHA antigen and used as a negative control for the assay. Sensitised and non-sensitised RBC were then washed with saline three times (10 min, 500g) and resuspended in 10 ml saline. Serum from patients and controls (100 µl) along with control serum (known IHA-positive and IHA-negative kindly provided by Dr Robert Norton, The Townsville Hospital, Townsville, Australia) was diluted in saline (300 µl) and heat-inactivated by incubating in a water bath at 54 °C for 30 min. Cooled serum was pre-absorbed by incubating with 10 % RBC (100 µl) for 5 min at room temperature then centrifuged to remove RBC (5000 G,

5 min). Serum samples (25 µl) were serially diluted 2-fold in saline in a micro-titre plate (Nunc, Thermo Fisher Scientific Pty Ltd, Scoresby, Australia) then incubated with sensitised or non-sensitised RBC (25 µl) for 4 hr at room temperature. The highest dilution where haemagglutination occurred for each individual was recorded as the IHA titre (Table 7.2). A titre of ≤1:40 is considered negative (Lazzaroni *et al.*, 2008; Harris *et al.*, 2009). All serum from IHA-negative patients and controls were negative for *B. pseudomallei* antibodies according to the IHA (Table 7.3).

Table 7.2 IHA results for IHA-negative patients and controls at the time of study

Patient	IHA Titre	Control	IHA Titre
1	<5	1	<5
2	<5	2	<5
3	<5	3	<5
4	<5	4	1:5
5	<5	5	1:10
6	<5	6	1:5

7.2.1.3 Isolation of PBMC

Peripheral blood mononuclear cells were isolated by density centrifugation using Ficoll-Paque (Section 3.4.3). Isolated PBMC were cultured using single strength culture media (Appendix 1) in 96 well plates (10^5 cells/well) for assessing T cell proliferation (Section 7.2.3) or in 24 well plates (10^5 cells/well) for assessing T cell subset activation (Section 7.2.4) and IFN-γ cytokine production and secretion (Section 7.2.5).

7.2.2 Preparation of *B. pseudomallei* lysates

A panel of eight *B. pseudomallei* lysates were prepared from eight clinical *B. pseudomallei* isolates. The six clinical isolates cultured from the IHA-negative patients during their admission were obtained from the Townsville Hospital collection (Section 3.3.2). Two other characterised and sequenced isolates, NCTC 13178 (high virulence) and NCTC 13179 (low virulence; Section 3.3.1) were also included since they

have previously been used to assess CMI response to *B. pseudomallei* (Ketheesan *et al.*, 2002; Govan and Ketheesan, 2004; Barnes and Ketheesan, 2007; Lazzaroni *et al.*, 2008). These lysates are a crude cocktail of *B. pseudomallei* antigens prepared by sonication of bacteria as previously described (Section 3.3.6). Each of the lysates were numbered as shown in the Table 7.3.

Table 7.3 *B. pseudomallei* lysates

Lysate #	*B. pseudomallei* isolate
1	From Patient 1
2	From Patient 2
3	From Patient 3
4	From Patient 4
5	From Patient 5
6	From Patient 6
78	NCTC 13178
79	NCTC 13179

7.2.3 Detection of T cell recall proliferation in response to *B. pseudomallei* lysate

Triplicate wells of PBMC isolated from IHA-negative patients and controls were stimulated with the eight different *B. pseudomallei* lysates (1µg/ml) and phytohaemagglutinin (PHA, positive control; 10µg/ml; Appendix 1) according to previously published methods (Barnes *et al.*, 2004; Lazzaroni *et al.*, 2008). Cultures were then incubated at 37 °C with 5 % CO_2. On days 4-7 of culture, proliferation was determined by measuring H^3-thymidine incorporation (0.25 µCi/well for 4 hr; Appendix 1). From the data generated (counts per minute (CPM)), stimulation indices (SI) were calculated (Appendix 3). The highest SI recorded over the four time points was selected and expressed as the mean maximum SI (± SEM) to compare the proliferative response of T cells from IHA-negative patients to controls.

7.2.4 Analysis of T cell subset activation in response to *B. pseudomallei* lysate

Isolated PBMC from IHA-negative patients and controls were stimulated in triplicate wells with *B. pseudomallei* lysates (prepared from NCTC 13179 or the Patient's own isolate) or phorbol myristate acetate (PMA, 50ng/ml; Sigma-Aldrich, Sydney, Australia) and calcium ionomycin (1µg/ml; Sigma-Aldrich, Sydney, Australia) as a positive control then incubated overnight at 37 °C with 5 % CO_2 according to previously published methods (Lazzaroni *et al.*, 2008). PBMC were phenotyped as activated $CD4^+$ ($CD3^+/CD4^+/CD69^+$) or $CD8^+$ ($CD3^+/CD8^+/CD69^+$) T cells using a combination of the following monoclonal antibodies (1µg/10^6 cells each; BD Biosciences, North Ryde, Australia): anti-human CD3 PerCP (isotype mouse $IgG_{1\kappa}$), anti-human CD69 PE (isotype mouse $IgG_{1\kappa}$) and anti-human CD4 FITC isotype mouse $IgG_{1\kappa}$) or anti-human CD8 FITC (isotype mouse $IgG_{1\kappa}$); or appropriate isotype controls. PBMC were stained with the appropriate antibody combination and incubated on ice for 30 mins. Following labelling, PBMC were washed with sodium azide buffer (SAB; Appendix 1), resuspended in 2 % paraformaldehyde (ProSciTech, Kirwan, Australia) and analysed by flow cytometry as previously described (Chapter 3.5). The percentage of activated $CD4^+$ and $CD8^+$ T cells was determined and data expressed as the change in activation (% ± SEM) above unstimulated cells.

7.2.5 Assessment of IFN-γ cytokine production by PBMC stimulated with *B. pseudomallei* lysate

The culture supernatants of PBMC from IHA-negative patients and controls stimulated with either *B. pseudomallei* lysates (NCTC 13179 or Patient's own isolate) or PMA and calcium ionomycin for determining T cell subset activation (Section 7.2.4) was collected after overnight culture and stored at -80 °C. The quantity of IFN-γ in cell free culture supernatants was later determined using a cytometric bead array (CBA™) human IFN-γ cytokine kit (BD Biosciences, North Ryde, Australia) according to the manufacturer's instructions. Data was expressed as the concentration (pg/ml ± SEM) of IFN-γ in the culture supernatant.

7.2.6 Statistical analysis

Statistical analysis of data was performed using GraphPad Prism 6 Software and reported in Appendix 2. Comparison of *B. pseudomallei*-specific T cell proliferation, T cell subset activation and IFN-γ production between patients and controls in response to different stimulants was analysed for significance using a 2way ANOVA with recommended Sidak's multiple comparisons posthoc test.

7.3 Results

7.3.1 *B. pseudomallei*-specific T cell response of persistently IHA-negative patients

In response to *in vitro* stimulation with *B. pseudomallei* lysate, T cells from IHA-negative patients demonstrated significantly increased *B. pseudomallei*-specific proliferation compared to controls (Figure 7.2). T cells from IHA-negative patients proliferated in response to the lysate prepared from their corresponding *B. pseudomallei* isolate, as well as to other *B. pseudomallei* isolates. Together, these findings provide evidence of *B. pseudomallei*-specific T cell memory in persistently IHA-negative patients who have recovered from melioidosis. In addition, comparable *B. pseudomallei*-specific recall responses were observed, regardless of the *B. pseudomallei* isolate used for antigen stimulation.

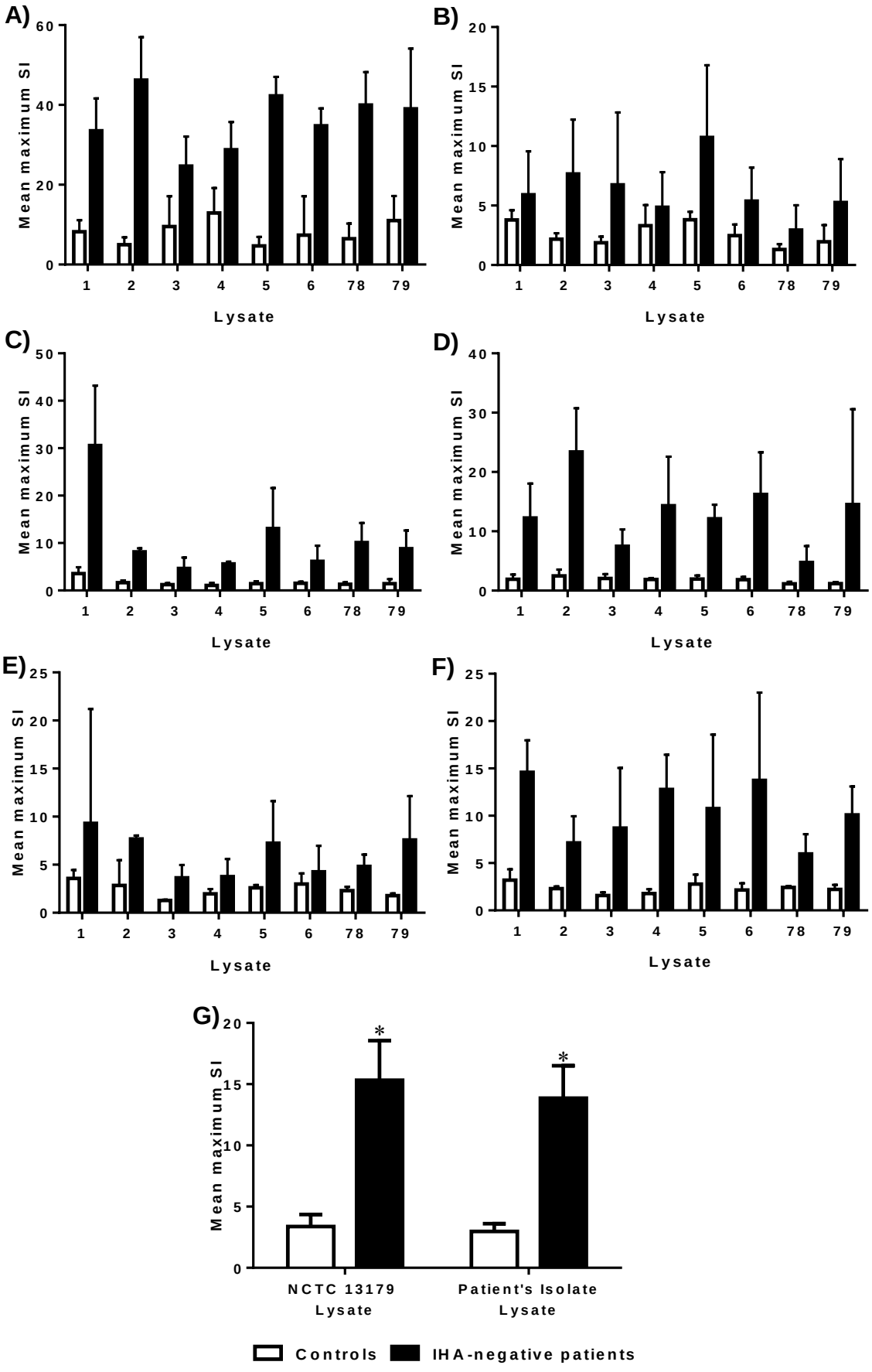
A)
B)
C)
D)
E)
F)
G)
Mean maximum SI
Lysate
1
2
3
4
5
6
78
79
NCTC 13179 Lysate
Patient's Isolate Lysate
Controls
IHA-negative patients
*

Figure 7.2 T cell proliferation from IHA-negative patients in response to *B. pseudomallei* lysates
Proliferation of T cells from IHA-negative patients and controls in response to *B. pseudomallei* lysates was assessed by determining H^3-thymidine incorporation. *B. pseudomallei*-specific proliferation of T cells from each of the IHA-negative patients was higher compared to T cells from controls (Graphs A to F depict the individual results for IHA-negative patients 1 to 6 with their matched control). G) Combined patient results. *B. pseudomallei*-specific T cell recall responses from IHA-negative patients were similar for *B. pseudomallei* lysates prepared from the patient's own bacterial isolate or from NCTC 13179. Bars depict the mean ± SD (A-F) and the mean ± SEM (G) of six experiments with one patient and control. * = $P \leq 0.05$, determined using a 2way ANOVA with multiple comparisons tests.

7.3.2 Activation of *B. pseudomallei*-specific $CD4^+$ and $CD8^+$ T cell subsets in IHA-negative patients

Previous studies have suggested that $CD4^+$ T cells are the dominant T cell subset responding to *B. pseudomallei* infection (Haque *et al.*, 2006b). Therefore, the activation of $CD4^+$ and $CD8^+$ T cells in response to *in vitro* stimulation with *B. pseudomallei* lysate was assessed by flow cytometry. In comparison to controls, blood from IHA-negative patients demonstrated increased activation of $CD4^+$ T cell following stimulation with *B. pseudomallei* lysate (Figure 7.3A). In contrast, expression of the T cell activation marker, CD69, was decreased on $CD8^+$ T cells from IHA-negative patients following stimulation with *B. pseudomallei* lysate (Figure 7.3B). Consistent with proliferation data, similar trends in $CD8^+$ and $CD4^+$ T cell activation were observed by T cells from IHA-negative patients following stimulation with antigens derived from the patient's own *B. pseudomallei* isolate or NCTC 13179.

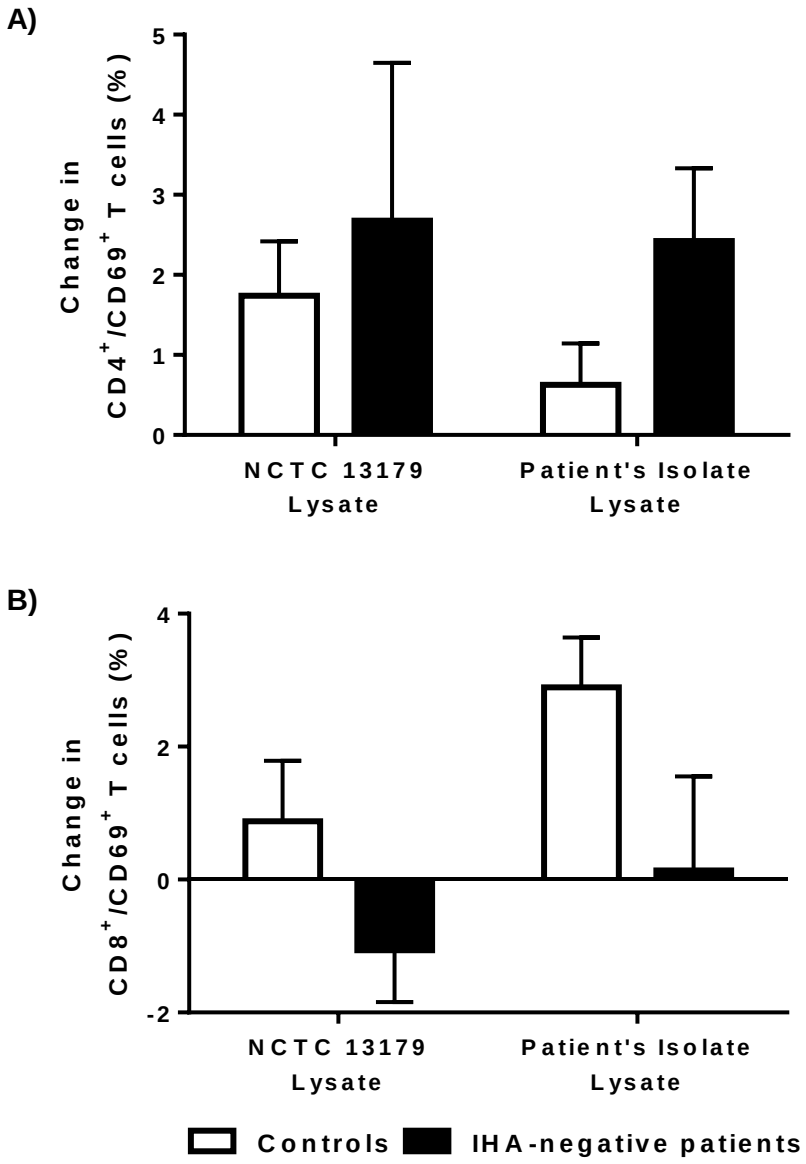

Figure 7.3 $CD4^+$ and $CD8^+$ T cell subset activation in response to *B. pseudomallei* lysate

The activation of A) $CD4^+$ and B) $CD8^+$ T cells from IHA-negative patients and controls following stimulation with *B. pseudomallei* lysates was assessed. IHA-negative patients exhibited a dominant $CD4^+$ memory T cell response demonstrated by increased $CD4^+$ (A) and decreased $CD8^+$ (B) T cell activation when challenged with *B. pseudomallei* antigen *in vitro*, although these trends were not significant. Bars depict the mean ± SEM of six experiments with one patient and control.

7.3.3 IFN-γ cytokine production by *B. pseudomallei*-specific T cells from IHA-negative patients

A strong IFN-γ response by antigen-specific T cells is important for clearance of *B. pseudomallei* infection through promotion of inflammation and activation of bacterial killing mechanisms by immune cells such as macrophages. Therefore, the culture supernatants of T cells from IHA-negative patients and controls following stimulation with *B. pseudomallei* lysate (NCTC 13179) was assessed for IFN-γ concentration. In response to *in vitro* stimulation with *B. pseudomallei* lysate, T cells from IHA-negative patients released more *B. pseudomallei*-specific IFN-γ compared to controls (Figure 7.4).

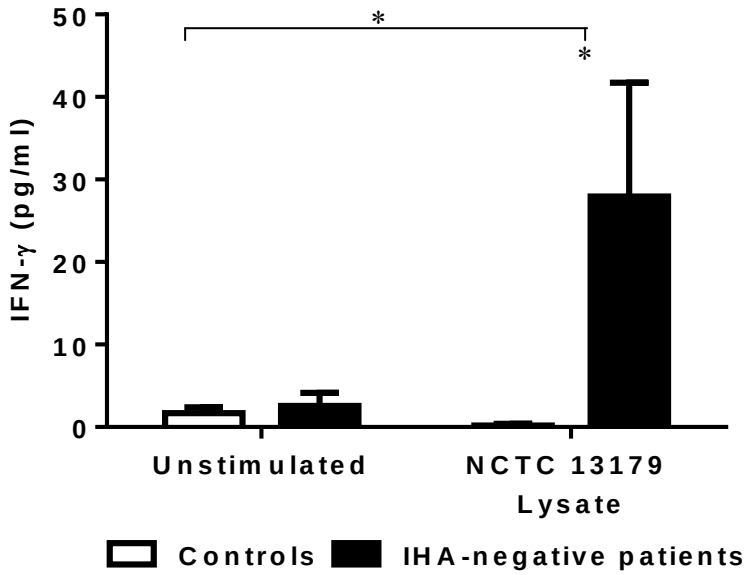

Figure 7.4 *B. pseudomallei*-specific IFN-γ release by T cells from IHA-negative patients
The concentration of IFN-γ in culture supernatants of T cells from IHA-negative patients and controls following stimulation with *B. pseudomallei* lysate prepared from the *B. pseudomallei* isolate NCTC 13179 was quantified using a CBA™ human IFN-γ cytokine kit. *B. pseudomallei*-specific T cells from IHA-negative patients stimulated with *B. pseudomallei* lysate demonstrated significantly increased IFN-γ production in comparison to T cells from controls. Bars depict the mean ± SEM of six experiments with one patient and control. * = P≤ 0.05, determined using a 2way ANOVA with multiple comparisons tests.

7.4 Discussion

Currently the IHA is the most commonly used diagnostic aid for demonstrating *B. pseudomallei* exposure in conjunction with bacterial culture. Although the IHA is a rapid test compared to culture, positive IHA test results are not indicative of active melioidosis, as infected patients may not have developed an antibody response if tested to early after infection. Furthermore, patients previously exposed to *B. pseudomallei* but who have recovered and cleared the infection will test IHA positive. In addition, the techniques used for the IHA and preparation of antigen are poorly standardised, thus the IHA is notorious for providing results with poor sensitivity and specificity (Norton *et al.*, 2012). Consequently, the IHA fails to detect *B. pseudomallei*-specific antibody responses in approximately 10 % of patients with culture-confirmed melioidosis (Harris *et al.*, 2009). These IHA-negative patients demonstrate persistently negative IHA results on admission, during infection and post-recovery from melioidosis. Therefore, the current study investigated *B. pseudomallei*-specific T cell responses in the peripheral blood of IHA-negative patients, who have recovered from culture-confirmed melioidosis, as an alternative method for demonstrating exposure and the development of adaptive immune responses to *B. pseudomallei*. T cell proliferation in response to *B. pseudomallei* antigen confirmed that IHA-negative patients had indeed developed a *B. pseudomallei*-specific T cell memory.

Published studies on the adaptive CMI responses in patients with melioidosis have previously used T cell proliferation assays to demonstrate the development of *B. pseudomallei*-specific T cell memory (Ketheesan *et al.*, 2002; Barnes *et al.*, 2004; Govan and Ketheesan, 2004). These studies confirmed that patients who have recovered from melioidosis (culture-confirmed, IHA-positive) or who have been exposed to melioidosis, develop *B. pseudomallei*-specific T cells. Therefore, the current study employed the T cell proliferation assay to determine whether *B. pseudomallei*-specific T cell memory could be demonstrated in IHA-negative patients who have recovered from melioidosis. Despite being persistently IHA-negative, T cells from IHA-negative patients who had recovered from melioidosis demonstrated strong *B. pseudomallei*-specific proliferation following *in vitro* challenge with *B. pseudomallei* antigen. In fact,

IHA-negative patients have a *B. pseudomallei*-specific T cell recall response to antigen preparations derived from different *B. pseudomallei* isolates. These findings support the hypothesis that IHA-negative patients have developed an adaptive immune response to *B. pseudomallei*, including a *B. pseudomallei*-specific antibody response. Rather, an unknown inherent flaw exists for the IHA that prevents the assay for detecting *B. pseudomallei*-specific antibody responses in a subset of melioidosis patients. Recently, improved immunoassay formats were developed that demonstrate the presence of antibodies against *B. pseudomallei* LPS in the serum of IHA-negative patients (Cooper *et al.*, 2013; Sorenson *et al.*, 2013). The 2-dimensional immunoarray developed not only remarkably improved sensitivity (100 %) and specificity (87.1 %) but was able to distinguish the LPS genotype of the *B. pseudomallei* infecting strain. Interestingly, although Australian *B. pseudomallei* isolates predominantly display LPS genotype A, only 50 % of IHA-negative patients had been infected with LPS genotype A *B. pseudomallei* strains. The findings of Cooper *et al*. (2013) and Sorenson *et al.* (2013) highlights the implications of *B. pseudomallei* strain types when preparing antigens for antibody detection assays. Notably, the findings of the current study demonstrate that strain type does not influence *B. pseudomallei*-specific T cell recall responses. This novel finding is advantageous and provides further impetus for the development of a CMI-based diagnostic assay for melioidosis.

In response to intracellular bacterial infections, $CD4^+$ and $CD8^+$ T cell subsets provide distinct effector functions. Activated $CD8^+$ T cells elicit direct cytotoxic effects against infected cells. Studies have demonstrated that the activation of cytolytic effector function by $CD8^+$ T cells is essential for acquired long-term immunity against intracellular bacteria such as *Listeria monocytogenes* (Bajenoff *et al.*, 2010; Henry *et al.*, 2010). In contrast, activated $CD4^+$ T cells produce distinct cytokine profiles that polarise effector immune responses specific for the pathogen. During bacterial infections with microbes such as *Mycobacterium tuberculosis*, $CD4^+$ T cells play a critical role in the control of the bacteria and the development of protection against recrudescence (Kaufmann *et al.*, 2005). The importance of $CD4^+$ T cell immunity against *M. tuberculosis* is highlighted by the observation that individuals with

$CD4^+$ T cell deficiency, such as HIV-induced T cell depletion, are unable to control *M. tuberculosis* and develop active infection (Geldmacher *et al.*, 2012). In previous studies by Ketheesan *et al.* (2002) and Tippayawat *et al.* (2009), patients who had recovered from culture-confirmed melioidosis demonstrated a mixed $CD4^+$ and $CD8^+$ T cell recall response to *B. pseudomallei* antigens (Ketheesan *et al.*, 2002).

Murine models of melioidosis have enabled further characterisation of the role of T cells in protection against *B. pseudomallei* infection (Haque *et al.*, 2006b; Barnes and Ketheesan, 2007). Mice with T cell deficiency demonstrated significantly increased mortality following *B. pseudomallei* infection (Haque *et al.*, 2006b). The ability to selectively deplete $CD4^+$ or $CD8^+$ T cells in mice enabled investigation of the role of T cell subsets in protection against *B. pseudomallei* infection. Depletion of $CD4^+$ T cells, but not $CD8^+$ T cells, significantly decreased the mean survival time of *B. pseudomallei* infected mice, confirming that $CD4^+$ T cells were essential for protection against *B. pseudomallei* (Haque *et al.*, 2006b). In the current study, $CD4^+$ T cells were the predominant T cell subset driving the *B. pseudomallei*-specific T cell recall response of IHA-negative patients. IHA-negative patients demonstrated an increase in $CD4^+$ T cell activation and a decrease in $CD8^+$ T cell activation in response to stimulation with *B. pseudomallei* antigen.

Activated $CD4^+$ T cells differentiate into T_h cells that can promote macrophage and neutrophil bactericidal activity, provide B cell help and activate $CD8^+$ T cell cytotoxicity (Muraille and Leo, 1998; O'Shea and Paul, 2010). The cytokines produced by different subsets of T_h cells play a pivotal role by polarising immune responses to a particular pathogen (O'Shea and Paul, 2010). In response to intracellular bacterial infections, T_h1 cells produce IFN-γ along with other cytokines that activate inflammatory responses. In the current study, *B. pseudomallei*-specific T cells from IHA-negative patients were observed to produce IFN-γ upon reactivation with *B. pseudomallei* antigen. Assessment of IFN-γ production by T cells in response to stimulation with *B. pseudomallei* antigen may therefore be a potential strategy for confirming exposure and monitoring the development of protective CMI responses in patients with

melioidosis. Similar diagnostic assays are currently used, such as the QuantiFERON TB Gold assay that is used to confirm prior exposure to *Mycobacterium tuberculosis* (Mori *et al*., 2004). Identification of purified antigens for stimulating *B. pseudomallei*-specific IFN-γ release would be preferable to using a crude lysate antigen, like the current study, to develop an IFN-γ release-based assay for melioidosis. Although Tippayawat *et al.* (2009) alluded to the use of T cell assays for identifying candidate vaccine antigens; their study may in fact have inadvertently identified potential antigens for a *B. pseudomallei*-specific CMI response assay. Tippayawat *et al.* (2009) found IFN-γ release was higher in IHA-positive patients who have recovered from melioidosis compared to healthy controls using both used an ELISA and ELISPOT assay. However, only the ELISA format was able to detect significant difference in IFN-γ release between IHA-positive patients who have recovered from clinical melioidosis compared to IHA-positive controls who have presumably been exposed but have not developed clinical melioidosis. Following on from determining antigen and assay formats, confirmation of the assay sensitivity and specificity would be paramount. Initial investigations using 'spike' samples would be useful to test the sensitivity of the assay and demonstrate that the assay does not cross-react with other *Burkholderia* species. Should the assay prove to be both highly sensitive and specific for *B. pseudomallei,* subsequent investigation using clinical samples from patients with *B. pseudomallei*-culture proven melioidosis, patients with non-melioidosis bacterial infections and healthy patient serum in *B. pseudomallei* endemic regions would then be required. Future studies to determine the efficacy of a *B. pseudomallei*-specific IFN-γ release-based assay to predict patient outcome by testing patient samples collected sequentially at admission and at regular intervals during the recovery would also be advantageous.

In summary, the adaptive CMI responses of IHA-negative patients predominantly consisted of $CD4^+$ T cells with IFN-γ production. IHA-negative patients have developed *B. pseudomallei*-specific T cell memory that is cross-reactive as demonstrated by the ability of patient memory T cells to be reactivated by lysates produced from different *B. pseudomallei* isolates. Overall, the current study demonstrates that in response to *B. pseudomallei*, IHA-negative patients developed a protective $CD4^+$ T_h1 type adaptive

immune response that upon recall challenge produce IFN-γ. These findings are in accordance with previously published data (Ketheesan *et al.*, 2002; Barnes, 2004). Given the limitations of the IHA, together with the findings of this study, assessment of *B. pseudomallei*-specific CMI responses is an underutilised assay for investigating the development of adaptive immune responses specific for *B. pseudomallei* in patients with melioidosis. Efforts focussed on the development of an IFN-γ release based CMI assay to demonstrate exposure to *B. pseudomallei* and to monitor patient recovery would be beneficial.

CHAPTER 8
GENERAL DISCUSSION

Since melioidosis was first described by Whitmore and Krishnaswami in 1911, the incidence and distribution of the disease has increased worldwide (Dance, 2000). Melioidosis is now considered an important infectious disease in tropical regions and is highly endemic in Northern Australia and Thailand. As soil and surface water in tropical regions is conducive for environmental persistence of *B. pseudomallei*, the lack of proven endemicity in other tropical regions probably reflects the inability to diagnose patients with melioidosis or detect environmental *B. pseudomallei* (Currie et al., 2008). Confirmation of endemicity relies upon case reporting and detection of environmental *B. pseudomallei*, both of which require specialised skill sets, including clinical awareness of melioidosis, diagnostic tools and skilled laboratory technicians to confirm patient diagnosis, along with soil and water testing to identify the presence of environmental *B. pseudomallei.* The presence of this infectious disease in tropical regions lacking appropriate protocols for identifying, diagnosing and treating melioidosis is a serious cause for concern, particularly since high mortality rates are observed despite clinician awareness in known endemic regions such as Thailand (Chaowagul *et al.*, 1989; Currie *et al.*, 2000b; Limmathurotsakul *et al.*, 2010). A number of factors contribute to the high mortality rates associated with melioidosis, such as the use of non-standardised and unreliable diagnostic assays, delays in culture-confirmation of presumptive, resistance of *B. pseudomallei* to common antimicrobials used for treating Gram-negative bacterial sepsis and a lack in understanding the mechanisms contributing to the rapid progression of melioidosis to a fulminating systemic disease (Wuthiekanun *et al.*, 2004; Cheng and Currie, 2005). Furthermore, *B. pseudomallei* is an efficient intracellular pathogen that can invade and persist within host cells. Thus the activation of host cell-mediated immune responses is crucial for controlling the infection (Lazar Adler *et al.*, 2009).

Dendritic cells are important immune cells that play a central role in the development of protective immunity (Steinman, 2008). Immature DC stationed throughout peripheral tissues or circulating within the blood, express a wide variety of pattern recognition

receptors (PRR) which enables them to recognise and internalise invading pathogens (Banchereau *et al.*, 2000). The subsequent maturation (up-regulation of antigen presenting molecules, co-stimulatory molecules and cytokine production) of pathogen-activated DC and migration to secondary lymphoid tissue plays a crucial role in the activation and polarisation of adaptive immune responses (Villadangos and Schnorrer, 2007). Different DC subsets, identified by their unique phenotype, contribute to the complexity of DC function by exhibiting preferential effector functions (Heath and Carbone, 2009). Thus the role of DC during infection is multifactorial by providing inflammatory responses, antigen presentation, T cell activation and also modulation of other innate and adaptive immune cells via cytokine signalling. While a limited number of studies have determined *in vitro* responses of conventional DC to *B. pseudomallei*, the role of DC and different DC subsets during *B. pseudomallei* infection is still largely undefined (Charoensap *et al.*, 2008; Williams *et al.*, 2008; Charoensap *et al.*, 2009; Horton *et al.*, 2012).

Plasmacytoid DC (pDC) are a subset of DC that specialise in rapidly producing large quantities of type I interferons (IFN), leading to the activation of NK and cytotoxic T cell cytolytic activity and modulation of other immune cells, such as other DC and macrophages to produce pro-inflammatory cytokines (Decker *et al.*, 2005). However, excessive type I IFN production has the potential to overstimulate anti-bacterial responses, causing pathological damage and inflammatory syndromes in the host (Reizis *et al.*, 2011a). Although excessive pro-inflammatory cytokine production is observed in patients with melioidosis sepsis, the signalling pathways driving this detrimental imbalance are undefined (Wiersinga *et al.*, 2007a). The findings of the current study are the first to describe the bactericidal activity of pDC against *B. pseudomallei*. Interestingly, an association was observed between increased type I IFN production and murine pDC generated from *B. pseudomallei*-susceptible BALB/c mice which develop an acute form of melioidosis similar to patients with melioidosis septic shock.

These findings provide novel insight into the role of pDC during *B. pseudomallei* infection and suggest that pDC and type I IFN may contribute to the hyper-inflammatory

immune responses that underlie the susceptibility of BALB/c mice toward *B. pseudomallei*. However, suitable *in vivo* studies are required to determine the true relevance these findings, including determination of whether pDC are recruited to the site of *B. pseudomallei* infection in resistant (C57BL/6) and susceptible (BALB/c) hosts and whether ablation of pDC negatively or positively impacts on disease progression. There is also accumulating evidence that pDC are not limited to immunomodulation but also develop a mature phenotype with the attributes of professional antigen presenting cells similar to conventional DC (Villadangos and Schnorrer, 2007). Furthermore, pDC have been shown to infiltrate *Legionella pneumophila* infection sites where they contribute to bacterial control without producing type I IFN (Ang *et al.*, 2010). As the effector function of pDC no longer appears to be limited to type I IFN production, the ability of pDC to modulate other innate immune cells during the early phases of *B. pseudomallei* infection and subsequently activate *B. pseudomallei*-specific T cell responses also requires investigation.

The central role of DC in both the modulation of inflammatory responses and the activation and tailoring of adaptive immune responses make them an ideal target for therapeutic and vaccine strategies (Steinman, 2008). Early studies by Healey *et al.* (2005) and Elvin *et al.* (2006) employed DC as vaccine vectors for *B. pseudomallei* antigens. Tippayawat *et al.* (2011) also utilised DC as vectors to deliver potential vaccine antigen to T cells *in vitro*. However, the use of DC as a vaccine vector or the development of strategies to target *B. pseudomallei* vaccine antigens to DC *in vivo* have failed to progress due to our limited knowledge of the role of DC and different DC subsets during *B. pseudomallei* infection and the signalling pathways that trigger the development of protective immune responses. Our understanding of DC and *B. pseudomallei* interactions is limited to a relatively small number of *in vitro* studies, which demonstrate that highly phagocytic, immature DC internalise and kill *B. pseudomallei*, triggering DC maturation and increased pro-inflammatory cytokine production (Williams *et al.*, 2008; Charoensap *et al.*, 2009; Horton *et al.*, 2012). Although these studies demonstrate maturation of *B. pseudomallei*-infected DC, which is crucial for efficient activation of naïve T cells, DC-*B. pseudomallei* studies have not

investigated the migration capacity of these mature, antigen-loaded DC which is equally important for T cell activation (Comerford *et al.*, 2013). Modulation or interference of DC migration, as observed in *Yersinia pestis*-infected DC, affects the ability of the host to mount a protective immune response (Velan *et al.*, 2006). In the current study, it was demonstrated that *B. pseudomallei* did not impair DC maturation or migration *in vitro*, rather migratory DC were found to harbour live bacteria. Based on these observations, it was hypothesised that *B. pseudomallei* would not impair the ability of DC to migrate *in vivo* to secondary lymphoid organs for antigen presentation and activation of *B. pseudomallei*-specific T cell responses but may contribute to the dissemination of *B. pseudomallei*.

Evidence of other intracellular bacteria, such as *Francisella tularensis* and *Listeria monocytogenes*, using DC as a 'Trojan Horse' to facilitate systemic dissemination has been reported (Pron *et al.*, 2001; Bar-Haim *et al.*, 2008). The factors that contribute to the rapid progression of *B. pseudomallei* from a localised infection to septicaemia and infection of multiple organs are largely unknown. It was recently reported that the transmigration of adoptively transferred, *B. pseudomallei*-infected monocyte/neutrophils enabled the bacteria to colonise the brain of mice (Liu *et al.*, 2013). In the current study, the *in vivo* migration of *B. pseudomallei*-infected DC was found to inadvertently facilitate systemic dissemination of the bacteria, enabling *B. pseudomallei* colonisation of the spleen. Importantly, these studies suggest inadvertent trafficking of *B. pseudomallei* by migrating DC may be a key mechanism underlying the rapid progression from a localised to systemic form of the disease.

It is tempting to speculate that therapeutic strategies targeting the migratory function of DC may be beneficial by limiting the dissemination of *B. pseudomallei*. Other studies have found that inhibiting DC migration is beneficial for hosts infected with *Pseudomonas aeruginosa*, *F. tularensis* or *Streptococcus pneumoniae* by resulting in DC accumulation at the site of infection, reducing the bacterial burden and limiting bacterial dissemination (Bar-Haim *et al.*, 2008; Eppert *et al.*, 2010; Rosendahl *et al.*, 2013). However, DC migration to secondary lymphoid tissue is also crucial for initiation of

protective adaptive immune responses. For example, following infection with *L. monocytogenes* or *M. tuberculosis*, impaired DC migration is detrimental as the host fails to activate protective T cells, resulting in increased bacterial burden and susceptibility to infection (Kursar *et al.*, 2005; Olmos *et al.*, 2010). Therefore, further investigation of the contribution of DC migration in breaking the control of balance between disease progression and the development of protection is paramount.

Future investigation of *B. pseudomallei* interactions with different DC subsets, to determine which subset/s play a central role at the site of infection and the dissemination of *B. pseudomallei*, could also provide important information on the type of adaptive immune responses that are initiated following infection with *B. pseudomallei*. Although, additional *in vivo* investigations were outside the scope of the current study, analysis of DC phenotype to differentiate DC subsets may have identified a particular DC subset involved in the dissemination of *B. pseudomallei* or activating protective *B. pseudomallei*-specific T cells. The ability of DC to present antigen and the type of T cell responses activated by DC exposed to live *B. pseudomallei* compared to ingestion of apoptotic *B. pseudomallei*-infected cells also requires investigation. Other studies suggest that ingestion of apoptotic, infected neutrophils suppresses antigen presentation by DC (Ribeiro-Gomes *et al.*, 2012). This is an unexplored but interesting avenue for future research as neutrophils are rapidly recruited to sites of *B. pseudomallei* infection (Easton *et al.*, 2007).

Understanding the host-pathogen interactions during *B. pseudomallei* infection will not only enable strategic development of improved therapeutics and efficacy of vaccines to reduce the mortality rates associated with melioidosis, they will be instrumental for vaccine development. However, early and accurate diagnosis of melioidosis is also critical for improving patient prognosis, which is currently hampered by unreliable diagnostic assays, that are low in specificity and sensitivity, and delays in culture-confirmation of presumptive diagnosis. The development of a rapid diagnostic assay that can accurately confirm active *B. pseudomallei* infection faces a number of additional challenges associated with the poor socioeconomic status of countries where

this disease is endemic. The assay also needs to be cost effective to obtain, have a stable shelf life and require minimal training and specialised equipment.

The IHA is a cost-effective diagnostic aid commonly used to detect antibodies in patient serum as a means of demonstrating exposure to *B. pseudomallei*. However, the IHA is known to have considerable limitations, including the inability to differentiate between active infection and past exposure to *B. pseudomallei,* has poor sensitivity and specificity, and persistently fails to detect antibodies in a subset of patients with culture-confirmed melioidosis, "IHA-negative patients" (Cheng *et al.*, 2006a; Harris *et al.*, 2009). Furthermore, the *B. pseudomallei*-specific antibody response of a patient with melioidosis is not predictive of clinical outcome (Ho *et al.*, 1997). Evidence that the development of a strong T cell response is protective in infected individuals and possibly protects an exposed individual from developing clinical melioidosis, suggests that the assessment of *B. pseudomallei*-specific T cell responses would be useful to demonstrate exposure and monitor recovery in patients with melioidosis (Ketheesan *et al.*, 2002; Barnes *et al.*, 2004; Govan and Ketheesan, 2004). In the current study, *B. pseudomallei*-specific T cell recall responses in IHA-negative patients were assessed to determine whether such an assay had benefits over *B. pseudomallei*-specific antibody detection for the confirmation of previous exposure to *B. pseudomallei*. Upon recall challenge *ex vivo*, IHA-negative patients demonstrated a *B. pseudomallei*-specific T cell memory response with a strong IFN-γ release. These findings provide evidence that T cell assays are superior to the IHA for demonstrating *B. pseudomallei* exposure and the development of adaptive immunity, especially in the 10 % of patients with melioidosis who are persistently IHA-negative.

Studies by Tippayawat *et al.* (2009) and Tippayawat *et al.* (2011) used similar T cell assays to demonstrate that terminally differentiated T memory cells from patients who have recovered from melioidosis produce IFN-γ when rechallenged *in vitro* with *B. pseudomallei* antigens. Although, Tippayawat *et al.* (2009) alluded to the use of T cell assays for identifying candidate vaccine antigens, we propose that T cell assays may be beneficial in a clinical setting. The development of a commercially available

IFN-γ detection-based assay to demonstrate exposure and the development of adaptive immune responses in patients with melioidosis would be beneficial and potentially predictive of clinical outcome. A similar assay, the QuantiFERON-TB Gold, is currently used for qualitatively diagnosing exposure to tuberculosis. The format of the commercially produced QuantiFERON-TB Gold, that is approved for use by the U. S. Food and Drug Administration, could be modified for melioidosis using *B. pseudomallei* antigens. However, to generate interest for the commercial production of a melioidosis-specific CMI assay requires further investigations to first refine appropriate T cell antigens from the crude *B. pseudomallei* lysate used in the current study. Subsequently, the development of an 'in house' version that can be adopted by clinicians to demonstrate the clinical suitability and potential of the assay to distinguish active from past infections and/or predict patient outcome based on the strength of the patient's *B. pseudomallei*-specific T cell-mediated IFN–γ response. Thus, a melioidosis-specific CMI assay may continue to be beneficial for monitoring and predicting patient outcome, even as rapid detection assays for *B. pseudomallei* infection become available.

In summary, the collective findings of the current study have contributed significant advancements in our understanding of the host-pathogen interactions during *B. pseudomallei* infection. A novel role for specialised type I IFN-producing pDC that internalise and kill *B. pseudomallei in vitro* was identified. Previous studies to define the innate immune responses to *B. pseudomallei* have overlooked the involvement of pDC. The findings of the current study along, with increasing evidence describing both beneficial and detrimental roles for pDC and type I IFN production suggest the need for future studies on their ability to modulate immune responses during *B. pseudomallei* infection. Furthermore, the current study is the first to demonstrate that migratory DC facilitate *B. pseudomallei* dissemination and contribute to improving our knowledge on the interactions between DC and *B. pseudomallei.* Unlike some intracellular bacteria, *B. pseudomallei* does not impair DC maturation or migration. Rather, the bacterium hijacks DC migration to facilitate systemic dissemination. Future studies using methods for ablation of DC, blocking of DC migration or phenotyping of DC would enable determination of the role of DC migration and the contribution of different DC subsets

in dissemination, as opposed to the development of protective immune responses against *B. pseudomallei*. The current study has also confirmed the utility of T cell assays for demonstrating the development of protective adaptive immune responses in patients with melioidosis, particularly when current antibody based tests such as the IHA fail to detect *B. pseudomallei*-specific antibodies. Given the central role of CMI response for the control of *B. pseudomallei* infection, it is anticipated that there will be growing interest in research focused on characterising the key cell types involved in initiating T cell responses, as well as the utilisation of this knowledge for improving strategies for vaccination, therapeutic management and diagnosis. The findings of the current study support research into the development of an IFN-γ based detection assay for confirming exposure and monitoring the recovery of patients with melioidosis.

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APPENDIX 1

REAGENTS

A1.1 Agars

A1.1.1 Ashdown agar

Agar Technical No. 3 (Oxiod Limited, Thermo Fisher Scientific, Adelaide, Aust.)	12 g
Tryptone (Oxiod Limited, Thermo Fisher Scientific, Adelaide, Aust.)	12 g
Glycerol (Merck Chemicals Ltd, Victoria, Aust.)	32 ml
Crystal violet – 0.1 % aqueous	4 ml
Neutral red – 1 % aqueous	4 ml
Single Distilled Water	800 ml

Combine ingredients and boil for 15mins. Autoclave at 121 °C for 15 min, cool to 50 °C and add 6.4 ml of 1 mg/ml of gentamicin. Pour into petri plates and store at 4 °C.

A1.1.2 Blood agar

Blood agar base no.2 (Oxiod Limited, Thermo Fisher Scientific, Adelaide, Aust.)	39.5 g
dH_2O	1000 ml
Blood (sheep) (James Cook University, Townsville, Aust.)	35 ml

Combine agar and water then heat with frequent agitation and boil for one minute to completely dissolve the medium. Autoclave at 121 °C for 15 min. Cool to 45 – 50 °C then add 5-10 % (v/v) of sterile defibrinated blood. Pour into petri plates and store at 4 °C.

A1.2 Cell Culture Media and Reagents

A1.2.1 β(2)-Mercaptoethanol (55 mM)

β(2)-Mercaptoethanol (14.2M) (Sigma-Aldrich, Sydney, Aust.)	38.5 µl
diH_2O	9.97 ml

Aliquot into 1 ml microfuge tubes and store at -70 °C. For DC media, add 500 µl to 500 ml RPMI to give final concentration of 50 µM.

A1.2.2 Concanavalin A

Concanavalin A (Sigma-Aldrich, Sydney, Aust.)	5 mg
RPMI 1640 (Invitrogen, Mulgrave, Aust.)	50 ml

Combine, filter sterilise using 0.22 μm filter, aliquot into 1 ml microfuge tubes then store at -70 °C. For use, add 10 μl to 200 μl cell cultures to give a final concentration of 2 μg/ml.

A1.2.3 Culture media - Ag8653 myeloma

RPMI-1640 (Invitrogen, Mulgrave, Aust.)	
Penicillin (Invitrogen, Mulgrave, Aust.)	100 U/ml
Streptomycin (Invitrogen, Mulgrave, Aust.)	100 μl/ml
L-Glutamine (Sigma-Aldrich, Sydney, Aust.)	2 mM
HEPES buffer (Gibco, Life Technologies Australia Pty Ltd, Mulgrave, Aust.)	2.5 mM
HI-FBS (Invitrogen, Mulgrave, Aust.)	7.5 %

A1.2.4 Culture media - BMDC media

RPMI-1640 (Invitrogen, Mulgrave, Aust.)	
Penicillin (Invitrogen, Mulgrave, Aust.)	100 U/ml
Streptomycin (Invitrogen, Mulgrave, Aust.)	100 μl/ml
L-Glutamine (Sigma-Aldrich, Sydney, Aust.)	2 mM
β-Mercaptoethanol (Sigma-Aldrich, Sydney, Aust.)	50 μM
HI-FBS (Invitrogen, Mulgrave, Aust.)	10 %

A1.2.5 Culture media - double strength

RPMI-1640 (Invitrogen, Mulgrave, Aust.)	
Penicillin (Invitrogen, Mulgrave, Aust.)	100 U/ml
Streptomycin (Invitrogen, Mulgrave, Aust.)	100 µl/ml
L-Glutamine (Sigma-Aldrich, Sydney, Aust.)	4 mM
HEPES buffer (Gibco, Life Technologies Australia Pty Ltd, Mulgrave, Aust.)	5 mM
HI-FBS (Invitrogen, Mulgrave, Aust.)	20 %

A1.2.6 Culture media - pDC media

RPMI-1640 (Invitrogen, Mulgrave, Aust.)	
Penicillin (Invitrogen, Mulgrave, Aust.)	100 U/ml
Streptomycin (Invitrogen, Mulgrave, Aust.)	100 µl/ml
L-Glutamine (Sigma-Aldrich, Sydney, Aust.)	2 mM
β-Mercaptoethanol (Sigma-Aldrich, Sydney, Aust.)	50 µM
HEPES buffer (Gibco, Life Technologies Australia Pty Ltd, Mulgrave, Australia)	2.5 mM
HI-FBS (Invitrogen, Mulgrave, Aust.)	10 %

A1.2.7 Culture media - single strength

RPMI-1640 (Invitrogen, Mulgrave, Aust.)	
Penicillin (Invitrogen, Mulgrave, Aust.)	100 U/ml
Streptomycin (Invitrogen, Mulgrave, Aust.)	100 µl/ml
L-Glutamine (Sigma-Aldrich, Sydney, Aust.)	2 mM
HEPES buffer (Gibco, Life Technologies Australia Pty Ltd, Mulgrave, Australia)	2.5 mM
HI-FBS (Invitrogen, Mulgrave, Aust.)	10 %

A1.2.8 *Escherichia coli* lipopolysaccharide (LPS, 1 mg/ml)

E. coli LPS (Sigma-Aldrich, Sydney, Aust.)	5 mg
RPMI (Invitrogen, Mulgrave, Aust.)	5 ml

Combine, filter sterilise using 0.22 μm filter, aliquot into 1 ml microfuge tubes then store at -70 °C. For use, add 10 μl (stock) to 10 ml RPMI to give 1 μg/ml then add 10 μl to 200 μl cell cultures to give a final concentration of 50 ng/ml.

A1.2.9 Heat-inactivated foetal bovine serum (HI-FBS)

Foetal bovine serum (FBS) (Sigma-Aldrich, Sydney, Aust.)	500 ml

Aliquot into 10 ml tubes then incubate tubes in 56 °C water bath for 25 min. Cool to room temperature then store at -20 °C.

A1.2.10 ^{3}H-Thymidine stock solution

^{3}H-Thymidine (26Ci/mM) (GE Healthcare Australia Pty Ltd, Rydalmere, Aust.)	1 ml
PBS, pH7.2	39 ml

Combine and store at 4 ºC. Use 10 μl stock solution in 200 μl cell cultures to give a final concentration of 0.25 μCi/well.

A1.2.11 Kanamycin (1 mg/ml)

Stock (10 mg/ml) (Sigma-Aldrich, Sydney, Aust.)	10 ml
PBS	90 ml

Combine, filter sterilise using 0.22 μm filter and aliquot into 10 ml tubes. Store at -20 °C. Add to culture to give final concentration of 250 μg/ml.

A1.2.12 L-Glutamine (15 μg/ml)

L-Glutamine (Sigma-Aldrich, Sydney, Aust.)	0.6 g
RPMI (Invitrogen, Mulgrave, Aust.)	40 ml

Combine, filter sterilise using 0.22 μm filter and aliquot into 5 ml tubes. Store at -20 °C.

A1.2.13 Phytohaemagglutinin

PHA lectin (Sigma-Aldrich, Sydney, Aust.)	5 mg
RPMI 1640 (Invitrogen, Mulgrave, Aust.)	25 ml

Combine, filter sterilise using 0.22 μm filter, aliquot into 1 ml microfuge tubes and store at -70 °C. For use, add 10 μl to 200 μl cell cultures to give a final concentration of 10 μg/ml.

A1.2.14 RPMI (pen/strep)

RPMI-1640 (Invitrogen, Mulgrave, Aust.)	
Penicillin (Invitrogen, Mulgrave, Aust.)	100 U/ml
Streptomycin (Invitrogen, Mulgrave, Aust.)	100 μl/ml

Combine ingredients and store at 4 °C.

A1.3 General Solutions

A1.3.1 Breaking buffer

Leupeptin (Sigma-Aldrich, Sydney, Aust.)	0.2 μg/ml
Pepstatin (Sigma-Aldrich, Sydney, Aust.)	0.2 μg/ml
DNase (Sigma-Aldrich, Sydney, Aust.)	50 Kuntz units

Combine ingredients in sterile PBS, aliquot into 1 ml microfuge tubes and store at -20 °C

A1.3.2 Crystal violet 0.1 %

Crystal Violet (Sigma-Aldrich, Sydney, Aust.)	0.1 g
dH_2O	100 ml

Combine and autoclave at 121°C for 15 min.

A1.3.2 Gentamicin (1 mg/ml)

Gentamicin solution (10 mg/ml) (Invitrogen, Mulgrave, Aust.)	10 ml
dH_2O	90 ml

Combine and filter sterilise using 0.22 μm filter. Store at 4 °C.

A1.3.3 Neutral red 1 %

Neutral Red (Sigma-Aldrich, Sydney, Aust.)	1 g
dH_2O	100 ml

Combine and autoclave at 121 °C for 15 min.

A1.3.4 Phosphate buffered saline (PBS) pH 7.2

NaCl (Sigma-Aldrich, Sydney, Aust.)	8 g
Na_2HPO_4 (Merck Chemicals Ltd, Victoria, Aust.)	0.64 g
KCl (Sigma-Aldrich, Sydney, Aust.)	0.20 g
KH_2PO_4 (Sigma-Aldrich, Sydney, Aust.)	0.16 g

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

A1.3.5 Saline 0.9 %

NaCl (Sigma-Aldrich, Sydney, Aust.)	0.9 g
dH_2O	100 ml

Combine ingredients and autoclave at 121 °C for 15 min.

A1.3.6 Sodium azide buffer

PBS, pH 7.2	
HI-FBS	1 %
Sodium Azide (Ajax Finechem, Thermo Fisher Scientific, Adelaide, Aust.)	0.1 % (w/v)

Combine ingredients and store at 4 °C.

A1.3.7 Sodium citrate buffer

Tri-sodium citrate (Sigma-Aldrich, Sydney, Aust.)	147 mg
0.9 % Saline	50 ml

Combine ingredients and adjust to pH 4.5. Autoclave at 121 °C for 15 min.

A1.3.8 TNT buffer

Tris-HCL, pH7.5 (Merck Chemicals Ltd, Victoria, Aust.)	0.1 M
NaCl (Sigma-Aldrich, Sydney, Aust.)	0.15 M
Tween20 (Sigma-Aldrich, Sydney, Aust.)	0.05%

Combine ingredients in dH_2O.

A1.3.9 Triton-X (0.1 %)

Triton-X (Sigma-Aldrich, Sydney, Aust.)	100 µl
dH_2O	99.9 ml

Combine and autoclave at 121 °C for 15 min.

APPENDIX 2

STATISTICAL ANALYSES

A2.1 Statistical Analysis of Data from Chapter 4

A2.1.1 *B. pseudomallei* internalisation by human pDC – Figure 4.8

Ordinary 1way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Donor 1 vs. Donor 2	2.728	1.25	1.478	-0.1517 to 3.108	0.0815
Donor 1 vs. Donor 3	2.728	1.94	0.7883	-1.208 to 2.785	0.6675
Donor 1 vs. Donor 4	2.728	1.887	0.8417	-1.155 to 2.838	0.6218
Donor 2 vs. Donor 3	1.25	1.94	-0.69	-2.686 to 1.306	0.7494
Donor 2 vs. Donor 4	1.25	1.887	-0.6367	-2.633 to 1.360	0.7911
Donor 3 vs. Donor 4	1.94	1.887	0.05333	-2.252 to 2.359	0.9999

A2.1.2 *B. pseudomallei* internalisation by murine pDC & cDC – Figure 4.9

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
NCTC 13178 - NCTC 13179					
C57BL/6 pDC	26200	4022	22178	-11635 to 55990	0.337
BALB/c pDC	24615	8756	15859	-17953 to 49672	0.6553
C57BL/6 cDC	29363	5015	24348	-9464 to 58161	0.2506
BALB/c cDC	50830	9356	41474	7662 to 75287	0.0101

2way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
NCTC 13178					
C57BL/6 pDC vs. BALB/c pDC	26200	24615	1585	-37514 to 40685	> 0.9999
C57BL/6 pDC vs. C57BL/6 cDC	26200	29363	-3163	-42262 to 35936	> 0.9999
C57BL/6 pDC vs. BALB/c cDC	26200	50830	-24630	-63729 to 14470	0.5621
BALB/c pDC vs. C57BL/6 cDC	24615	29363	-4748	-43848 to 34351	> 0.9999
BALB/c pDC vs. BALB/c cDC	24615	50830	-26215	-65314 to 12885	0.4678
C57BL/6 cDC vs. BALB/c cDC	29363	50830	-21467	-60566 to 17633	0.7484
NCTC 13179					
C57BL/6 pDC vs. BALB/c pDC	4022	8756	-4733	-43833 to 34366	> 0.9999
C57BL/6 pDC vs. C57BL/6 cDC	4022	5015	-992.6	-40092 to 38107	> 0.9999
C57BL/6 pDC vs. BALB/c cDC	4022	9356	-5333	-44433 to 33766	> 0.9999
BALB/c pDC vs. C57BL/6 cDC	8756	5015	3741	-35359 to 42840	> 0.9999
BALB/c pDC vs. BALB/c cDC	8756	9356	-600	-39699 to 38499	> 0.9999
C57BL/6 cDC vs. BALB/c cDC	5015	9356	-4341	-43440 to 34759	> 0.9999

A2.1.3 Intracellular survival of *B. pseudomallei* in murine pDC & cDC – Figure 4.10

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
NCTC 13178 - NCTC 13179					
C57BL/6 pDC	187.8	231.1	-43.33	-1650 to 1563	> 0.9999
BALB/c pDC	1383	581.1	802.2	-804.2 to 2409	0.5909
C57BL/6 cDC	674.4	807.8	-133.3	-1740 to 1473	0.9992
BALB/c cDC	3139	2111	1028	-578.6 to 2634	0.3526

2way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
NCTC 13178					
C57BL/6 pDC vs. BALB/c pDC	187.8	1383	-1196	-2900 to 509.1	0.3072
C57BL/6 pDC vs. C57BL/6 cDC	187.8	674.4	-486.7	-2191 to 1218	0.9672
C57BL/6 pDC vs. BALB/c cDC	187.8	3139	-2951	-4656 to -1246	0.0001
BALB/c pDC vs. C57BL/6 cDC	1383	674.4	708.9	-995.8 to 2414	0.8312
BALB/c pDC vs. BALB/c cDC	1383	3139	-1756	-3460 to -50.87	0.0406
C57BL/6 cDC vs. BALB/c cDC	674.4	3139	-2464	-4169 to -759.8	0.0016
NCTC 13179					
C57BL/6 pDC vs. BALB/c pDC	231.1	581.1	-350	-2055 to 1355	0.9939
C57BL/6 pDC vs. C57BL/6 cDC	231.1	807.8	-576.7	-2281 to 1128	0.9278
C57BL/6 pDC vs. BALB/c cDC	231.1	2111	-1880	-3585 to -175.3	0.0239
BALB/c pDC vs. C57BL/6 cDC	581.1	807.8	-226.7	-1931 to 1478	0.9995
BALB/c pDC vs. BALB/c cDC	581.1	2111	-1530	-3235 to 174.7	0.0993
C57BL/6 cDC vs. BALB/c cDC	807.8	2111	-1303	-3008 to 401.4	0.2203

A2.1.4 IFN-α produced by human pDC – Figure 4.11A

Ordinary 1way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Uninfected vs. CpG ODN 2216	69	2941	-2872	-4441 to -1303	0.0016
Uninfected vs. NCTC 13178	69	183	-114	-1683 to 1455	0.9776
CpG ODN 2216vs. NCTC 13178	2941	183	2758	1189 to 4327	0.0022

A2.1.5 IFN-β produced by human pDC – Figure 4.11B

Ordinary 1way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Uninfected vs. CpG ODN 2216	93.72	1017	-923.4	-1645 to -201.5	0.015
Uninfected vs. NCTC 13178	93.72	141.3	-47.6	-769.4 to 674.2	0.9815
CpG ODN 2216 vs. NCTC 13178	1017	141.3	875.8	153.9 to 1598	0.0198

A2.1.6 IFN-α produced by uninfected pDC – Table 4.2

Mann Whitney test

Column B vs. Column A	BALB/c pDC vs. C57BL/6 pDC
Mann Whitney test	
P value	0.0108
Exact or approximate P value?	Exact
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	69 , 51
Mann-Whitney U	6
Difference between medians	
Median of column A	60.11, n=6
Median of column B	0.0, n=9
Difference: Actual	-60.11
Difference: Hodges-Lehmann	-48.44

A2.1.7 IFN-β produced by uninfected pDC – Table 4.2

Mann Whitney test

Column B vs. Column A	BALB/c pDC vs. C57BL/6 pDC
Mann Whitney test	
P value	0.3
Exact or approximate P value?	Exact
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	13 , 8
Mann-Whitney U	2
Difference between medians	
Median of column A	2.000, n=3
Median of column B	0.0, n=3
Difference: Actual	-2
Difference: Hodges-Lehmann	-2

A2.1.8 IFN-α produced by murine pDC – Figure 4.12A

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
C57BL/6 pDC - BALB/c pDC					
CpG ODN 2216	42.23	22.49	19.74	-10.97 to 50.45	0.313
NCTC 13178	2.066	23.76	-21.69	-47.72 to 4.328	0.1273
NCTC 13179	0.4782	34.81	-34.34	-60.36 to -8.314	0.0062

2way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
C57BL/6 pDC					
NCTC 13178 vs. CpG ODN 2216	-40.17	11.71	-40.17	-68.59 to -11.74	0.0038
NCTC 13179 vs. CpG ODN 2216	-41.76	11.71	-41.76	-70.18 to -13.33	0.0026
NCTC 13179 vs. NCTC 13178	-1.588	10.47	-1.588	-27.01 to 23.84	0.9874
BALB/c pDC					
NCTC 13178 vs. CpG ODN 2216	1.266	11.2	1.266	-25.92 to 28.45	0.993
NCTC 13179 vs. CpG ODN 2216	12.32	11.2	12.32	-14.86 to 39.50	0.5191
NCTC 13179 vs. NCTC 13178	11.05	10.47	11.05	-14.37 to 36.48	0.5466

A2.1.9 IFN-β produced by murine pDC – Figure 4.12B

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
C57BL/6 pDC - BALB/c pDC					
CpG ODN 2216	46.9	16	30.9	-19.25 to 81.04	0.3035
NCTC 13178	78.96	60.53	18.43	-31.72 to 68.58	0.6975
NCTC 13179	17.14	85.31	-68.17	-118.3 to -18.02	0.0081

2way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
C57BL/6 pDC					
NCTC 13178 vs. CpG ODN 2216	78.96	46.9	32.06	-24.80 to 88.92	0.4753
NCTC 13179 vs. CpG ODN 2216	17.14	46.9	-29.76	-86.62 to 27.10	0.5546
NCTC 13179 vs. NCTC 13178	17.14	78.96	-61.82	-118.7 to -4.959	0.0304
BALB/c pDC					
NCTC 13178 vs. CpG ODN 2216	60.53	16	44.53	-12.33 to 101.4	0.1672
NCTC 13179 vs. CpG ODN 2216	85.31	16	69.3	12.44 to 126.2	0.0143
NCTC 13179 vs. NCTC 13178	85.31	60.53	24.77	-32.09 to 81.63	0.7303

A2.1.10 Change in MHC class II expression on murine pDC – Figure 4.15A

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
C57BL/6 pDC - BALB/c pDC					
CpG ODN 2216	6.478	2.929	3.549	-5.283 to 12.38	0.6911
NCTC 13178	-1.6	-6.1	4.5	-4.016 to 13.02	0.4812
NCTC 13179	2.822	-5.95	8.772	0.2559 to 17.29	0.0417

2way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
C57BL/6 pDC					
CpG ODN 2216 vs. NCTC 13178	6.478	-1.6	8.078	0.003952 to 16.15	0.0499
CpG ODN 2216 vs. NCTC 13179	6.478	2.822	3.656	-4.418 to 11.73	0.5203
NCTC 13178 vs. NCTC 13179	-1.6	2.822	-4.422	-12.50 to 3.652	0.3872
BALB/c pDC					
CpG ODN 2216 vs. NCTC 13178	2.929	-6.1	9.029	0.1644 to 17.89	0.0451
CpG ODN 2216 vs. NCTC 13179	2.929	-5.95	8.879	0.01443 to 17.74	0.0495
NCTC 13178 vs. NCTC 13179	-6.1	-5.95	-0.15	-8.714 to 8.414	0.999

A2.1.11 Change in CD86 expression on murine pDC – Figure 4.15B

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
C57BL/6 pDC - BALB/c pDC					
CpG ODN 2216	12.71	6.483	6.231	-5.239 to 17.70	0.4524
NCTC 13178	0.3	6.071	-5.771	-16.79 to 5.249	0.4835
NCTC 13179	7.371	9.557	-2.186	-13.21 to 8.835	0.946

2way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
C57BL/6 pDC					
CpG ODN 2216 vs. NCTC 13178	12.71	0.3	12.41	1.658 to 23.17	0.0207
CpG ODN 2216 vs. NCTC 13179	12.71	7.371	5.343	-5.413 to 16.10	0.4521
NCTC 13178 vs. NCTC 13179	0.3	7.371	-7.071	-17.83 to 3.684	0.2553
BALB/c pDC					
CpG ODN 2216 vs. NCTC 13178	6.483	6.071	0.4119	-10.78 to 11.61	0.9955
CpG ODN 2216 vs. NCTC 13179	6.483	9.557	-3.074	-14.27 to 8.121	0.7812
NCTC 13178 vs. NCTC 13179	6.071	9.557	-3.486	-14.24 to 7.270	0.7097

A2.2 Statistical Analysis of Data from Chapter 5

A2.2.1 CD11c expression on BMDC – Figure 5.2B

2way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
6 hr					
Uninfected vs. *B. pseudomallei*	92.13	93.1	-0.9667	-2.105 to 0.1718	0.1114
Uninfected vs. LPS	92.13	92.58	-0.45	-1.589 to 0.6885	0.6111
B. pseudomallei vs. LPS	93.1	92.58	0.5167	-0.6218 to 1.655	0.5236
12 hr					
Uninfected vs. *B. pseudomallei*	91.97	93.2	-1.233	-2.372 to -0.09482	0.0308
Uninfected vs. LPS	91.97	92.78	-0.8167	-1.955 to 0.3218	0.2047
B. pseudomallei vs. LPS	93.2	92.78	0.4167	-0.7218 to 1.555	0.6552
18 hr					
Uninfected vs. *B. pseudomallei*	92.48	94.45	-1.967	-3.105 to -0.8282	0.0003
Uninfected vs. LPS	92.48	94.6	-2.117	-3.255 to -0.9782	0.0001
B. pseudomallei vs. LPS	94.45	94.6	-0.15	-1.289 to 0.9885	0.9463
24 hr					
Uninfected vs. *B. pseudomallei*	92.62	93.88	-1.267	-2.405 to -0.1282	0.0258
Uninfected vs. LPS	92.62	93.45	-0.8333	-1.972 to 0.3052	0.1922
B. pseudomallei vs. LPS	93.88	93.45	0.4333	-0.7052 to 1.572	0.6332

A2.2.2 MHC class II expression on BMDC – Figure 5.2D

2way ANOVA with tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
6 hr					
Uninfected vs. *B. pseudomallei*	20.63	30.17	-9.533	-12.54 to -6.524	< 0.0001
Uninfected vs. LPS	20.63	36.57	-15.93	-18.94 to -12.92	< 0.0001
B. pseudomallei vs. LPS	30.17	36.57	-6.4	-9.409 to -3.391	< 0.0001
12 hr					
Uninfected vs. B. pseudomallei	24.87	47.17	-22.3	-25.31 to -19.29	< 0.0001
Uninfected vs. LPS	24.87	47.63	-22.77	-25.78 to -19.76	< 0.0001
B. pseudomallei vs. LPS	47.17	47.63	-0.4667	-3.476 to 2.543	0.9209
18 hr					
Uninfected vs. *B. pseudomallei*	47.17	71.1	-23.93	-26.94 to -20.92	< 0.0001
Uninfected vs. LPS	47.17	71.63	-24.47	-27.48 to -21.46	< 0.0001
B. pseudomallei vs. LPS	71.1	71.63	-0.5333	-3.543 to 2.476	0.8981
24 hr					
Uninfected vs. *B. pseudomallei*	45.73	76.23	-30.5	-33.51 to -27.49	< 0.0001
Uninfected vs. LPS	45.73	75.77	-30.03	-33.04 to -27.02	< 0.0001
B. pseudomallei vs. LPS	76.23	75.77	0.4667	-2.543 to 3.476	0.9209

A2.2.3 CD86 expression on BMDC – Figure 5.2F

2way ANOVA with tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
6 hr					
Uninfected vs. *B. pseudomallei*	20.47	33.3	-12.83	-15.81 to -9.861	< 0.0001
Uninfected vs. LPS	20.47	57.17	-36.7	-39.67 to -33.73	< 0.0001
B. pseudomallei vs. LPS	33.3	57.17	-23.87	-26.84 to -20.89	< 0.0001
12 hr					
Uninfected vs. *B. pseudomallei*	21.87	62.47	-40.6	-43.57 to -37.63	< 0.0001
Uninfected vs. LPS	21.87	71.03	-49.17	-52.14 to -46.19	< 0.0001
B. pseudomallei vs. LPS	62.47	71.03	-8.567	-11.54 to -5.595	< 0.0001
18 hr					
Uninfected vs. *B. pseudomallei*	39.97	71.2	-31.23	-34.21 to -28.26	< 0.0001
Uninfected vs. LPS	39.97	75.77	-35.8	-38.77 to -32.83	< 0.0001
B. pseudomallei vs. LPS	71.2	75.77	-4.567	-7.539 to -1.595	0.0022
24 hr					
Uninfected vs. *B. pseudomallei*	42.93	86.77	-43.83	-46.81 to -40.86	< 0.0001
Uninfected vs. LPS	42.93	86.67	-43.73	-46.71 to -40.76	< 0.0001
B. pseudomallei vs. LPS	86.77	86.67	0.1	-2.872 to 3.072	0.9961

A2.2.4 CCR7 expression on BMDC – Figure 5.2H

2way ANOVA with tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
6 hr					
Uninfected vs. *B. pseudomallei*	20.47	51.57	-31.1	-35.00 to -27.20	< 0.0001
Uninfected vs. LPS	20.47	50	-29.53	-33.43 to -25.64	< 0.0001
B. pseudomallei vs. LPS	51.57	50	1.567	-2.330 to 5.463	0.5815
12 hr					
Uninfected vs. *B. pseudomallei*	27.03	55.97	-28.93	-32.83 to -25.04	< 0.0001
Uninfected vs. LPS	27.03	51.27	-24.23	-28.13 to -20.34	< 0.0001
B. pseudomallei vs. LPS	55.97	51.27	4.7	0.8036 to 8.596	0.016
18 hr					
Uninfected vs. *B. pseudomallei*	35.83	57.33	-21.5	-25.40 to -17.60	< 0.0001
Uninfected vs. LPS	35.83	52.57	-16.73	-20.63 to -12.84	< 0.0001
B. pseudomallei vs. LPS	57.33	52.57	4.767	0.8702 to 8.663	0.0145
24 hr					
Uninfected vs. *B. pseudomallei*	39.07	59.8	-20.73	-24.63 to -16.84	< 0.0001
Uninfected vs. LPS	39.07	61	-21.93	-25.83 to -18.04	< 0.0001
B. pseudomallei vs. LPS	59.8	61	-1.2	-5.096 to 2.696	0.7252

A2.2.5 Intracellular survival of *B. pseudomallei* within BMDC – Figure 5.5

Ordinary 1way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
6 hr vs. 12 hr	4.461	3.861	0.5992	0.01814 to 1.180	0.0434
6 hr vs. 18 hr	4.461	3.05	1.411	0.8301 to 1.992	0.0002
6 hr vs. 24 hr	4.461	2.184	2.277	1.696 to 2.858	< 0.0001
12 hr vs. 18 hr	3.861	3.05	0.8119	0.2308 to 1.393	0.0089
12 hr vs. 24 hr	3.861	2.184	1.678	1.097 to 2.259	< 0.0001
18 hr vs. 24 hr	3.05	2.184	0.8659	0.2848 to 1.447	0.0061

A2.2.6 *In vitro* migration of LPS-stimulated BMDC – Figure 5.6

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Media - CCL19					
Unstim	3.816	13.62	-9.809	-20.78 to 1.162	0.1068
2 hr	4.956	12.44	-7.485	-20.92 to 5.951	0.6207
4 hr	5.888	22.27	-16.38	-25.34 to -7.421	< 0.0001
6 hr	4.924	18.72	-13.79	-22.75 to -4.835	0.0004
12 hr	6.755	21.58	-14.82	-25.79 to -3.851	0.0026
18 hr	6.538	28	-21.46	-32.43 to -10.49	< 0.0001
24 hr	6.536	25.38	-18.84	-27.80 to -9.883	< 0.0001

2way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Media					
Unstim vs. 2 hr	3.816	4.956	-1.14	-14.61 to 12.34	> 0.9999
Unstim vs. 4 hr	3.816	5.888	-2.072	-13.07 to 8.930	0.9975
Unstim vs. 6 hr	3.816	4.924	-1.108	-12.11 to 9.895	> 0.9999
Unstim vs. 12 hr	3.816	6.755	-2.939	-14.99 to 9.114	0.9898
Unstim vs. 18 hr	3.816	6.538	-2.722	-14.77 to 9.330	0.9933
Unstim vs. 24 hr	3.816	6.536	-2.72	-13.72 to 8.283	0.9891
2 hr vs. 4 hr	4.956	5.888	-0.9324	-13.48 to 11.61	> 0.9999
2 hr vs. 6 hr	4.956	4.924	0.03206	-12.51 to 12.58	> 0.9999
2 hr vs. 12 hr	4.956	6.755	-1.799	-15.27 to 11.68	0.9996
2 hr vs. 18 hr	4.956	6.538	-1.582	-15.06 to 11.89	0.9998
2 hr vs. 24 hr	4.956	6.536	-1.58	-14.12 to 10.96	0.9997
4 hr vs. 6 hr	5.888	4.924	0.9645	-8.876 to 10.81	> 0.9999
4 hr vs. 12 hr	5.888	6.755	-0.8666	-11.87 to 10.14	> 0.9999
4 hr vs. 18 hr	5.888	6.538	-0.6499	-11.65 to 10.35	> 0.9999
4 hr vs. 24 hr	5.888	6.536	-0.6476	-10.49 to 9.193	> 0.9999
6 hr vs. 12 hr	4.924	6.755	-1.831	-12.83 to 9.171	0.9988
6 hr vs. 18 hr	4.924	6.538	-1.614	-12.62 to 9.388	0.9994
6 hr vs. 24 hr	4.924	6.536	-1.612	-11.45 to 8.229	0.9989
12 hr vs. 18 hr	6.755	6.538	0.2167	-11.84 to 12.27	> 0.9999
12 hr vs. 24 hr	6.755	6.536	0.219	-10.78 to 11.22	> 0.9999
18 hr vs. 24 hr	6.538	6.536	0.002318	-11.00 to 11.00	> 0.9999
CCL19					
Unstim vs. 2 hr	13.62	12.44	1.184	-12.29 to 14.66	> 0.9999
Unstim vs. 4 hr	13.62	22.27	-8.642	-19.64 to 2.360	0.2231
Unstim vs. 6 hr	13.62	18.72	-5.091	-16.09 to 5.911	0.8015
Unstim vs. 12 hr	13.62	21.58	-7.952	-20.00 to 4.101	0.4267
Unstim vs. 18 hr	13.62	28	-14.37	-26.43 to -2.322	0.0092
Unstim vs. 24 hr	13.62	25.38	-11.75	-22.75 to -0.7494	0.0285
2 hr vs. 4 hr	12.44	22.27	-9.826	-22.37 to 2.718	0.226
2 hr vs. 6 hr	12.44	18.72	-6.275	-18.82 to 6.270	0.7374
2 hr vs. 12 hr	12.44	21.58	-9.136	-22.61 to 4.339	0.393
2 hr vs. 18 hr	12.44	28	-15.56	-29.03 to -2.083	0.0132
2 hr vs. 24 hr	12.44	25.38	-12.94	-25.48 to -0.3910	0.0388
4 hr vs. 6 hr	22.27	18.72	3.551	-6.290 to 13.39	0.9294
4 hr vs. 12 hr	22.27	21.58	0.6904	-10.31 to 11.69	> 0.9999
4 hr vs. 18 hr	22.27	28	-5.732	-16.73 to 5.271	0.6991
4 hr vs. 24 hr	22.27	25.38	-3.11	-12.95 to 6.731	0.9621
6 hr vs. 12 hr	18.72	21.58	-2.861	-13.86 to 8.142	0.9858
6 hr vs. 18 hr	18.72	28	-9.283	-20.29 to 1.719	0.1556
6 hr vs. 24 hr	18.72	25.38	-6.661	-16.50 to 3.180	0.3951
12 hr vs. 18 hr	21.58	28	-6.422	-18.47 to 5.630	0.6765
12 hr vs. 24 hr	21.58	25.38	-3.8	-14.80 to 7.202	0.9422
18 hr vs. 24 hr	28	25.38	2.622	-8.380 to 13.62	0.991

A2.2.7 *In vitro* migration of *B. pseudomallei*-infected BMDC – Figure 5.7

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Media - CCL19					
Uninfected	3.592	13.88	-10.29	-22.06 to 1.478	0.1136
6 hr	1.029	12.93	-11.9	-33.69 to 9.898	0.5643
12 hr	1.076	18.07	-16.99	-38.79 to 4.804	0.1974
18 hr	9.032	30.77	-21.74	-43.53 to 0.05776	0.0509
24 hr	6.233	30.84	-24.6	-34.26 to -14.94	< 0.0001

2way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Media					
Uninfected vs. 6 hr	3.592	1.029	2.563	-16.31 to 21.44	> 0.9999
Uninfected vs. 12 hr	3.592	1.076	2.515	-16.36 to 21.39	> 0.9999
Uninfected vs. 18 hr	3.592	9.032	-5.44	-24.32 to 13.44	0.9945
Uninfected vs. 24 hr	3.592	6.233	-2.641	-13.22 to 7.941	0.9983
6 hr vs. 12 hr	1.029	1.076	-0.04727	-23.92 to 23.83	> 0.9999
6 hr vs. 18 hr	1.029	9.032	-8.003	-31.88 to 15.87	0.9829
6 hr vs. 24 hr	1.029	6.233	-5.204	-23.25 to 12.85	0.9945
12 hr vs. 18 hr	1.076	9.032	-7.956	-31.83 to 15.92	0.9836
12 hr vs. 24 hr	1.076	6.233	-5.156	-23.21 to 12.89	0.9949
18 hr vs. 24 hr	9.032	6.233	2.799	-15.25 to 20.85	> 0.9999
CCL19					
Uninfected vs. 6 hr	13.88	12.93	0.9577	-18.54 to 20.45	> 0.9999
Uninfected vs. 12 hr	13.88	18.07	-4.184	-23.68 to 15.31	0.9995
Uninfected vs. 18 hr	13.88	30.77	-16.89	-36.38 to 2.610	0.1362
Uninfected vs. 24 hr	13.88	30.84	-16.95	-29.85 to -4.058	0.0031
6 hr vs. 12 hr	12.93	18.07	-5.141	-29.02 to 18.74	0.9995
6 hr vs. 18 hr	12.93	30.77	-17.84	-41.72 to 6.033	0.2917
6 hr vs. 24 hr	12.93	30.84	-17.91	-36.79 to 0.9656	0.0745
12 hr vs. 18 hr	18.07	30.77	-12.7	-36.58 to 11.17	0.7447
12 hr vs. 24 hr	18.07	30.84	-12.77	-31.65 to 6.107	0.4263
18 hr vs. 24 hr	30.77	30.84	-0.06734	-18.94 to 18.81	> 0.9999

A2.2.8 Effect of kanamycin treatment on BMDC migration – Figure 5.8

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Untreated - Kanamycin-treated					
Uninfected	7.051	9.561	-2.51	-9.843 to 4.822	0.6095
B. pseudomallei	18.07	20.25	-2.181	-9.513 to 5.152	0.6843

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Uninfected - *B. pseudomallei*					
Untreated	7.051	18.07	-11.02	-18.35 to -3.685	0.0067
Kanamycin-treated	9.561	20.25	-10.69	-18.02 to -3.356	0.0079

A2.2.8 BMDC migration toward CCL19 &/or CCL21 – Figure 5.9

2way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
CCL19					
Uninfected vs. LPS	7.278	13.88	-6.602	-12.74 to -0.4658	0.0319
Uninfected vs. *B. pseudomallei*	7.278	17.74	-10.46	-16.60 to -4.326	0.0003
LPS vs *B. pseudomallei*	13.88	17.74	-3.86	-9.996 to 2.277	0.2965
CCL21					
Uninfected vs. LPS	7.824	13.94	-6.119	-12.26 to 0.01724	0.0508
Uninfected vs. *B. pseudomallei*	7.824	11.81	-3.99	-10.13 to 2.146	0.2731
LPS vs *B. pseudomallei*	13.94	11.81	2.129	-4.008 to 8.265	0.6877
CCL19 + CCL21					
Uninfected vs. LPS	9.976	12.76	-2.788	-7.541 to 1.965	0.3466
Uninfected vs. *B. pseudomallei*	9.976	15.46	-5.483	-10.03 to -0.9320	0.014
LPS vs *B. pseudomallei*	12.76	15.46	-2.695	-7.246 to 1.856	0.3397

2way ANOVA with Sidak's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Uninfected					
CCL19 vs. CCL21	7.278	7.824	-0.5458	-6.682 to 5.591	0.9756
CCL19 vs. CCL19+21	7.278	9.976	-2.698	-8.186 to 2.791	0.4736
CCL21 vs. CCL19+21	7.824	9.976	-2.152	-7.640 to 3.337	0.6204
LPS					
CCL19 vs. CCL21	13.88	13.94	-0.06278	-6.199 to 6.074	0.9997
CCL19 vs. CCL19+21	13.88	12.76	1.117	-4.372 to 6.605	0.8788
CCL21 vs. CCL19+21	13.94	12.76	1.18	-4.309 to 6.668	0.8658
B. pseudomallei					
CCL19 vs. CCL21	17.74	11.81	5.926	-0.2107 to 12.06	0.0307
CCL19 vs. CCL19+21	17.74	15.46	2.282	-3.033 to 7.596	0.5646
CCL21 vs. CCL19+21	11.81	15.46	-3.644	-8.959 to 1.670	0.2368

A2.2.9 BMDC migration following exposure to *B. pseudomallei* isolates of high and low virulence – Figure 5.10

Ordinary 1way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Uninfected vs. NCTC 13179	8.984	14.76	-5.775	-10.14 to -1.413	0.007
Uninfected vs. NCTC 13178	8.984	12.31	-3.33	-8.590 to 1.930	0.0181
NCTC 13179 vs. NCTC 13178	14.76	12.31	2.445	-2.648 to 7.538	0.4782

A2.2.10 Migrated BMDC harbour live *B. pseudomallei* – Figure 5.11

Mann Whitney test

Column B vs. Column A	*B. pseudomallei* -infected BMDC vs. *B. pseudomallei* alone
Mann Whitney test	
P value	0.0238
Exact or approximate P value?	Exact
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	6 , 39
Mann-Whitney U	0
Difference between medians	
Median of column A	12.83
Median of column B	106.3
Difference: Actual	93.5
Difference: Hodges-Lehmann	84.75

A2.3 Statistical Analysis of Data from Chapter 6

A2.3.1 *In vivo* migration of BMDC exposed to *B. pseudomallei* for 18 & 24 hr – Figure 6.5

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Uninfected - *B. pseudomallei*					
18 hr	0.1495	0.5631	-0.4136	-0.7827 to -0.04446	0.0303
24 hr	0.03929	0.7024	-0.6631	-1.032 to -0.2939	0.0023

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
18 hr - 24 hr					
Uninfected	0.1495	0.03929	0.1102	-0.2589 to 0.4793	0.6825
B. pseudomallei	0.5631	0.7024	-0.1393	-0.5084 to 0.2298	0.5522

A2.3.2 Effect of time post-footpad injection – Figure 6.6

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Uninfected - *B. pseudomallei*					
18 hr	0.00665	0.01667	-0.01002	-0.08850 to 0.06847	0.9803
24 hr	0.03929	0.7024	-0.6631	-0.7415 to -0.5846	< 0.0001
36 hr	0.07512	0.1741	-0.099	-0.1775 to -0.02051	0.0132

2way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Uninfected					
18 hr vs. 24 hr	0.00665	0.03929	-0.03264	-0.1082 to 0.04295	0.5023
18 hr vs. 36 hr	0.00665	0.07512	-0.06847	-0.1441 to 0.007115	0.0774
24 hr vs. 36 hr	0.03929	0.07512	-0.03583	-0.1114 to 0.03975	0.4403
B. pseudomallei					
18 hr vs. 24 hr	0.01667	0.7024	-0.6857	-0.7613 to -0.6101	< 0.0001
18 hr vs. 36 hr	0.01667	0.1741	-0.1574	-0.2330 to -0.08187	0.0003
24 hr vs. 36 hr	0.7024	0.1741	0.5282	0.4527 to 0.6038	< 0.0001

A2.3.3 BMDC *in vivo* migration – Figure 6.7

1way ANOVA with Tukey's multiple comparison test

Tukey's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Uninfected vs. *B. pseudomallei*	0.1272	0.5017	-0.3745	-0.6203 to -0.1288	0.0061
Uninfected vs. LPS	0.1272	0.2623	-0.1352	-0.2643 to -0.006062	0.0411
B. pseudomallei vs. LPS	0.5017	0.2623	0.2394	-0.01182 to 0.4906	0.0609

A2.3.4 Organ loads following BMDC migration *in vivo* – Figure 6.8

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
B. pseudomallei -infected BMDC - *B. pseudomallei* alone					
pLN	2.379	2.39	-0.01158	-0.6327 to 0.6095	> 0.9999
iLN	0.1281	0.183	-0.05493	-0.6761 to 0.5662	0.9998
Spleen	2.138	1.281	0.857	0.2359 to 1.478	0.0023
Lung	0.9863	0.1165	0.8697	0.2486 to 1.491	0.0019
Blood	0.2519	0	0.2519	-0.3692 to 0.8730	0.8231

A2.3.5 Effect of collagenase digestion on *B. pseudomallei* viability – Figure 6.9

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Collagenase Digested - Undigested					
Extracellular	6.197	6.116	0.08139	-0.08692 to 0.2497	0.4233
Intracellular	5.562	5.579	-0.01712	-0.1854 to 0.1512	0.9598

A2.3.6 Internalisation of *B. pseudomallei* by endogenous DC in the footpad – Figure 6.11

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
DC - Non-DC					
2 hr	21.7	0.7956	20.91	3.144 to 38.67	0.0188
4 hr	8.572	0.3504	8.222	-9.540 to 25.98	0.4896

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
2 hr - 4 hr					
DC	21.7	8.572	13.13	-4.633 to 30.89	0.1764
Non-DC	0.7956	0.3504	0.4452	-17.32 to 18.21	0.9978

A2.3.7 Disseminated *B. pseudomallei* in endogenous DC in the pLN - Figure 6.12A

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
DC - Non-DC					
24 hr	6.261	0.2339	6.027	-2.689 to 14.74	0.216
48 hr	0	0.00568	-0.005681	-8.722 to 8.710	> 0.9999

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
24 hr - 48 hr					
DC	6.261	0	6.261	-2.455 to 14.98	0.193
Non-DC	0.2339	0.00568	0.2282	-8.488 to 8.944	0.9976

A2.3.8 Disseminated *B. pseudomallei* in endogenous DC in the spleen - Figure 6.12B

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
DC - Non-DC					
24 hr	1.699	0.06435	1.635	-6.872 to 10.14	0.8497
48 hr	8.076	0.5995	7.477	-1.030 to 15.98	0.0431

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
24 hr - 48 hr					
DC	1.699	8.076	-6.377	-14.88 to 2.130	0.1421
Non-DC	0.06435	0.5995	-0.5352	-9.042 to 7.972	0.9824

A2.3.9 Time point for assessing *B. pseudomallei*-specific T cell responses in pLN – Figure 6.13A

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Unstimulated - Hk-*B. pseudomallei*					
Day 7	1.16	2.599	-1.439	-3.082 to 0.2041	0.0928
Day 14	2.188	2.468	-0.2798	-1.923 to 1.363	0.9555
Day 21	1.867	3.84	-1.973	-3.616 to -0.3301	0.018

A2.3.10 Time point for assessing *B. pseudomallei*-specific T cell responses in spleen – Figure 6.13B

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Unstimulated - Hk-*B. pseudomallei*					
Day 7	1.701	1.699	0.002336	-1.313 to 1.318	> 0.9999
Day 14	2.555	3.654	-1.099	-2.414 to 0.2170	0.1132
Day 21	1.685	3.577	-1.891	-3.207 to -0.5757	0.0054

A2.3.11 Effect of kanamycin in cell culture media on T cell proliferation – Figure 6.14

Unpaired t test with Welch's correction

Column B vs. Column A	Kanamycin Treated vs. Untreated
Unpaired t test with Welch's correction	
P value	0.0028
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
Welch-corrected t, df	t=8.842 df=3.054
How big is the difference?	
Mean ± SEM of column A	17.50 ± 2.021, n=3
Mean ± SEM of column B	37.76 ± 1.079, n=3
Difference between means	20.26 ± 2.291
95% confidence interval	13.04 to 27.47
R squared	0.9624

A2.3.12 Effect of kanamycin in isolation media only at killing bacteria while isolating T cell – Figure 6.15A

Unpaired t test with Welch's correction

Column B vs. Column A	Kanamycin Treated vs. Untreated
Unpaired t test with Welch's correction	
P value	0.001
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
Welch-corrected t, df	t=31.58 df=2.000
How big is the difference?	
Mean ± SEM of column A	3.411 ± 0.1080
Mean ± SEM of column B	0.0 ± 0.0
Difference between means	-3.411 ± 0.1080
95% confidence interval	-3.876 to -2.946
R squared	0.998

A2.3.13 Effect of kanamycin in isolation media only on T cell proliferation – Figure 6.15B

Unpaired t test with Welch's correction

Column B vs. Column A	Kanamycin Treated vs. Untreated
Unpaired t test with Welch's correction	
P value	0.716
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
Welch-corrected t, df	t=0.4119 df=2.281
How big is the difference?	
Mean ± SEM of column A	8.605 ± 1.001, n=3
Mean ± SEM of column B	10.21 ± 3.770, n=3
Difference between means	1.606 ± 3.900
95% confidence interval	-13.34 to 16.56
R squared	0.06923

A2.3.14 Comparison of survival curves following *in vivo* migration of BMDC – Figure 6.16A

Comparison of Survival Curves

Log-rank (Mantel-Cox) test (recommended)	
Chi square	4.273
df	2
P value	0.1181
P value summary	ns
Are the survival curves sig different?	No
Logrank test for trend (recommended)	
Chi square	3.281
df	1
P value	0.0701
P value summary	ns
Sig. trend?	No
Gehan-Breslow-Wilcoxon test	
Chi square	4.269
df	2
P value	0.1183
P value summary	ns
Are the survival curves sig different?	No

A2.3.15 Comparison of T cell proliferation following *in vivo* migration of BMDC – Figure 6.16B

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
pLN					
Uninfect vs. *Bps*-infect BMDC	1.12	1.7	-0.58	-1.355 to 0.1948	0.2516
Uninfect vs. *Bps* alone	1.12	1.03	0.09	-0.6848 to 0.8648	0.9998
Bps-infected BMDC vs. *Bps* alone	1.7	1.03	0.67	-0.1048 to 1.445	0.1265
Spleen					
Uninfect vs. *Bps*-infect BMDC	1.1	1.5	-0.4	-0.8649 to 0.06491	0.1301
Uninfect vs. *Bps* alone	1.1	1.4	-0.3	-0.7649 to 0.1649	0.4178
Bps-infected BMDC vs. *Bps* alone	1.5	1.4	0.1	-0.3649 to 0.5649	0.9932

A2.3.16 Organ loads in *B. pseudomallei* infected mice (10^6 & 10^4 CFU) – Figure 6.17A

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
10^6 CFU *B. pseudomallei* - 10^4 CFU *B. pseudomallei*					
pLN	2.706	2.39	0.3161	-0.6709 to 1.303	0.8182
Spleen	2.952	1.281	1.672	0.6846 to 2.659	0.0003
Lung	1.54	0.1165	1.423	0.4362 to 2.410	0.0024

A2.3.17 T cell proliferation responses in *B. pseudomallei* infected mice (10^6 CFU) – Figure 6.17B

Unpaired t test with Welch's correction

Column B vs. Column A	10^6 CFU *B. pseudomallei* vs. Uninfected
Unpaired t test with Welch's correction	
P value	0.0454
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
Welch-corrected t, df	t=2.633 df=5.106
How big is the difference?	
Mean ± SEM of column A	26.64 ± 4.217, n=5
Mean ± SEM of column B	134.8 ± 40.86, n=6
Difference between means	108.1 ± 41.07
95% confidence interval	3.209 to 213.1
R squared	0.5758

A2.4 Statistical Analysis of Data from Chapter 7

A2.4.1 T cell proliferation from IHA-negative patients - Figure 7.2G

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
IHA-neg Patient - Control					
NCTC 13179	15.32	3.372	11.95	4.921 to 18.98	0.0005
Patient's Isolate	13.85	2.976	10.88	3.846 to 17.91	0.0015

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
NCTC 13179 - Patient's Isolate					
IHA-neg Patient	15.32	13.85	1.471	-5.559 to 8.500	0.866
Control	3.372	2.976	0.3955	-6.634 to 7.425	0.9896

A2.4.2 CD4 T cell activation - Figure 7.3A

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
IHA-neg Patient - Control					
NCTC 13179	2.675	1.74	0.935	-3.274 to 5.144	0.8245
Patient's Isolate	2.423	0.6275	1.795	-2.414 to 6.004	0.5067

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
NCTC 13179 - Patient's Isolate					
IHA-neg Patient	2.675	2.423	0.2525	-3.957 to 4.462	0.9858
Control	1.74	0.6275	1.113	-3.097 to 5.322	0.7624

A2.4.3 CD8 T cell activation - Figure 7.3B

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
IHA-neg Patient - Control					
NCTC 13179	-1.065	0.8767	-1.942	-5.359 to 1.475	0.3354
Patient's Isolate	0.1367	2.892	-2.755	-6.172 to 0.6619	0.1267

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
NCTC 13179 - Patient's Isolate					
IHA-neg Patient	-1.065	0.1367	-1.202	-4.619 to 2.215	0.6464
Control	0.8767	2.892	-2.015	-5.432 to 1.402	0.3102

A2.4.4 IFN-g production - Figure 7.4

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
IHA-neg Patient - Control					
Unstimulated	2.55	1.69	0.86	-22.40 to 24.12	0.9953
NCTC 13179	27.94	0.1889	27.75	3.852 to 51.65	0.0204

2way ANOVA with Sidak's multiple comparison test

Sidak's multiple comparisons test	Mean 1	Mean 2	Mean Diff.	95% CI of diff.	Adjusted P Value
Unstimulated - NCTC 13179					
IHA-neg Patient	2.55	27.94	-25.39	-48.65 to -2.129	0.0304
Control	1.69	0.1889	1.501	-22.40 to 25.40	0.9866

APPENDIX 3

FORMULAS

A3.1 Formulas used in Chapter 4

A3.1.1 Percentage of human pDC in PBMC

Human pDC (%) = (cell concentration of pDC / cell concentration of PBMC) X 100

A3.1.2 Calculation of the total number of PBMC

Total # of PBMC isolated = original PBMC (cells/ml) X original volume (ml)

A3.1.2 Estimation of potential human pDC yield from PBMC

Potential yield (pDC/ml of blood) =

(# of PBMC isolated X % of pDC in PBMC) / original volume of blood

A3.1.3 Purity of isolated human pDC

Purity (%) = (# of pDC events counted / # of total cell events counted) X 100

A3.2 Formulas used in Chapter 5

A3.2.1 Enumeration of BMDC following *in vitro* migration

Number of migrated cells = cell concentration (cells/ml) x sample volume (ml)

A3.2.2 Enumeration of BMDC following *in vivo* migration

A3.2.2.1 The concentration of $CFSE^+/CD11c^+$ BMDC

Concentration (Cells/μl) = (number of cell events / number of CBB added) X (# of bead events / volume of sample (μl))

A3.2.2.2 The total number of $CFSE^+/CD11c^+$ BMDC per popliteal lymph node

Total cell number = concentration (cells/μl) X original sample volume (μl)

A.3.2.2.3 The percentage of injected BMDC that migrated to the popliteal lymph node

Migrated (%) = Total cell number / number of cells injected X 100

A3.3 Formulas used in Chapter 6

A3.3.1 The purity of endogenous DC isolated

Purity (%) = ($CD11c^+/CD45^+$ concentration / Total Concentration) X 100

A3.4 Formulas for Calculating Bacterial Internalisation and Survival Percentages in Chapters 4, 5 and 6

A3.4.1 Internalisation of *B. pseudomallei*

Internalised bacteria (%) = (average # of intracellular bacteria at 4 hr / number of bacteria added to well) X 100

A3.4.2 Intracellular survival of *B. pseudomallei*

Bacteria survival (%) = (average # of intracellular bacteria at 24 hr / average # of intracellular bacteria at 4 hr) X 100

A3.5 Formula for Calculating Stimulation Indices as a Measure of Mononuclear Cell Proliferation used in Chapter 6 & 7

Stimulation Indexes (SI) = stimulated CPM / unstimulated CPM.

Mean maximum SI = the highest average SI recorded over 4 time points between day 4 to 7 of stimulation.

APPENDIX 4

RAW PROLIFERATION DATA FROM CHAPTER 6

A4.1 Raw CPM data for Figure 6.13A

Spleen CPM		Replicate	Unstimulated	*B. pseudomallei* lysate (1μg/ml)	ConA (2μg/ml)
Day 7	**Unstimulated BMDC**	**1**	1418	2801	2734
		2	955	2125	2638
		3	1266	1883	2931
		Mean	1213	2270	2768
		SD	236	476	149
		SEM	136	275	86
	Hk-*B.pseudomallei*-stimulated BMDC	**1**	736	1377	1849
		2	900	1339	3697
		3	927	1637	1783
		Mean	854	1451	2443
		SD	103	162	1086
		SEM	60	94	627
Day 14	**Unstimulated BMDC**	**1**	337	927	569
		2	411	1216	1058
		3	204	1750	681
		Mean	317	1298	769
		SD	105	418	256
		SEM	61	241	148
	Hk-*B.pseudomallei*-stimulated BMDC	**1**	366	1267	2085
		2	415	1798	1664
		3	240	1211	1237
		Mean	340	1425	1662
		SD	90	324	424
		SEM	52	187	245
Day 21	**Unstimulated BMDC**	**1**	1146	1684	5359
		2	807	948	4520
		3	747	779	6025
		Mean	900	1137	5301
		SD	215	481	754
		SEM	124	278	435
	Hk-*B.pseudomallei*-stimulated BMDC	**1**	330	2865	5359
		2	267	3733	4520
		3	496	2648	6025
		Mean	364	3082	5301
		SD	118	574	754
		SEM	68	331	435

A4.1 Raw CPM data for Figure 6.13B

Popliteal lymp node CPM		Replicate	Unstimulated	*B. pseudomallei* lysate (1μg/ml)	ConA (2μg/ml)
Day 7	**Unstimulated BMDC**	**1**	559	1589	5928
		2	1078	3562	5947
		3	910	1625	3576
		Mean	849	2259	5150
		SD	265	1129	1363
		SEM	153	652	787
	Hk-*B.pseudomallei*-stimulated BMDC	**1**	1227	1673	1679
		2	1696	1725	2556
		3	898	2376	2107
		Mean	1274	1925	2114
		SD	401	392	439
		SEM	232	226	253
Day 14	**Unstimulated BMDC**	**1**	150	281	1573
		2	150	726	888
		3	251	484	1106
		Mean	184	497	1189
		SD	58	223	350
		SEM	34	129	202
	Hk-*B.pseudomallei*-stimulated BMDC	**1**	1428	6123	3965
		2	1000	4346	4307
		3	1467	6201	5121
		Mean	1298	5557	4464
		SD	259	1049	594
		SEM	150	606	343
Day 21	**Unstimulated BMDC**	**1**	190	485	918
		2	447	391	1064
		3	200	464	1232
		Mean	279	447	1071
		SD	146	49	157
		SEM	84	28	91
	Hk-*B.pseudomallei*-stimulated BMDC	**1**	101	1774	616
		2	143	237	722
		3	110	1056	574
		Mean	118	1022	637
		SD	22	769	76
		SEM	13	444	44

A4.1 Raw CPM data for Figure 6.14

Untreated cell culture media	Replicate	Unstimulated	ConA (2μg/ml)
	1	179	3407
	2	281	2710
	3	122	4068
	Mean	194	3395
	SD	81	679
	SEM	47	392
Kanamycin treated cell culture media	**Replicate**	**Unstimulated**	**ConA (2μg/ml)**
	1	154	5476
	2	171	5903
	3	119	5385
	Mean	148	5588
	SD	27	277
	SEM	15	160

A4.1 Raw CPM data for Figure 6.15B

Untreated isolation media	Replicate	Unstimulated	ConA (2μg/ml)
	1	162	1329
	2	120	1296
	3	128	903
	Mean	137	1176
	SD	22	237
	SEM	13	137
Kanamycin treated isolation media	**Replicate**	**Unstimulated**	**ConA (2μg/ml)**
	1	119	1417
	2	166	2626
	3	174	644
	Mean	153	1562
	SD	30	999
	SEM	17	577

A4.1 Raw CPM data for Figure 6.16B

Spleen mononuclear cells

	Uninfected BMDC			*B.pseudomallei*- infected BMDC			*B.pseudomallei alone*		
Replicate	Unstimulated	*B. pseudomallei* lysate (1μg/ml)	ConA (2μg/ml)	Unstimulated	*B. pseudomallei* lysate (1μg/ml)	ConA (2μg/ml)	Unstimulated	*B. pseudomallei* lysate (1μg/ml)	ConA (2μg/ml)
1	1153	1269	11294	1211	1143	4494	1170	1767	9320
2	1321	1501	10272	1122	2273	2884	1105	1732	6238
3	1341	1759	15372	1042	976	6561	958	1728	6980
4	1379	2299	14826	1120	923	3260	1024	1714	7460
5	1156	1572	9250	1134	1041	3815	927	1748	7485
6	1238	1608	10546	1113	1007	5179	856	1721	7689
7	984	1260	2534	1093	917	5888	1126	1713	7363
8	1162	1228	2662	1070	1080	5083	1024	1708	10735
9	1288	1916	19849	819	1199	5308	890	952	4799
10	1656	2929	12620	856	1108	4153	913	867	6293
11	947	897	4715	862	1006	3229	812	1186	3138
12	1051	1063	4464	833	1061	2498	779	1201	10077
13	985	967	1276	822	1078	5005	771	1290	2905
14	940	1011	4113	1189	10814	4355	763	1411	3262
15	1015	827	8565	1194	947	3146	741	1288	2320
16	955	892	4207	896	930	3146	756	1194	2488
17	952	921	1152	756	1065	3745	773	1212	2675
18	1000	1468	4003	824	917	4287	772	1280	3719
19	1135	2536	8340	798	7705	3328	644	1396	2607
20	1172	1901	6174	717	7980	3882	722	1247	3034
21	1150	2497	6641	928	2361	5128	665	1371	5083
22	1319	2217	4125	1034	2188	4235	722	1475	7891
23	901	1487	4236	976	1969	2673	700	1418	7214
24	1076	1285	3229	933	2289	2302	644	1085	3215
25	1147	1387	2799	1027	2682	4108	625	1148	3182
26	1063	1377	2481	1076	2599	3351	637	1111	3446
27	945	2223	7275	945	2946	3320			
28	1184	1957	79836	990	2891	3906			
29	1066	2227	9095						
30	1024	2151	4197						
Average	1124	1621	9338	978	2325	4010	828	1383	5408
STDEV	168	570	14074	143	2443	1044	160	270	2595
SEM	31	104	2570	26	446	191	29	49	474

Popliteal lymph node mononuclear cells

	Uninfected BMDC			*B.pseudomallei*- infected BMDC			*B.pseudomallei alone*		
Replicate	Unstimulated	*B. pseudomallei* lysate (1μg/ml)	ConA (2μg/ml)	Unstimulated	*B. pseudomallei* lysate (1μg/ml)	ConA (2μg/ml)	Unstimulated	*B. pseudomallei* lysate (1μg/ml)	ConA (2μg/ml)
1	1036	2026	9157	1231	4293	17174	1084	1384	6147
2	1143	1965	9151	1251	3447	18379	979	1364	5588
3	1157	2319	10857	1230	2334	15367	1064	1335	6626
4	838	865	2106	775	1114	9712	861	868	7176
5	930	846	2486	769	1288	9197	852	842	6910
6	992	848	2940	795	1199	8470	839	813	6028
7	1054	853	2224	993	1072	3505	860	822	3553
Average	1021	1389	5560	1006	2107	11686	934	1061	6004
STDEV	114	677	3943	229	1302	5413	106	281	1210
SEM	47	257	1493	95	493	2051	44	107	458

A4.1 Raw CPM data for Figure 6.17B

	Replicate	Unstimulated	*B. pseudomallei* lysate (1μg/ml)	*ConA* (2μg/ml)
Uninfected	**1**	10.75	97.5	414.75
	2	1	58.75	857.5
	3	4.75	77.5	891
	4	1	108	1715.75
	5	1	151	1236.75
	Average	3.7	98.55	1023.15
	STDEV	4.262481672	34.88749776	484.864369
	SEM	1.906239754	15.60216331	216.837938
10^6 CFU	**1**	1	1	1558.5
	2	1	48.75	2023.25
	3	9	306.5	7380
	4	1	291.5	3084.75
	5	1	231.5	1917.75
	6	1	1	2417.75
	7	1	17	7837.25
	8	1	115.5	3482.5
	Average	2	126.59375	3712.71875
	STDEV	2.828427125	131.0979689	2487.16348
	SEM	1	46.35013141	879.345081

APPENDIX 5
PUBLICATIONS RESULTING FROM THIS THESIS

A5.1 Publication of data from Chapter 4

Williams, N.L., Morris, J.L., Rush, C.M. and Ketheesan, N. (2014) Plasmacytoid dendritic cell bactericidal activity against *Burkholderia pseudomallei. Microbes Infect* (Epub ahead of print)

A5.2 Publication of data from Chapters 5 & 6

Williams, N.L., Morris, J.L., Rush, C.M. and Ketheesan, N. (2014) Migration of dendritic cells facilitates systemic dissemination of *Burkholderia pseudomallei*. *Infect Immun* 82: 4233-40

A5.3 Publication of data from Chapter 7

Harris, P.N., Williams, N.L., Morris, J.L., Ketheesan, N. and Norton, R.E. (2011) Evidence of *Burkholderia pseudomallei*-specific immunity in patient sera persistently nonreactive by the indirect haemagglutination assay. *Clin Vaccine Immunol* 18: 1288-91

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Short communication

Plasmacytoid dendritic cell bactericidal activity against *Burkholderia pseudomallei*

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Abstract

Melioidosis sepsis, caused by *Burkholderia pseudomallei*, is associated with high mortality due to an overwhelming inflammatory response. Plasmacytoid dendritic cells (pDC) are potent producers of type I interferons (IFN). This study investigated whether pDC and type I IFN play a role during the early stages of *B. pseudomallei* infection. Human and murine pDC internalised and killed *B. pseudomallei* as efficiently as murine conventional DC (cDC). pDC derived from *B. pseudomallei*-susceptible (BALB/c) mice demonstrated poor intracellular killing and increased IFN-alpha compared to pDC derived from *B. pseudomallei*-resistant (C57BL/6) mice. This is the first evidence of pDC bactericidal activity against *B. pseudomallei* infection.

Keywords: *Burkholderia pseudomallei*; Melioidosis; Dendritic cells; Interferon-alpha; Interferon-beta

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Infection and Immunity

Migration of Dendritic Cells Facilitates Systemic Dissemination of Burkholderia pseudomallei

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Migration of Dendritic Cells Facilitates Systemic Dissemination of *Burkholderia pseudomallei*

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***Burkholderia pseudomallei*, the etiological agent for melioidosis, is an important cause of community-acquired sepsis in northern Australia and northeast Thailand. Due to the rapid dissemination of disease in acute melioidosis, we hypothesized that dendritic cells (DC) could act as a vehicle for dissemination of *B. pseudomallei*. Therefore, this study investigated the effect of *B. pseudomallei* infection on DC migration capacity and whether migration of DC enabled transportation of *B. pseudomallei* from the site of infection. *B. pseudomallei* stimulated significantly increased migration of bone marrow-derived DC (BMDC), both *in vitro* and *in vivo*, compared to uninfected BMDC. Furthermore, migration of BMDC enabled significantly increased *in vitro* trafficking of *B. pseudomallei* and *in vivo* dissemination of *B. pseudomallei* to secondary lymphoid organs and lungs of C57BL/6 mice. DC within the footpad infection site of C57BL/6 mice also internalized *B. pseudomallei* and facilitated dissemination. Although DC have previously been shown to kill intracellular *B. pseudomallei in vitro*, the findings of this study demonstrate that *B. pseudomallei*-infected DC facilitate the systemic spread of this pathogen.**

Melioidosis is a tropical infection caused by the intracellular bacterium, *Burkholderia pseudomallei* (1). In regions of northern Australia and northeast Thailand where the organism is endemic, *B. pseudomallei* infection is an important cause of community-acquired sepsis, which is associated with fatality rates of up to 77% (2–4). During the early phases of infection, macrophages and neutrophils provide essential innate defense against *B. pseudomallei*. However, macrophages also provide a potential intracellular niche for *B. pseudomallei* persistence and replication (5–11).

Dendritic cells (DC) are professional antigen-presenting cells that are responsible for linking the innate and adaptive immune responses (12, 13). Highly phagocytic, immature DC contribute to innate immune responses by internalizing and killing pathogens at the site of infection. DC-pathogen interaction triggers DC maturation, a process whereby DC upregulate expression of migration receptors (CC-chemokine receptor 7 [CCR7]), antigen-presenting molecules (major histocompatibility complex class I [MHC-I] and MHC-II), and T cell costimulatory molecules (CD80 and CD86) (12, 13). Mature DC also demonstrate increased production of cytokines: interleukin-6 (IL-6), IL-12, and tumor necrosis factor alpha (TNF-α) (12). Migration of mature, antigen-loaded DC from the site of infection to secondary lymphoid organs is coordinated by the CCR7 ligands, CCL19 and CCL21 (14). Within secondary lymphoid organs, elevated expression of antigen-presenting and costimulatory molecules on mature DC promotes efficient activation of naive T cells and development of pathogen-specific effector T cells (12).

Investigations of the functional responses of DC toward *B. pseudomallei* have shown internalization and killing of intracellular *B. pseudomallei* by DC occurs *in vitro* (15–19). DC maturation was triggered by *B. pseudomallei*, demonstrated by increased expression of molecules for antigen presentation and costimulation, along with increased cytokine production (15, 17). Furthermore, host susceptibility to *B. pseudomallei* infection was associated with altered DC functional responses (15). Despite significant maturation and IL-12 production in bone marrow-derived DC (BMDC) from *B. pseudomallei*-susceptible BALB/c mice, bactericidal activity against intracellular *B. pseudomallei* was lower than observed for BMDC from *B. pseudomallei*-resistant C57BL/6 mice (15). Both human and murine DC stimulated with heat-killed *B. pseudomallei* are capable of activating naive T cells to initiate *B. pseudomallei*-specific T cell responses and reactivating memory *B. pseudomallei*-specific T cells to produce IFN-γ *in vitro* (16, 19–22).

The migration of DC from the site of *B. pseudomallei* infection to secondary lymphoid organs is an important factor for the development of *B. pseudomallei*-specific adaptive immune responses. Similar to other intracellular bacteria, *B. pseudomallei* could potentially interfere with mechanisms driving DC migration, thereby subsequently impairing activation of *B. pseudomallei*-specific adaptive immune responses. The virulence of *Yersinia pestis*, the causative agent of the human bubonic or pneumonic plague, is in part, attributed to the bacterium's ability to impair DC migration. Cytoskeleton rearrangement is impaired in DC infected with *Y. pestis*, rendering them unable to adhere to surfaces and migrate toward CCL19 (23). Alternatively, other intracellular pathogens, such as *Listeria monocytogenes*, *Francisella tularensis*, and *Streptococcus pneumoniae*, use DC migration as a "Trojan horse" to facilitate dissemination (24–26). Blocking of DC migration or depletion of DC correlated with enhanced capacity to restrict systemic dissemination, significantly reduced bacterial loads, and improved resistance to *F. tularensis* and *S. pneumoniae* infection (25, 26). DC migration has also been shown to facilitate the rapid dissemination of *Bacillus anthracis* spores from the lungs to the thoracic lymph nodes (using transgenic mice developed to specifically express green fluorescent protein [GFP] in DC only

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[$CX_3CR1^{+/GFP}$]) (27, 28). Similarly, *B. pseudomallei* is an intracellular pathogen capable of persistence within different host cells and rapid systemic spread to multiple organs within hours of exposure (6, 29, 30). Recently, colonization of the brain in a murine model of neurological melioidosis was found to occur via the transmigration of *B. pseudomallei*-infected $CD11b^+$ phagocytes across endothelial cells (31). Therefore, a mechanism whereby *B. pseudomallei* uses DC as a vehicle for dissemination is plausible due to the rapid progression of melioidosis from a localized infection to septicemia and involvement of multiple organs. Therefore, the current study investigated (i) the effect of *B. pseudomallei* infection on DC migration capacity *in vitro* and (ii) whether *in vivo* migration of DC enables transportation of *B. pseudomallei* from the site of infection.

MATERIALS AND METHODS

BMDC culture. BMDC were cultured according to published methods (15, 32). Bone marrow (BM) was isolated from the femurs and tibias of C57BL/6 mice (8 to 12 weeks old; James Cook University Small Animal Breeding Facility). All animal studies were conducted according to the guidelines and institutional ethics approval provided by James Cook University Animal Ethics Committee (A1601). Isolated BM cells (2×10^5 cells/ml) were cultured in DC media (RPMI 1640 with 10% heat-inactivated fetal bovine serum [HI-FBS], 1.5 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol; Invitrogen) supplemented with 10% supernatant from Ag8653 myeloma cells transfected with the gene encoding murine GM-CSF (GM-CSF supernatant; cell line kindly provided by B. Stockinger, NMRI, Mill Hill, London, United Kingdom). On day 3, cultures were fed 10 ml of DC medium supplemented with 10% GM-CSF supernatant and, on day 6, 50% of the medium was replaced. On day 10 of culture, BMDC were harvested by gentle pipetting to remove nonadherent BMDC and then seeded into six-well plates (Nunc) at 10^6 cells/ml in DC medium.

Infection of BMDC. A previously characterized clinical *B. pseudomallei* isolate of low virulence (NCTC 13179) was used throughout the present study for investigating the effect of *B. pseudomallei* infection on DC migration (30). Cultured BMDC (10^6 cells/ml in DC media) were infected with log-phase *B. pseudomallei* (cultured in tryptic soy broth (Acumedia) then washed with phosphate-buffered saline [PBS]) at a multiplicity of infection (MOI) 1:1 and incubated in 5% CO_2 at 37°C as previously described (15, 33). *E. coli* lipopolysaccharide (LPS; 50 ng/ml; Sigma-Aldrich) was used as a positive stimulant to enable comparison and show positive DC migration (34). To determine whether *B. pseudomallei* isolates of high and low virulence differentially influenced the migration of BMDC *in vitro*, where specified, BMDC were also infected with NCTC 13178, a highly virulent clinical *B. pseudomallei* isolate at an MOI of 1:1 (30). Antibiotic protection of BMDC was required to enable enumeration of intracellular bacteria only and prevent uncontrolled extracellular bacterial replication from affecting BMDC viability. *B. pseudomallei* is resistant to a large range of antibiotics but isolates used in the present study are known to be sensitive to kanamycin (15, 35). Therefore, kanamycin (250 μg/ml; Sigma-Aldrich) was added after 4 h of infection, to eliminate extracellular bacteria.

Flow cytometric analysis of BMDC phenotype. Expression of CD11c, CD86, MHC class II, and CCR7 on BMDC was analyzed by flow cytometry. Uninfected and *B. pseudomallei*-infected BMDC were fluorescently stained with a combination of anti-mouse CD11c fluorescein isothiocyanate (FITC; BD Biosciences), anti-mouse CD86 PE (BD Biosciences), anti-mouse CCR7 PerCP-Cy5.5 (eBioscience), and anti-mouse I-A/I-E PE (MHC class II; BD Biosciences). All flow cytometry was performed using fluorescence-activated cell sorting (FACS) with a FACSCalibur apparatus and CellQuest software (BD Biosciences), and postacquisition analysis was performed using FlowJo software (Tree Star, Inc.).

Assessment of intracellular *B. pseudomallei* within BMDC. Intracellular *B. pseudomallei* were released from BMDC by lysing cells with 0.1% Triton-X in sterile deionized water. The lysates were serially diluted (10-fold) in PBS (pH 7.2), and 10-μl aliquots of each dilution were plated in triplicate on Ashdown agar. Agar plates were incubated for 48 h at 37°C prior to enumerating bacteria.

***In vitro* BMDC migration assay.** Mature DC expressing CCR7 migrate toward CCL19 and/or CCL21 (14, 36). To determine whether *B. pseudomallei* stimulated *in vitro* migration of BMDC, we assessed the movement of uninfected and *B. pseudomallei*-infected BMDC from the upper chamber of a Transwell plate (Corning; Sigma-Aldrich), through a 5-μm-pore polycarbonate membrane toward a chemokine in the lower chamber. Prior to conducting the assay, the Transwell plates were equilibrated by filling the upper and lower chambers with RPMI 1640 containing 1% HI-FBS and then incubated for 1 h at 37°C in 5% CO_2. Uninfected, *B. pseudomallei*-infected and LPS-stimulated BMDC were seeded into the upper chamber (10^6 cells/well). Chemokines, CCL19 (100 ng/ml; Peprotech [Abacus ALS]) and/or CCL21 (100 ng/ml; Peprotech), were added to the lower chambers where appropriate, and cultures incubated at 37°C in 5% CO_2 for 2 h (25, 36). The migrated BMDC were collected from the lower chambers, fixed with 4% paraformaldehyde (ProSciTech), washed, and resuspended in 300 μl of PBS (pH 7.2). Countbright absolute counting beads (Invitrogen) were added immediately prior to performing flow cytometric acquisition. The number of Countbright beads and cell events counted was then used to determine the cell concentration and the total number of migrated cells.

***In vivo* BMDC migration assay.** The *in vivo* migration of mature *B. pseudomallei*-infected BMDC from the footpad injection site to the draining popliteal lymph node (pLN) was assessed. Uninfected, *B. pseudomallei*-infected and LPS-stimulated BMDC were fluorescently stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; 8 μM [Molecular Probes]), incubated at 37°C for 10 min with gentle inverting. The reaction was stopped by adding RPMI 1640 containing 10% HI-FBS. Washed CFSE-labeled BMDC were rested for 1 h at 37°C, and then 10^6 BMDC (40 μl) were injected into the left hind footpads of C57BL/6 mice. At designated time points (18, 24, or 36 h) postinjection (p.i.) of BMDC, the draining pLN was harvested, macerated, and digested with collagenase (type IV, 400 U/ml; Gibco/Life Technologies Australia Pty, Ltd.) for 30 min at 37°C to release the DC. Washed pLN cells were stained with anti-mouse CD11c biotin (BD Biosciences) and streptavidin-PE (BD Biosciences). Countbright beads were added to samples prior to analysis. BMDC that had migrated from the footpad to the pLN were identified as $CFSE^+$/$CD11cPE^+$ cells by flow cytometry. The percentage of $CFSE^+/CD11c^+$ BMDC that migrated to the pLN was calculated.

Assessment of *B. pseudomallei* dissemination by BMDC. The dissemination of *B. pseudomallei* by BMDC was assessed following footpad injection of *B. pseudomallei*-infected BMDC or *B. pseudomallei* alone. Previous data indicate that BMDC internalize between 1 and 10% *B. pseudomallei* when infected at an MOI of 1:1. Over 24 h, BMDC exhibit killing of intracellular *B. pseudomallei*. Consequently, the number of intracellular bacteria within BMDC infected with *B. pseudomallei* at an MOI of 1:1 can range from 10^2 to 10^4 CFU (15). Therefore, to compare cell-dependent versus independent dissemination of *B. pseudomallei*, the left hind footpad of C57BL/6 mice was injected with 10^6 *B. pseudomallei*-infected BMDC (10^4 CFU of intracellular bacteria) or 10^4 CFU *B. pseudomallei* (NCTC 13179). The actual number of intracellular *B. pseudomallei* within 10^6 BMDC injected into mice was subsequently determined to be 2×10^2 CFU/10^6 BMDC. On day 1 p.i., mice were euthanized to harvest the draining pLN, inguinal LN (iLN), spleen, lung, and blood. Organs were macerated in PBS (pH 7.2); serial 10-fold dilutions of tissue homogenates were then performed, and aliquots were plated in triplicate on Ashdown agar. Agar plates were incubated for 48 h at 37°C prior to enumerating the CFU.

Internalization by infection site DC and dissemination of *B. pseudomallei*. Internalization of *B. pseudomallei* by skin DC at the site of infection (footpad) and dissemination of *B. pseudomallei* to the draining

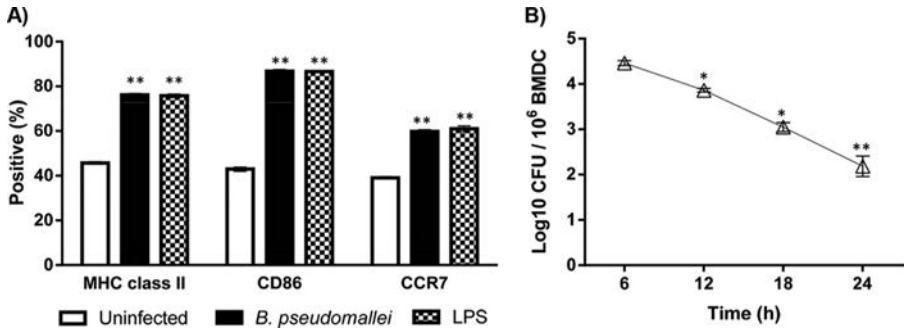

FIG 1 Phenotype and bacterial load of BMDC infected with *B. pseudomallei*. (A) The expression of MHC-II, CD86, and CCR7 on uninfected, *B. pseudomallei*-infected, and LPS-stimulated BMDC was determined by flow cytometry. The expression of DC maturation markers, MHC-II, CD86, and CCR7 on BMDC was significantly increased following 24 h of infection with *B. pseudomallei* (NCTC13179) or LPS. (B) The intracellular survival of *B. pseudomallei* within BMDC significantly decreased between 6 and 24 h. The data depict the mean ± SEM of two experiments. *, $P \leq 0.05$; **, $P \leq 0.01$.

pLN and spleen was investigated by determining the bacterial burden of DC ($CD11c^+$) and non-DC ($CD11c^-$) cell fractions within each organ. *B. pseudomallei* (NCTC 13179; 10^6 CFU) was injected into the left hind footpad of C57BL/6 mice. Footpads were excised at 2 and 4 h p.i. ($n = 3$ per time point). The draining pLN and spleen were harvested 24 and 48 h p.i. ($n = 3$ per time point). Each organ was macerated and digested with collagenase as described above. DC ($CD11c^+$) and non-DC ($CD11c^-$) fractions were prepared using anti-mouse CD11c biotin (BD Biosciences) and streptavidin-magnetic nanoparticles (BD Biosciences). Intracellular *B. pseudomallei* were released by lysing DC and non-DC cell fractions with 0.1% Triton-X. Lysates were serially diluted (10-fold), and aliquots were plated in triplicate on Ashdown agar. After 48 h of culture, intracellular bacteria within DC ($CD11c^+$) and non-DC ($CD11c^-$) cell fractions were enumerated. An aliquot of each cell fraction was reserved to assess the purity of DC ($CD11c^+$) fractions. DC and non-DC fractions were fluorescently stained with anti-mouse CD11c FITC (BD Biosciences) and anti-mouse CD45 PerCP (BD Biosciences), and the percentage of $CD45^+$/$CD11c^+$ cells were determined by flow cytometry. The average purity (± the standard errors of the mean [SEM]) of DC fractions isolated from the footpad, pLN, spleen, and lung was 93.4% ± 1.9%, 85.3% ± 3.2%, 91.3% ± 1.1%, and 87.9% ± 5.3%, respectively.

Statistical analysis. Statistical analysis of data was performed using GraphPad Prism 6 Software. Comparisons between time points and stimulation groups were tested for significance using a two-way analysis of variance (ANOVA) with Tukey's post hoc multiple-comparison test. The intracellular survival of *B. pseudomallei* within BMDC over time (6, 12, 18, and 24 h) was tested using a one-way ANOVA with Turkey's post hoc multiple-comparison test. Differences between tested groups were considered significant if the *P* value was ≤0.05, and this is indicated in the figures by asterisks (*, $P \leq 0.05$; **, $P \leq 0.01$).

RESULTS

Maturation and bactericidal activity of *B. pseudomallei*-infected BMDC. Increased expression of DC maturation markers (MHC-II, CD86, and CCR7) on BMDC occurred following *in vitro* infection with *B. pseudomallei* (NCTC13179; Fig. 1A). CD11c was consistently expressed on uninfected and infected BMDC throughout the 24-h culture period (data not shown). After 24 h, *B. pseudomallei*-infected and LPS-stimulated (positive control) BMDC demonstrated significantly increased MHC-II and CD86 expression compared to uninfected BMDC (Fig. 1A). In response to *B. pseudomallei* infection and LPS stimulation, the level of CCR7 significantly increased on BMDC. DC maturation coincided with bactericidal activity against intracellular *B. pseudomallei* (NCTC 13179), with significantly decreased intracellular survival of *B. pseudomallei* between 6 and 24 h (Fig. 1B). After 24 h of coculture, the number of surviving bacteria was 2.18 ± 0.23 $\log_{10}$ CFU (2×10^2 CFU)/10^6 BMDC.

Migrated BMDC traffic *B. pseudomallei in vitro*. BMDC infected with *B. pseudomallei* (NCTC 13179) for 18 or 24 h demonstrated significantly increased migration compared to uninfected BMDC (Fig. 2A). *B. pseudomallei*-infection or LPS stimulation of BMDC elicited similar migration responses *in vitro* (Fig. 2B). BMDC migration in the presence of the chemokines CCL19 and CCL21 alone (Fig. 2B) or in combination (data not shown) was significantly increased compared to chemokine-free media. However, *B. pseudomallei*-infected BMDC migration toward CCL19 was significantly higher than CCL21. Comparison of BMDC migration in response to *B. pseudomallei* isolates of high (NCTC 13178) and low (NCTC 13179) virulence indicated that *B. pseudomallei* virulence did not affect BMDC migration (Fig. 2C). Therefore, the low-virulence *B. pseudomallei* isolate, NCTC 13179, was used for the *in vivo* migration studies. The movement of *B. pseudomallei* from the upper chamber to the lower chamber of an *in vitro* migration assay in a cell-dependent (*B. pseudomallei*-infected BMDC) compared to an independent (*B. pseudomallei* alone) manner was subsequently determined. Persistence of intracellular *B. pseudomallei* within migrated, mature BMDC was demonstrated *in vitro*, which suggests that *B. pseudomallei* could potentially use DC migration to facilitate dissemination in a cell-dependent manner *in vivo* (Fig. 2D). Overall, *B. pseudomallei* did not impair the *in vitro* migratory capacity of BMDC. Furthermore, migration of BMDC facilitated *in vitro* trafficking of *B. pseudomallei*.

Mature *B. pseudomallei*-infected BMDC migrate from the skin to the pLN. *In vivo* migration of *B. pseudomallei*-infected BMDC ($CFSE^+$/$CD11c^+$) from the skin to the draining pLN was assessed following footpad injection of mice (Fig. 3A, B, and C). BMDC infected *in vitro* with *B. pseudomallei* for 18 and 24 h prior to footpad injection, demonstrated significant *in vivo* migration compared to uninfected BMDC (migrated $CFSE^+$/$CD11c^+$ BMDC [%] at 18 h: uninfected [0.15 ± 0.06] versus *B. pseudomallei*-infected [0.56 ± 0.18], $P \leq 0.05$; migrated $CFSE^+$/$CD11c^+$ BMDC [%] at 24 h: uninfected [0.08 ± 0.04] versus *B. pseudomallei*-infected [0.71 ± 0.02], $P \leq 0.01$). Peak *in vivo* migration of *B. pseudomallei*-infected BMDC, from the injection site to the pLN, was observed at 24 h p.i. (migrated $CFSE^+$/$CD11c^+$ BMDC [%] at 18 h p.i. = 0.02 ± 0.01, 24 h p.i. = 0.71 ± 0.02, and 36 h p.i. = 0.18 ± 0.02). Using the optimal *in vitro* infection time (24 h of culture) and *in*

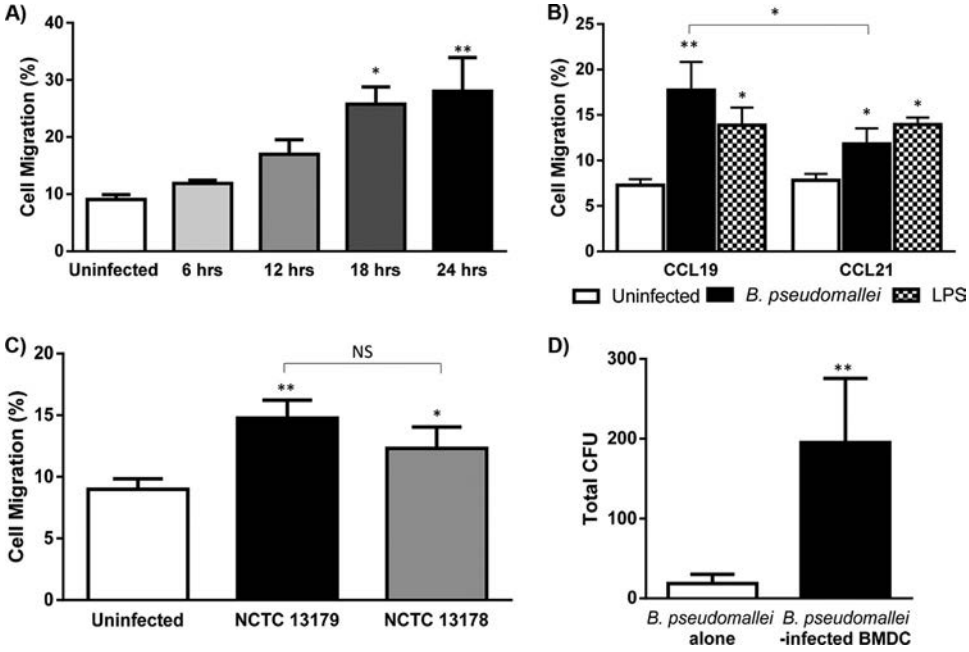

FIG 2 Migration of BMDC infected with *B. pseudomallei*. The *in vitro* migration of uninfected, *B. pseudomallei*-infected, and LPS-stimulated BMDC toward CCR7 ligands CCL19 and CCL21 was assessed. (A) *B. pseudomallei*-infected (NCTC 13179) BMDC cultured for 18 to 24 h *in vitro* demonstrated significantly increased migration in the presence of CCL19 compared to uninfected BMDC. (B) Significant numbers of *B. pseudomallei*-infected (NCTC 13179) and LPS-stimulated BMDC migrated toward the CCR7 ligands, CCL19 (100 ng/ml), and CCL21 (100 ng/ml). The migration of *B. pseudomallei*-infected BMDC toward CCL19 was significantly greater compared to CCL21. (C) The migration of BMDC infected with isolates of high (NCTC 13178) and low (NCTC 13179) virulence for 24 h was comparable. (D) Subsequently, the movement of *B. pseudomallei* from the upper chamber to the lower chamber of an *in vitro* migration assay in a cell-dependent (*B. pseudomallei*-infected BMDC) compared to an independent (*B. pseudomallei* alone) manner was determined. The presence of viable intracellular *B. pseudomallei* within BMDC that migrate *in vitro* demonstrates the propensity of DC to traffic *B. pseudomallei*. Bars in panels A to C depict mean ± SEM of three separate experiments. Bars in panel D depict the mean ± standard deviation of one experiment with six replicate wells. *, $P \leq 0.05$; **, $P \leq 0.01$; NS, not significant.

vivo assay time (24 h p.i.), it was observed that *B. pseudomallei*-infected (NCTC 13179) and LPS-stimulated BMDC elicited similar migration responses *in vivo*, which were significantly higher than that observed for uninfected BMDC (Fig. 3D). Although *B. pseudomallei* infection caused significantly increased migration of BMDC to the pLN, <1% of the BMDC injected into the footpad were detected in the draining pLN 24 h p.i.

BMDC migration facilitates *B. pseudomallei* dissemination. To compare cell-dependent versus independent dissemination of *B. pseudomallei*, the organ bacterial loads were enumerated 24 h after footpad of injection of *B. pseudomallei*-infected BMDC or *B. pseudomallei* alone (NCTC 13179). Dissemination of *B. pseudomallei* to the draining pLN, iLN, spleen, lung, and blood occurred after injection of *B. pseudomallei*-infected BMDC or *B. pseudomallei* alone (Fig. 3E). As shown previously, the number of intracellular *B. pseudomallei* within 10^6 BMDC injected into mice was 2×10^2 CFU/10^6 BMDC. Although mice injected with *B. pseudomallei* alone (10^4 CFU) actually received 50 times the number of CFU compared to *B. pseudomallei*-infected BMDC, the bacterial burden of the spleen and lung was significantly higher in C57BL/6 mice that received *B. pseudomallei*-infected BMDC compared to *B. pseudomallei* alone.

Infection site DC internalize *B. pseudomallei* and facilitate dissemination. Internalization of *B. pseudomallei* by skin DC at the infection site and dissemination of *B. pseudomallei* to secondary lymphoid tissues was subsequently investigated. C57BL/6 mice were infected with *B. pseudomallei* (NCTC 13179) via the footpad. The number of intracellular *B. pseudomallei* within skin DC ($CD11c^+$) and non-DC ($CD11c^-$) fractions isolated from the footpad at 2 and 4 h postinfection were enumerated (Fig. 4A). The ratio of intracellular *B. pseudomallei* within infection site skin DC was significantly higher than non-DC at 2 h postinfection. Dissemination of *B. pseudomallei* within DC to the draining pLN (6.26 ± 4.25 *B. pseudomallei*/10^3 DC) and spleen (Fig. 4B) was observed at 24 h postinfection. The ratio of *B. pseudomallei* to spleen DC increased between 24 and 48 h postinfection (Fig. 4B). However, by 48 h postinfection, the bacteria were cleared from the pLN (data not shown), and no intracellular *B. pseudomallei* was detected within DC and non-DC fractions in the lungs (data not shown). Overall, in support of our hypothesis, skin DC at the site of infection internalized *B. pseudomallei* and disseminated *B. pseudomallei* to secondary lymphoid tissue.

DISCUSSION

This is the first study to investigate *in vitro* and *in vivo* migration of DC following infection with *B. pseudomallei*. BMDC infected with *B. pseudomallei in vitro* developed a mature phenotype, indicated by increased expression of MHC-II and CD86, and elicited bactericidal activity against intracellular *B. pseudomallei*, as shown in previous studies (15, 17). Migration of DC from the site of infection to secondary lymphoid organs requires upregulation of CCR7, enabling recognition of migratory cues by CC-chemokines, CCL19 and CCL21 (14, 36). Intracellular bacteria such as *F. tularensis* stimulate DC migration by triggering DC maturation

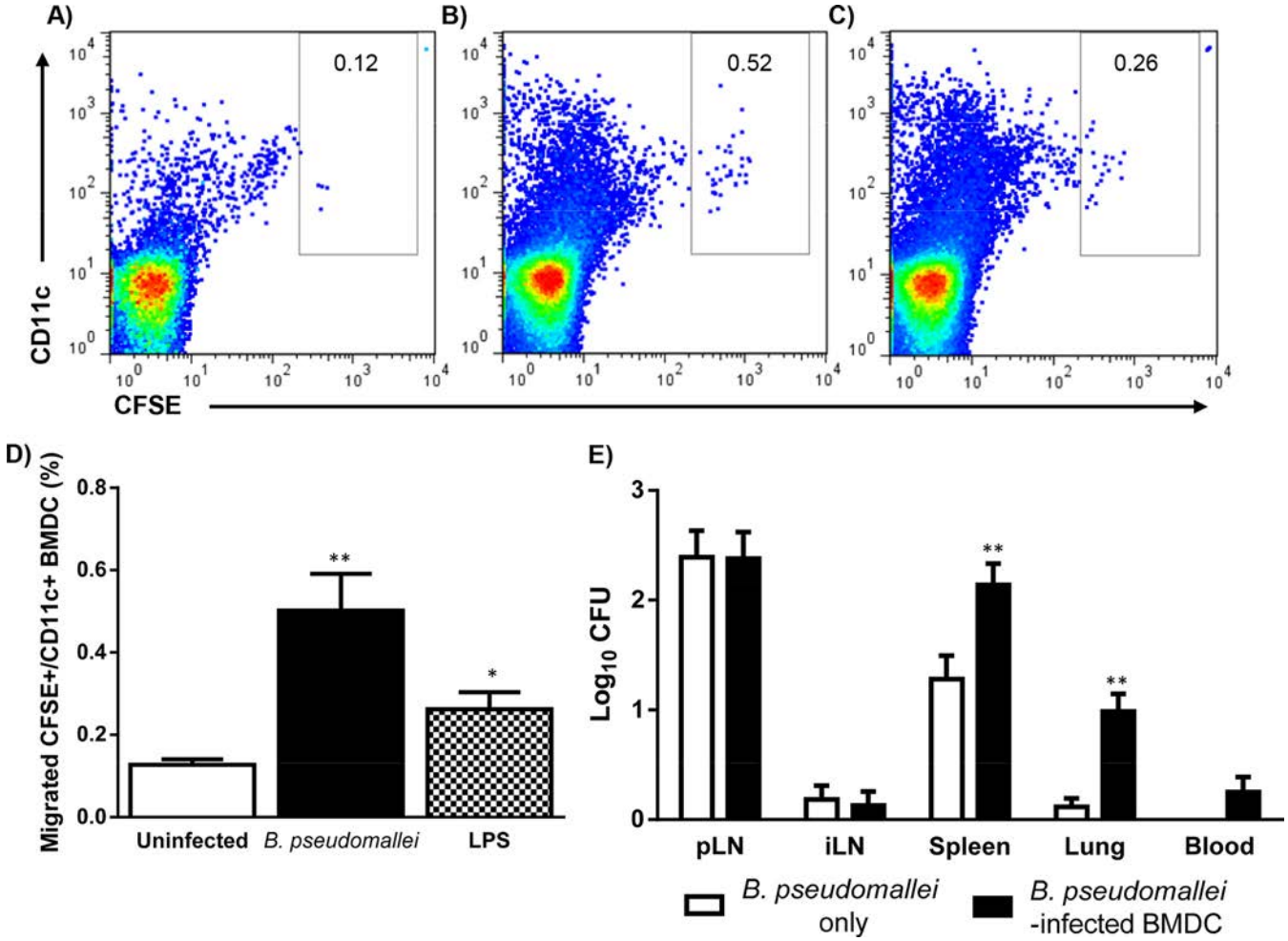

FIG 3 *In vivo* migration of *B. pseudomallei*-infected BMDC and dissemination of *B. pseudomallei*. The *in vivo* migration of *B. pseudomallei*-infected BMDC from the site of injection to the draining pLN was assessed using CFSE labeling and flow cytometry. Representative FACS plots demonstrate increased numbers of migrated BMDC ($CFSE^+/CD11c^+$) in the draining pLN 24 h after mice were injected with uninfected (A), *B. pseudomallei*-infected (NCTC 13179) (B), and LPS-stimulated (C) BMDC (stimulated for 24 h *in vitro*). (D) The migration of *B. pseudomallei*-infected BMDC into pLN was significantly greater compared to uninfected BMDC at 24 h p.i. No significant difference was observed between migration of *B. pseudomallei*-infected and LPS-stimulated BMDC. (E) Bacterial burden of the draining pLN, iLN, spleen, lung, and blood was assessed at 24 h after footpad injection of mice with *B. pseudomallei*-infected BMDC ($n = 5$) or *B. pseudomallei* alone ($n = 5$). The bacterial burdens of the spleens and lungs were significantly higher in C57BL/6 mice that received *B. pseudomallei*-infected BMDC compared to *B. pseudomallei* alone. Bacteria were not detected in the blood of mice infected with *B. pseudomallei* alone. Bars depict the mean ± SEM of three experiments. *, $P \leq 0.05$; **, $P \leq 0.01$.

and the upregulation of CCR7 expression (25). In contrast, *Brucella suis* infection does not activate DC maturation and increased CCR7 expression (37). In the present study, CCR7 expression was significantly increased on BMDC infected with *B. pseudomallei* compared to uninfected BMDC, suggesting that they have the capacity for migration. Although CCR7 expression is essential for DC migration, intracellular bacteria such as *Y. pestis* are known to impair the migration of $CCR7^+$ DC via mechanisms independent from the CCR7/CCL19/CCL21 axis (23).

In vitro migration of mature *B. pseudomallei*-infected BMDC toward the CCR7 ligands, CCL19 and CCL21, was subsequently demonstrated. Migration of BMDC infected with *B. pseudomallei* isolates of high (NCTC 13178) and low (NCTC 13179) virulence was comparable *in vitro*. Both CCL19 and CCL21 are expressed within lymphoid organs and bind to CCR7 with similar affinities. Studies have demonstrated that CCL19 is more potent at attracting DC migration and provides directional signaling for homing DC migration to T-cell-rich areas within secondary lymphoid organs (14, 36). In contrast, initiation of DC migration is via CCL21, the only CCR7 ligand expressed in peripheral tissues on high endothelial venules and lymphatic endothelial cells (14, 38). Therefore, the migration of *B. pseudomallei*-infected BMDC toward both CCL19 and CCL21 was investigated. In the present study, *B. pseudomallei* stimulated migration of DC toward both CCR7 ligands, although the chemokine CCL19 attracted significantly higher migration of *B. pseudomallei*-infected BMDC compared to CCL21. Importantly, the presence of viable intracellular *B. pseudomallei* within *in vitro*-migrated BMDC demonstrates the propensity of DC to traffic *B. pseudomallei*.

The findings of the *in vitro* assays were confirmed in subsequent *in vivo* migration studies by injecting mice with BMDC infected *in vitro* with *B. pseudomallei*. In the present study,

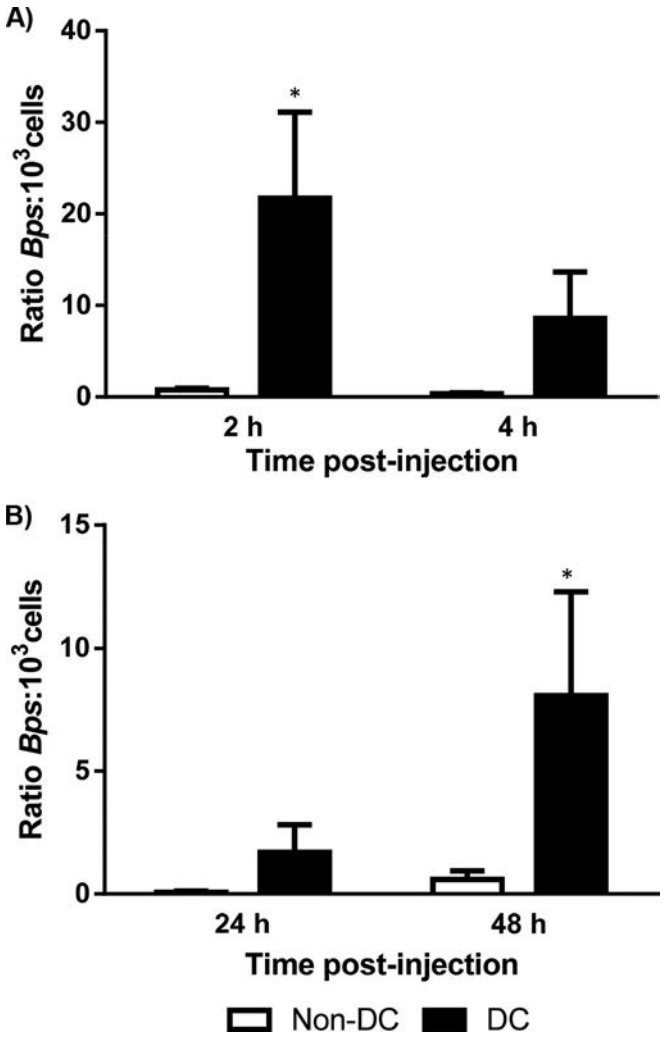

FIG 4 Internalization and dissemination of *B. pseudomallei* by DC. The internalization of *B. pseudomallei* (*Bps*) by skin DC at the footpad infection site and the dissemination of *B. pseudomallei* to the spleen was determined by quantifying the bacterial burden of DC ($CD11c^+$) and non-DC ($CD11c^-$) fractions for each organ. (A) Skin DC at the site of infection internalized *B. pseudomallei*. The ratio of intracellular *B. pseudomallei* within skin DC (*Bps*:10^3 cells) at the site of infection was significantly higher than non-DC at 2 h postinfection. (B) Dissemination of *B. pseudomallei* within DC to the spleen was observed at 24 and 48 h postinfection. At 48 h postinfection, the ratio of intracellular *B. pseudomallei* within DC (*Bps*:10^3 cells) was significantly higher than non-DC in the spleen. The increased ratio of *B. pseudomallei* to spleen DC (*Bps*:10^3 cells), at 48 h postinfection, indicates intracellular replication of *B. pseudomallei* within spleen DC. Bars depict mean ± SEM of three experiments. *, $P \leq 0.05$.

B. pseudomallei-infected BMDC demonstrated significant migration from the site of injection to the draining pLN in comparison to uninfected BMDC. However, <1% of *B. pseudomallei*-infected BMDC migrated to the draining LN, a relatively low number compared to published studies on DC migration (34). Martìn-Fontecha et al. (34) reported 3% migration for LPS-stimulated BMDC in BALB/c mice. However, differences in the *in vivo* migration observed between the present study and that by Martìn-Fontecha et al. may reflect variation in the migratory capacity of DC isolated from different mouse strains. *In vivo* migration of DC within BALB/c infected with *Leishmania major* was found to be 8 times higher than that observed in C57BL/6 mice (39). Comparison of the *in vivo* migratory response of *B. pseudomallei*-infected BMDC from C57BL/6 and BALB/c mice was not feasible due to the high susceptibility of BALB/c mice. The subcutaneous 50% lethal dose for BALB/c mice infected with the low-virulence strain of *B. pseudomallei* (NCTC 13179) used in the present study was 9×10^2 CFU compared to $>10^8$ CFU for *B. pseudomallei*-resistant C57BL/6 mice (30). This is further complicated by the impaired *B. pseudomallei* killing efficiency of BMDC derived from BALB/c mice (15). Therefore, BALB/c mice received a lethal higher dose of bacteria when injected with 10^6 BMDC compared to C57BL/6 mice (data not shown). Consequently, BALB/c mice may not be a suitable model for investigating the *in vivo* migration of BMDC infected with *B. pseudomallei*. There is scope for future studies to investigate the dissemination of *B. pseudomallei* by tissue resident DC in BALB/c mice infected with an appropriate low dose of bacteria. Overall, using the C57BL/6 model, migration of *B. pseudomallei*-infected BMDC exhibit *in vivo* migration, similar to LPS-stimulated BMDC, which was significantly increased compared to uninfected immature BMDC.

The dissemination of *B. pseudomallei* was facilitated by the migration of mature *B. pseudomallei*-infected BMDC from the site of injection. Despite relatively low numbers of migratory *B. pseudomallei*-infected BMDC, significant numbers of *B. pseudomallei* were isolated from the spleens and lungs of mice by 24 h p.i. Furthermore, the combined bacterial burden within pLNs, iLNs, spleens, and lungs of mice was higher than the original number of intracellular *B. pseudomallei* surviving within the injected BMDC. In the present study, total bacterial loads were determined in tissue homogenates of organs rather than within DC extracted from the organ. Therefore, it was not possible to distinguish whether *B. pseudomallei* replication has occurred within BMDC or after escape from DC. Regardless, these findings demonstrate that DC migration facilitated the dissemination of *B. pseudomallei*.

Prior to the present study, the role of skin DC at the site of *B. pseudomallei* infection had not been investigated. We found here that skin DC internalized *B. pseudomallei* within the footpad infection site and facilitated the dissemination of *B. pseudomallei* to the pLN and spleen. Since the percentage of DC within the footpad, pLN, and spleen ranges between 0.5 and 4% of the nucleated cell population, the ratio of *B. pseudomallei* per 10^3 cells was determined for DC and non-DC fractions from each organ (40). At the site of infection, the ratio of *B. pseudomallei* per 10^3 skin DC was significantly higher compared to non-DC at 2 h postinfection, although these numbers decreased by 4 h postinfection. Unlike the draining pLN, the ratio of *B. pseudomallei* per 10^3 spleen DC increased between 24 and 48 h. Collectively, our data demonstrate internalization of *B. pseudomallei* by skin DC at the site of infection and significant association of disseminated *B. pseudomallei* within DC in secondary lymphoid organs. Furthermore, DC within the spleen, but not the site of infection or draining LN, appears to provide an intracellular niche for *B. pseudomallei* replication.

A limitation of the present study is that extensive phenotyping of BMDC and tissue resident DC was not performed. Although the BMDC culture method used in the current study is well established for generating a nonadherent high $CD11c^+$ cell with DC morphology, it does not achieve 100% purity. Furthermore, whereas high CD11c expression is distinctive for DC, CD11c is not

exclusively expressed on DC (32, 41). Therefore, additional low frequencies of CD11c-expressing cell types, such as inflammatory macrophages, could potentially have been included within the tissue resident DC fractions. In addition, without extensive DC phenotyping it is not possible to distinguish migratory DC from tissue resident DC in the current study. Since it is plausible that *B. pseudomallei* spreads from migratory skin DC to resident spleen DC, future studies employing techniques for more extensive phenotyping will enable determination of which DC subset/s (migratory skin DC, resident spleen DC, or both) facilitate *B. pseudomallei* persistence in the spleen. Importantly, our findings identify $CD11c^+$ DC migration as a mechanism for *B. pseudomallei* cell-dependent dissemination. These findings warrant further characterization studies, possibly using transgenic mice with specific GFP expression in DC or extensive characterization of the DC phenotype, to confirm the beneficial as opposed to the detrimental role of DC subsets and indeed other phagocytic cells involved in the *in vivo* trafficking of *B. pseudomallei*.

In summary, we investigated the effect of *B. pseudomallei* on the migration of DC and their potential to inadvertently facilitate systemic dissemination of the bacteria. After *B. pseudomallei* infection, BMDC demonstrated significant *in vitro* and *in vivo* migration compared to uninfected BMDC. Although DC are capable of killing internalized *B. pseudomallei*, the findings presented here indicate that the persistence of small numbers of *B. pseudomallei* within DC enabled the dissemination of bacteria to other organs via DC migration. Furthermore, *B. pseudomallei* replicated inside spleen DC in mice contradicting our *in vitro* studies whereby murine BMDC were capable of killing intracellular *B. pseudomallei*. Previous *in vitro* studies using human and murine DC have consistently shown that DC grown under *in vitro* culture conditions and protected by antibiotics are capable of killing intracellular *B. pseudomallei* (15, 17). However, organ-specific differences in the ability of DC to kill intracellular *B. pseudomallei* were observed *in vivo*, in particular within the spleen, where DC were a site for *B. pseudomallei* persistence. This highlights the need for caution when interpreting *in vitro* studies and the requirement for subsequent studies using animal models to determine the true *in vivo* relevance of *in vitro* observations. Further studies are warranted to determine which DC subset(s) (migrating skin DC, resident spleen DC, or both) facilitates *B. pseudomallei* persistence in the spleen. Notably, this is the first evidence that DC migration facilitates dissemination of *B. pseudomallei* to secondary lymphoid organs. The migration of mature *B. pseudomallei*-infected DC to secondary lymphoid organs suggests that these cells have the potential to present antigen to naive T cells. Given that DC migration is central to the development of protective adaptive immune responses to infection, clarification of the contribution of DC migration to disease progression, as opposed to the development of protection, in *B. pseudomallei* infection is of paramount importance and may facilitate the identification of potential novel immunomodulatory therapies and preventative strategies.

ACKNOWLEDGMENT

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Evidence of *Burkholderia pseudomallei*-Specific Immunity in Patient Sera Persistently Nonreactive by the Indirect Hemagglutination Assay▽

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The indirect hemagglutination assay (IHA) is the most frequently used serological test to confirm exposure to *Burkholderia pseudomallei*. Patients with culture-confirmed disease often have a nonreactive IHA at presentation and occasionally fail to seroconvert on serial testing. We investigated whether using antigens derived from the cultured isolates of persistently IHA-nonreactive patient sera improved the sensitivity of the IHA. In addition, we assessed the antigen-specific lymphocyte response in this group of patients to a panel of *B. pseudomallei* antigens, including those derived from their own cultured isolates. Eleven patients with culture-proven melioidosis were identified as having persistently IHA-nonreactive sera. A modified IHA using erythrocytes sensitized with patient isolate-derived antigen tested against convalescent-phase serum was performed. The majority (82%) of sera showed a negative (≤1:5) result, one was borderline (1:20), and one was positive at the cutoff value (1:40). IHA-nonreactive sera were also tested by enzyme immunoassay (EIA), with 73% (8/11) demonstrating IgG positivity. In addition, lymphocytes isolated from persistently IHA-nonreactive patient sera demonstrated significantly increased proliferation in response to *B. pseudomallei* antigens compared to controls. These studies demonstrate the presence of *B. pseudomallei*-specific antibody by EIA and *B. pseudomallei*-specific lymphocytes in patient sera categorized as persistently nonreactive according to the IHA. New immunoassays are required and should incorporate *B. pseudomallei* antigens that are immunoreactive for this subset of IHA-nonreactive patient sera.

Melioidosis, a disease endemic to northern Australia and southeast Asia, is caused by the Gram-negative soil saprophyte *Burkholderia pseudomallei*. It causes significant morbidity and mortality, with a wide spectrum of clinical presentations (15, 19). The gold standard for the diagnosis of melioidosis is culture from clinical specimens. However, serological and possibly cellular assays may have an adjunctive role in certain situations, such as screening travelers returning from areas of endemicity with a febrile illness or aiding diagnosis in unusual presentations (e.g., chronic disease) or where specimens for culture may be unavailable (e.g., deep brain abscesses). It may also provide supportive information if melioidosis is suspected but the organism fails to grow. Simple, rapid, and reliable serological tests for melioidosis hold the possibility of identifying cases earlier and thereby improving outcomes, given that culture and identification of *B. pseudomallei* can be delayed. Furthermore, early diagnosis and selection of appropriate antimicrobial therapy may help reduce the significant mortality associated with the disease.

The indirect hemagglutination assay (IHA) has been the mainstay of serological testing for melioidosis over many years, and the technique has remained largely unchanged since first described over 40 years ago (12). Despite variable sensitivity and specificity, it remains the most commonly employed serological test, with titers of 1:40 or greater considered reactive in Australia (2). High background rates of seropositivity have been described in areas of endemicity and can reduce the utility of the test (16). Several patterns of serological responses have been described in previous studies that examined serial IHA testing over time, including late seroconversion and persistently reactive and persistently nonreactive tests as well as seroreversion (8, 11). The range of titers in seropositive specimens is often wide. A critical limitation of the assay is the lack of standardization between laboratories with respect to the antigens used. The IHA relies upon the agglutination of sheep red cells in the presence of serum antibodies to polysaccharide and lipopolysaccharide antigens derived from defined strains of *B. pseudomallei*. However, these antigens remain poorly characterized and are likely to be variable between isolates.

In the Townsville Hospital (Queensland, Australia) cohort of patients with culture-proven melioidosis, approximately 50% were seronegative by IHA at presentation (11). Furthermore, a significant proportion (approximately 10%) of these failed to seroconvert on serial testing over time, with no specific clinical features clearly associated with this phenomenon (11). We postulated that the limited number of *B. pseudomallei* strains used to provide sensitizing antigens for the IHA may account for this failure to detect the presence of antibody. Thus, in this study we aimed to employ antigens derived from *B. pseudomallei* isolates cultured from individual IHA-nonreactive patient

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serum samples to be tested in an IHA format against convalescent-phase sera from the same patients. IHA-nonreactive sera were also tested by enzyme immunoassay (EIA) to detect *B. pseudomallei*-specific IgG. We also performed lymphocyte proliferation assays to determine whether patients who had IHA-nonreactive sera had developed a *B. pseudomallei*-specific cell-mediated immune (CMI) response (13). Our hypothesis was that patients with persistently IHA-nonreactive sera may demonstrate seroreactivity if antigens derived from their own cultured isolates were used in the assay preparation. Since antibody detection may not necessarily reflect the totality of the patient's immune response or protective immunity, we aimed to additionally demonstrate evidence of specific CMI to *B. pseudomallei* in these individuals.

MATERIALS AND METHODS

Study setting and patient selection. Townsville Hospital, a tertiary referral center for tropical north Queensland, services a population of approximately 250,000. Melioidosis is a relatively common condition in this region, especially during the rainy season (approximately from December to February). We also receive isolates from patients admitted and treated in district hospitals. We retrospectively examined the records of all patients with culture-confirmed melioidosis from clinical specimens in our laboratory between January 1996 and January 2010. All patients showing a persistently nonreactive IHA (tested at baseline and at least 1 month after presentation) and for whom sera and bacterial isolates had been stored were included. All available patients with persistently IHA-nonreactive sera who had culture-proven melioidosis were requested to participate in further studies of cell-mediated immunity. Those enrolled were paired with an age- and sex-matched control group of healthy individuals from the same geographic region with no history of melioidosis and IHA-negative serology. Ethical approval for this study was obtained from the Townsville Health Service District Ethics Committee.

Indirect hemagglutination assay. The standard IHA was performed as previously described using sheep erythrocytes sensitized with antigens from five selected strains of *B. pseudomallei* (1). Serum samples were heat inactivated and then incubated with nonsensitized sheep red cells to remove nonspecific agglutinins. Sample sera were then titrated in microwell plates, and antigen-sensitized red cells were added. The presence of antibody was confirmed by red cell agglutination. A titer of ≥1:40 was considered positive. Nonsensitized ovine red cells were used as controls. The modified IHA used different sensitizing antigens, prepared as follows. Isolates of *B. pseudomallei* cultured from each of the patients with persistently IHA-nonreactive sera were taken from frozen storage (−80°C), incubated on Columbia horse blood agar in ambient air at 37°C for 24 h, and checked for purity. Antigens were then prepared from these isolates using a crude heat-killed and filtered extract. Back titrations against known IHA-reactive serum (at a titer of 1:80) were performed on each antigen to determine optimal antigen concentrations. Some antigen preparations demonstrated gross red cell hemolysis unless serially diluted. As a result, optimal concentrations of these antigens could not be determined, and a presumptive 1:40 antigen dilution was used. An IHA with red cells sensitized with patient-derived antigen tested against the patient's own convalescent-phase serum along with low-positive (1:80), high-positive (1:320), and negative (<1:5) controls was then performed using the method previously described by Ashdown (1).

Enzyme immunoassay IgG. The EIA to detect *B. pseudomallei* IgG was performed as previously described, with minor modifications (3). Briefly, antigens were prepared from eight defined strains of *B. pseudomallei* by heating, sonication, and filtration. Optimal antigen dilution was obtained by titration against known IgG-positive and -negative controls. EIA plates (Nunc, Copenhagen, Denmark) were coated with 100 μl of diluted antigen in borate-saline buffer and incubated overnight at 4°C. To eliminate nonspecific binding, the plates were then blocked using 5% bovine serum albumin by incubation at 35°C for 90 min and washed with phosphate-buffered saline (PBS) plus 0.05% Tween 20 solution (PBS-Tween). Samples were then tested in duplicate using 50 μl of patient serum diluted 1:100 with PBS-Tween and incubated at room temperature for 30 min. The plates were then washed, 50 μl of horseradish peroxidase-IgG conjugate (Panbio, Australia) was added, and then plates were incubated for a further 30 min. After another washing step, 100 μl of tetramethylbenzidine and hydrogen peroxide (TMB; Panbio, Australia) substrate was added. After 10 min the reaction was terminated using 1 M phosphoric acid, and the reactions were read by automated plate reader at 450/620 nm (Multiskan; Flow Laboratories, McLean, VA). All test sera were run with negative, low-positive, and high-positive controls. Results were expressed as enzyme immunoassay units (EIU) calculated from the following formula: EIU = (test absorbance − negative-control absorbance)/(low-positive-control absorbance − negative-control absorbance) × 100. EIU values of ≤25 were considered negative, values between 26 and 50 were considered equivocal, and values that were >50 were considered positive.

Lymphocyte proliferation assay. A panel of seven *B. pseudomallei* crude antigens were prepared from seven clinical *B. pseudomallei* strains comprised of six strains isolated from each of the patients with IHA-nonreactive sera who were participating in this study and one previously characterized *B. pseudomallei* isolate of low virulence, NCTC 13179 (13). Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density centrifugation over Ficoll-Paque Plus (GE Healthcare Biosciences, Australia) and cultured as previously described (5). Triplicate wells (1×10^5 PBMC/well) were stimulated with the seven *B. pseudomallei* antigens (1 μg/ml) and phytohemagglutinin ([PHA] positive control; 10 μg/ml). Cultures were then incubated at 37°C with 5% CO_2. Proliferative responses were assessed at 24-h intervals between 96 and 168 h of culture by determining [^{3}H]thymidine incorporation (1.25 μCi/ml for 4 h; GE Healthcare Biosciences, Australia) with outputs measured in counts per minute (cpm). Stimulation indexes (SI) were calculated ($SI = cpm_{stimulated}/cpm_{unstimulated}$). The highest SI recorded over the four time points was selected to compare the proliferative response of PBMC from IHA-nonreactive patient sera to samples from healthy controls.

RESULTS

During the study period from January 1996 to January 2010, 177 patients were identified with culture-confirmed melioidosis. Of those that had serial IHA testing performed, 14 had persistently nonreactive sera over time. Eleven of these patients had stored sera and bacterial isolates available and were included in the study. Six of the patients (two males and four females) with IHA-nonreactive sera were also available for CMI studies.

Indirect hemagglutination assay. During IHA antigen titration, 4 of the 11 *B. pseudomallei* antigen preparations showed gross red cells hemolysis unless serially diluted, possibly suggesting the presence of a hemolysin, a phenomenon that has been previously observed with *B. pseudomallei* (4). Nine samples showed a negative (≤1:5) result in the modified IHA using the individual patient-derived antigen, one was borderline (1:20), and one was positive at the cutoff value (1:40) (Table 1). All nonreactive test sera showed appropriate results in positive and negative controls.

Enzyme immunoassay IgG. All 11 patients with a nonreactive IHA were tested by EIA to detect *B. pseudomallei*-specific IgG. Of these, eight (73%) were positive on at least one convalescent-phase serum sample, two were negative, and one was equivocal (Table 1).

Lymphocyte proliferation. When stimulated with *B. pseudomallei* antigens, lymphocytes from IHA-nonreactive patient sera demonstrated significantly higher proliferation than lymphocytes from controls (Fig. 1). In addition, the *B. pseudomallei*-specific lymphocyte proliferative response of IHA-nonreactive patient sera was elicited in response to both NCTC 13179 and patient isolate-derived antigen.

DISCUSSION

Antigens derived from isolates of *B. pseudomallei* cultured from IHA-nonreactive patient sera did not improve assay sensitivity in a modified IHA. The initial hypothesis that the poor test characteristics of the IHA reflect the use of a limited array

TABLE 1. Comparison of IHA results from culture-positive persistently nonreactive patient sera by a standard IHA with an IHA using patient isolate-derived antigens and EIA IgG

Isolate no.	Standard IHA titer	Modified IHA titer (dilution)[a]	Control IHA titer (dilution)			EIA IgG
			Low positive (1:80)	High positive (1:320)	Nonsensitized red cells	
1	<1:5	<1:5 (1:10)	1:160	1:640	<1:5	Pos
2	<1:5	<1:5 (1:10)	1:80	1:320	<1:5	Equiv
3	<1:5	<1:5 (1:40)	1:80	1:80	<1:5	Neg
4	<1:5	<1:5 (1:40)	1:80	1:320	<1:5	Pos
5	1:5	<1:5 (1:40)	1:160	1:320	<1:5	Pos
6	<1:5	<1:5 (1:10)	1:80	1:1,280	<1:5	Pos
7	<1:5	<1:5 (1:40)	1:80	1:320	<1:5	Pos
8	1:5	1:40 (1:40)	1:320	1:1,280	<1:5	Pos
9	1:10	<1:5 (1:40)	1:40	1:160	<1:5	Pos
10	<1:5	1:5 (1:40)	1:160	1:320	<1:5	Neg
11	1:20	1:20 (1:10)	1:160	1:1,280	<1:5	Pos

[a] Patient isolate-derived antigen.

of sensitizing antigens was not supported by the findings. Although the use of specific antigens derived from individual patients could not demonstrate the presence of antibody in corresponding sera from the same patients, these antigens were recognized appropriately by sera from known seropositive controls. Thus, it seems unlikely that the apparent failure to seroconvert is a product of the isolate itself or the antigens that are presented within the IHA format. A possible explanation might be that these patients had not developed antibodies to the particular antigens that are presented in the IHA format. To our knowledge, characterization of the *B. pseudomallei* antigens displayed by erythrocytes in the IHA has not been investigated. Sera from patients in the current study (4 out of 5) demonstrated seroconversion for hepatitis B surface antibody after vaccination, making anergy an unlikely explanation for the lack of antibodies reactive toward *B. pseudomallei* antigens in the IHA.

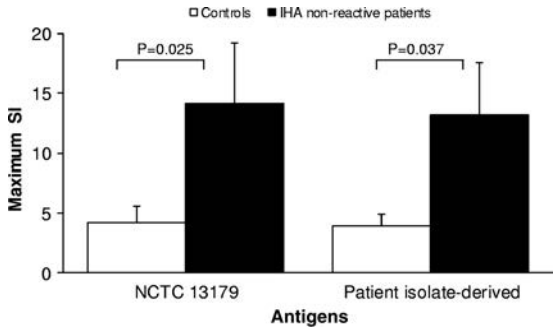

FIG. 1. Lymphocyte proliferation in IHA-nonreactive patient sera in response to *B. pseudomallei* antigens. PBMC isolated from the peripheral blood of IHA-nonreactive patient sera and controls were stimulated with *B. pseudomallei* antigens generated from either NCTC 13179 or individual isolates derived from the IHA-nonreactive patient sera. Proliferative responses were assessed at 24-h intervals between 96 and 168 h of culture by determining [^{3}H]thymidine incorporation. Results are expressed as mean maximum SIs ± standard error of the mean. Maximum SIs were significantly higher in PBMC from patients with IHA-nonreactive sera than in samples from healthy controls following stimulation with antigens from either the patients' own bacterial isolates or isolate NCTC 13179. No significant differences were observed between the maximum SIs of patient PBMC stimulated with either isolate.

Importantly, the majority (73%) of IHA-nonreactive samples were found to be IgG positive by EIA. This suggests that specific antibodies were produced in response to *B. pseudomallei* infection in this subset of individuals; however, these were not directed against or were unable to bind the specific epitopes presented on the surface of erythrocytes in the IHA. These findings provide further evidence to support previous studies demonstrating the poor sensitivity of the IHA and the superior sensitivity of the EIA in the diagnosis of melioidosis (3, 11). However, while the EIA may be more sensitive than the IHA in confirming the diagnosis of melioidosis, the tendency of sera from patients with melioidosis to remain reactive by EIA for long periods after acute infection means that this assay may not be an effective tool for monitoring disease progression. Its specificity for diagnosing acute infection may also be affected by high background seroreactivity in the population (20). The specificity of the EIA, using the same method and in the same population as in this study, has previously been shown to be 95% (3). However, a fundamental problem of most *B. pseudomallei* EIAs is that they are rarely validated outside the laboratory that originally developed the assay or tested in a wide variety of populations. A recent study from Thailand using Bayesian latent-class models to generate new cutoff values for EIAs that employed five different antigens has suggested that the sensitivity and specificity of these assays may be much improved when one considers that the gold standard of microbiological culture is in itself imperfect (14).

We have previously used lymphocyte proliferation assays to provide evidence for the development of *B. pseudomallei*-specific CMI responses in patients who have recovered from melioidosis (5, 13). Using the same technique, this study has demonstrated that patients with IHA-nonreactive sera develop a strong *B. pseudomallei*-specific CMI response. In accordance with the modified IHA used in this study, no additional benefit was provided by using patient isolate-derived antigen to stimulate lymphocytes isolated from IHA-nonreactive patient sera. Demonstration of specific CMI responses in patients with melioidosis continues to be an underutilized indicator of exposure to *B. pseudomallei*. Findings of Barnes et al. (5) suggest a tentative link between the development of a *B. pseudomallei*-specific CMI response and protection against disease progres-

sion (5). Due to the intracellular nature of *B. pseudomallei*, CMI responses may play a more important role than antibodies in recovery from infection and in the development of immunity to this pathogen. Therefore, the incorporation of antigens triggering strong CMI responses in diagnostic tests and assays for monitoring disease progression may prove to be more clinically relevant than the current assays based on antibody responses. Furthermore, the use of molecular diagnostic techniques, such as nucleic acid detection, holds promise for the rapid recognition of melioidosis (10, 17) although some assays have lacked sufficient sensitivity (6, 7).

Limitations of this study are acknowledged. First, the number of participants in this study was limited by the relatively small proportion of patients with confirmed melioidosis demonstrating persistently IHA-nonreactive sera. Furthermore, the surprisingly high rate of erythrocyte hemolysis seen in some IHA antigen preparations made determination of the optimal antigen concentration uninterpretable for these isolates. However, the high- and low-positive controls were reasonably concordant in almost all cases, making major dilution errors less likely.

To date, our understanding of the immunopathogenesis of *B. pseudomallei* is limited. Antigens involved in the development of protective specific immunity have not been identified. Additional investigations are warranted to better characterize the immunological responses in patients with melioidosis, particularly those that remain persistently IHA negative, and to understand the reasons for the observed limitations of serological testing. Such an understanding is critical to the development of new immunoassays and effective vaccines for melioidosis. Identification of immunodominant antigens will be essential to this process, and recent work has begun to elucidate this area (9, 18).

In summary, the use of extracts of *B. pseudomallei* isolated from patients with IHA-nonreactive sera and culture-confirmed melioidosis as sensitizing antigens in a modified IHA tested against the patients' own convalescent-phase sera did not improve assay sensitivity. Our data suggest that the poor sensitivity of the IHA does not reflect the limited choice of strains used in the antigen preparation for the assay. Furthermore, the majority of patients with IHA-nonreactive sera were seroreactive for IgG as measured by EIA. We have also demonstrated that patients with IHA-nonreactive sera developed a strong *B. pseudomallei*-specific adaptive CMI response. These findings identify additional shortcomings in the current standard serological assay used in the diagnosis of melioidosis. The immunodominant antigens of *B. pseudomallei* have not yet been identified. However, the procedures for the preparation of antigens used in the IHA, EIA, and lymphocyte proliferation assay are different. As such, variations in the immunogenicity and concentration of bacterial antigens present in each preparation may alter the immune response being measured. The results suggest that the *B. pseudomallei* antigens used in the IHA format may not be sufficiently immunogenic for a subset of patients with melioidosis. Further work to identify immunodominant antigens of *B. pseudomallei* is warranted to facilitate the development of more reliable and sensitive diagnostic assays.

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