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Investigation of dendritic cells as a vector for vaccine delivery

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Investigation of Dendritic Cells as a Vector for Vaccine Delivery

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Dedication

This thesis is dedicated to my wife Angie and three boys Corey, Oliver and Dylan for their love and support and the happiness they bring to my life.

Declaration

I confirm that this thesis is my own original work and that all contributions have been appropriately acknowledged. None of the material herein has been previously submitted for a degree or other qualification to this or any other university or institution.

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Publications

Healey, G.D., Elvin, S.J., Morton, M. & Williamson, E.D. Humoral and cell-mediated adaptive immune responses are required for protection against *Burkholderia pseudomallei* challenge and bacterial clearance post infection. *Infection and Immunity* **73** (9), 5945 – 5951.

(See appendix)

List of abbreviations

	2-ME	2-mercaptoethanol
A	**ABC**	Adenosine triphosphate-binding cassette
	ACDP	Advisory committee on dangerous pathogens
	ANOVA	Analysis of variance
	APC	Antigen presenting cell
	ATP	Adenosine triphosphate
B	**Bsa**	*Burkholderia* secretion apparatus
C	**CBA**	Cytometric bead array
	CCR	Chemokine receptor
	CD	Cluster of differentiation
	CI	Confidence interval
	CLIP	Class-II-associated Ii peptide
	CMI	Cell-mediated immunity
	Con A	Concanavalin A
D	**DC**	Dendritic cells
	DNA	Deoxyribonucleic acid
	DTH	Delayed type hypersensitivity
E	**ELISA**	Enzyme linked immunosorbant assays
	EPS	Exopolysaccharide
	ER	Endoplasmic reticulum
F	**FBS**	Foetal bovine serum
	Flt-3L	fms-like tyrosine kinase receptor ligand
G	**GM-CSF**	Granulocyte macrophage – colony stimulating factor

	GT	Granulocyte macrophage – colony stimulating factor + tumour necrosis factor - alpha
	GT4	Granulocyte macrophage – colony stimulating factor + tumour necrosis factor – alpha + interlukin 4
	GT12	Granulocyte macrophage – colony stimulating factor + tumour necrosis factor – alpha + interlukin 12
	GTP	Guanosine triphosphate
H	**HI**	Heat inactivated
	HK	Heat killed
	HLA	Human leukocyte antigen
	HNP -	Human neutrophil peptide -
I	**ID**	Intradermal
	IFN	Interferon
	IL -	Interlukin -
	IN	Intranasal
	IM	Intramuscular
	IP	Intraperitoneal
	iNOS	Inducible nitric oxide synthase
	ITAM	Immune tyrosine-based activation motif
L	**LAMP -**	Lysosome-associated membrane glycoprotein -
	LPS	Lipopolysaccharide
	LSM	Lymphocyte separation medium
M	**MACS**	Magnet assisted cell sorting
	MHC	Major histocompatibility complex
	MIP-	Macrophage inflammatory protein -

	MNGC	Multinucleate giant cells
	MPL + TDM	Monophosphoryl lipid A + Trehalose dicorynomycolate
	MRNA	Messenger ribosomal nucleic acid
N	NCTC	National collection of type cultures
	NF -	Nuclear factor -
P	PAMPs	Pathogen associated molecular patterns
	PBS	Phosphate buffered saline
	PDC	Plasmacytoid DC
	PRRs	Pathogen recognition receptors
	PSG	Penicillin / Streptomycin / Glutamine
R	ROI	Reactive oxygen intermediates
	RNA	Ribonucleic acid
	rRNA	Ribosomal ribonucleic acid
	RNI	Reactive nitrogen intermediates
	RPMI	Roswell Park Memorial Institute
S	SCF	Stem cell factor
T	TAP	Transporters associated with antigen processing
	TCR	T cell receptor
	TGF -	Transforming growth factor -
	Th	T helper
	TLR -	Toll-like receptor -
	TNF -	Tumour necrosis factor -

Abstract

Dendritic cells (DC) are an attractive target for both vaccine targeting and use as a delivery vehicle due to their ability to direct innate and adaptive immune responses. In this study a procedure was developed where DC were employed as a vehicle to induce cell-mediated immune responses to the Gram negative bacillus *Burkholderia pseudomallei*. This bacterium is the causative agent of melioidosis, a disease endemic to areas of south-east Asia and northern Australia, to which presently no licensed vaccine exists.

Purified DC were pulsed with heat-killed (HK) whole cell *B. pseudomallei* and used to immunise syngeneic mice. The use of this immunisation method elicited strong cellular immune responses against *B. pseudomallei*, which have historically been difficult to generate. Antibody responses following immunisation with DC were low. Booster immunisations of either a second dose of HK *B. pseudomallei* pulsed DC or HK *B. pseudomallei* in MPL + TDM adjuvant were administered to increase the immune response. Immunised animals were challenged with fully virulent *B. pseudomallei* and protection was demonstrated in animals immunised with a combination of HK *B. pseudomallei* pulsed DC and HK *B. pseudomallei* delivered in MPL + TDM adjuvant. The protective immune response demonstrated was then further elucidated to reveal a role for both cell-mediated and humoral immunity in protection against *B. pseudomallei* infection. The data also demonstrate the potential for DC as vaccine delivery vectors and as a screening mechanism for the evaluation of potential vaccine candidates.

Chapter 1

Introduction

1.1 Genus *Burkholderia*

1.1.1 The pseudomonads

The pseudomonads are a major group of chemoorganotropic aerobic gram-negative rods with three important genera, *Pseudomonas*, *Commamonas* and *Burkholderia*. Phylogenetically, the various genera of pseudomonads scatter within the purple bacteria. They are thought to be derived from ancestral phototropic bacteria, which dispensed with the property of photosynthesis in evolving to colonise habitats where the ability to carry out anaerobic photosynthesis was not a significant advantage, such as in soil or on the surfaces of animals (132).

The original description of the genus *Burkholderia* was in 1992 (234) for seven species belonging to the RNA homology group II of genus *Pseudomonas: P. cepacia, P. mallei, P. pseudomallei, P. caryophylli, P. gladioli, P. pickettii* and *P. solanacearum.* To date, 42 species have been identified as belonging to the genus *Burkholderia* on the basis of DNA-DNA homology, 16s rRNA base sequences and various phenotypical characteristics (160). While the majority of these species are saprophytes or plant pathogens (56) three, *B. cepacia, B. mallei* and *B. pseudomallei* can cause potentially fatal disease in humans (6).

1.1.2 *Burkholderia mallei*

Glanders, a zoonotic disease primarily of solipeds, which can be traced back almost two thousand years (73), is occasionally transmitted to humans (48). At the turn of the twentieth century glanders was prevalent world-wide due to the extensive use of solipeds. The general decline in their use, however, together with the introduction of

strict health enforcement procedures has led to a significant reduction in disease occurrence and it is now found primarily in Asia, Africa and the Middle East (5).

B. mallei was first isolated in 1882 by Loeffler and Schutz (19) and is a Gram-negative, non-motile, oxidase-positive, aerobic bacillus, 0.3-0.5 μm in width and 1-3 μm long and an obligate animal pathogen (51,160,220). Human susceptibility to it has not been studied in great detail although it has proven highly infectious in laboratory settings (29,73,95,193).

1.1.3 *Burkholderia pseudomallei*

First isolated by Whitmore and Krishnaswami (1912) from members of the population of Rangoon in Burma, exhibiting a glanders-like disease (225), *B. pseudomallei* has now been isolated from every continent on the globe (47). Confirmation of the taxonomic relatedness between *B. mallei* and *B. pseudomallei* vindicated Whitmore's original proposal of the specific epithet 'pseudomallei' (48) and later, Stanton and Fletcher (1921) suggested the term melioidosis, derived from the Greek μηλιξ, meaning glanders or distemper of asses (48). *B. pseudomallei* is a Gram-negative, motile, facultative anaerobic bacillus usually less than 2 μm in length (96) that is responsible for a broad spectrum of illnesses observed in both humans and animals (48,96) and is a common cause of human pneumonia and fatal bacteraemias in endemic areas (35).

1.1.3.1 Clinical manifestations

The manifestations of melioidosis vary greatly from an asymptomatic state to chronic illness to acute overwhelming septicaemia (217,229). Its non-pathognomonic symptoms (123), together with the need for relatively sophisticated laboratory facilities

(50), often complicate diagnosis leading to the disease being mistaken for malaria, plague, pneumonia or miliary tuberculosis (96,123).

The literature reports that the vast majority of infections appear to be sub-clinical (48), an observation supported by the fact that nearly 80% of children by the age of four years old (114) and up to 30% of adults (229) in endemic areas have antibodies to *B. pseudomallei*. Seroconversion during these mild and sub-clinical infections has been associated with a flu-like illness (7) although this is obviously difficult to confirm in every case. Since the description of the non-pathogenic *B. thailandensis* (192), which is antigenically cross reactive with *B. pseudomallei* (192) however, it is important to consider the possibility that the prevalence of seroconversion might in fact be due to *B. thailandensis* rather than *B. pseudomallei*, and that re-evaluation of this data may be appropriate.

Latency periods as long as 62 years between *B. pseudomallei* infection and disease have been reported (123,136), with relapses usually occurring at times of stress, such as acute infections or trauma (48). The mechanisms of persistence still require further elucidation although localised foci of melioidosis in the lung, liver and spleen have been reported in animals and the utilisation of intracellular survival mechanisms is thought likely to play a part (229). In a 5-year study conducted in Sappasitprasong Hospital in north-eastern Thailand, Chaowagul *et al* (1993) (34) showed that nearly all suspected cases of recrudescence were actual melioidosis relapses and also estimated the rate to be approximately 15.3% (95% CI, 10.5% - 22.3%) per year of follow-up. Difficulties in identifying individuals with latent infection however, means the exact numbers of seropositive people at risk from future relapse is unknown (48).

Only a small proportion of *B. pseudomallei* infections are sufficiently severe to come to medical attention. Sixty percent of these cases however, have positive blood cultures for *B. pseudomallei* and they usually present clinically as 'community-acquired sepsis syndrome' with a short history (1 day to 2 months) of pyrexia and rigors (35). The acute form of the disease can be divided into two groups, acute pulmonary, which is associated with pulmonary distress on presentation (22) and acute septicaemic forms. Approximately half of patients have evidence of a primary focus of infection (usually the lung, skin or subcutaneous tissues) and confusion, stupor, jaundice and diarrhoea may also be prominent features (48). Laboratory investigations usually reveal anaemia, neutrophilia, coagulopathy and renal and hepatic failure. Such patients deteriorate rapidly, developing widespread metastatic abscesses (particularly in the lungs, liver and spleen) and metabolic acidosis, which is accompanied by Kussmaul's breathing. The onset of septic shock is associated with mortality approaching 95% and the majority of patients die within 48 hours of hospital admission (35,48). Even with vigorous appropriate antibiotics and supportive therapy, mortality is still 40% or greater (229).

If patients survive the acute phase of the disease, the manifestations of multiple septic foci, resulting from bacteraemic dissemination become prominent. The most common foci of infection are the lungs, liver, spleen, skin and soft tissues (48). The nervous system can also become involved in a syndrome known as neurological melioidosis, which is characterised by peripheral motor weakness, brain stem encephalitis, aseptic meningitis and respiratory failure (48).

A sub-acute form of the disease also occurs, which has been described as a prolonged febrile illness (22). There is multiple abscess formation, although rarely on the brain,

and *B. pseudomallei* can be readily cultured from the blood, pus, urine and other bodily tissues and secretions in the later stages of disease (22,123).

1.1.3.2 Epidemiology

Endemic areas of melioidosis typically border 20° north and south of the equator although the incidence has been shown to be particularly high in Southeast Asia and northern Australia (35,47,48,49). The first indigenous case in Thailand was described in 1995 (47). It is now estimated that 20% of community acquired septicaemia and 40% of deaths associated with bacterial sepsis in north-east Thailand can be attributed to *B. pseudomallei* (35), and 2000-5000 patients per year present with melioidosis (55). In northern Australia, melioidosis has been recognised as endemic since 1949 (83) although the exact route by which it was introduced remains unclear. The disease is also becoming increasingly prevalent in China, where it has been routinely isolated since 1975 (236), and in the Indian subcontinent, and recent surveys have shown that the organism is generally much more prevalent than previously believed (47).

1.1.3.3 Reservoirs and transmission

Stanton and Fletcher (1932) first identified melioidosis in animals, and concluded that the disease was a zoonosis with a reservoir in rodents. This theory was later disproved by French workers in French Indochina who observed a prevalence of disease occurrence following contact with muddy waters and that rats were rarely infected. Later they proved that *B. pseudomallei* was in fact an environmental saprophyte that could be readily isolated from soil and surface water in endemic areas (30). In particular the organism is associated with rice paddy fields (being readily isolated from 68 – 78% of rice fields in Thailand) (48) and these habitats are thought to be the primary reservoirs from which susceptible hosts acquire infection (123).

Ecologically, *B. pseudomallei* is thought to persist in deeper clay layers during the dry season, rising to the surface after the annual rains and thus enhancing the potential for exposure of humans and animals (22). This supposition is one that fits with the marked seasonal variation seen with melioidosis whereby 75% of cases present during the rainy season in Thailand (201). It also fits with dramatic increases in incidence that frequently coincide with heavy monsoon rains in Australia (49), although as Dance (1998) (48) points out, this pattern implies that most cases are recently acquired. The incubation period of melioidosis (2 days to 62 years) (23,147,232) suggests that some other seasonal factor(s) might also precipitate relapses of latent infections. Two studies have contradicted the seasonal variation theories by paradoxically finding a higher isolation rate of *B. pseudomallei* during the dry season than the rainy season (23,232), although technical reasons, such as the processing of larger samples during the dry season (232) or the dilution of *B. pseudomallei* during the rainy season (49) were noted as possible explanations for the findings. These studies suggest that the seasonal variation seen in melioidosis presentation may be due to increased exposure during rice planting or harvesting, rather than an actual increase in the number of organisms present in the soil (49).

The most common route of infection for humans and animals is believed to be the inoculation or contamination of wounds or the mucine surfaces with soil or water (35,123). This tends to explain the prevalence of disease amongst rice farmers and their families who labour in the rice paddies without the benefits of protective clothing (35,123). However, specific episodes of exposure are only identified in approximately 6% of cases in Thailand (201) and between 19% and 51% of cases in Australia (49). Aerosols have also been proposed as a possible route of infection, and were thought to

be a source of infection (aerosolised dust and fomites) for helicopter crews during the Vietnam War (96). While only two cases of person-to-person spread have been described (137), occasional iatrogenic infections from contaminated injections as well as laboratory-acquired infections have together led to the classification of *B. pseudomallei* as an Advisory Committee on Dangerous Pathogens (ACDP) category 3 pathogen (1,116). There are currently no reports of disease transmission from animals to humans (122,123).

1.2   Host-pathogen interactions

For an invading pathogen to establish a successful infection it must first circumvent the plethora of innate (non-specific) and adaptive (specific) host defences that exist to combat the infective process. The role of the innate immune system is to prevent entry into the host or, if this occurs, to inhibit the establishment of infection. In addition however, the innate immune response is involved in the activation of adaptive immunity. This system provides a pathogen-specific defence mechanism aimed at stifling the infection process but also, importantly, establishing immunological memory such that future infections with the same pathogen are more efficiently combated (81).

As discussed, the inoculation or contamination of wounds or the mucine membranes is likely the most common route of *B. pseudomallei* infection (35,123). Both the mucine and epithelial membranes however, are usually highly effective barriers to pathogen entry. Breech past the epithelial membranes is usually only possible through existing wounds or via vectors such as biting insects and the mucine membranes, while certainly more vulnerable to invasion, incorporate a number of effective barriers to infection such as mucus, cilia and their normal flora, which compete with invading pathogens for

adhesion sites and nutrients essential for the establishment of infection. Should breech occur, the resulting physiological response is inflammation.

1.2.1 The innate immune response

1.2.1.1 Acute inflammation

The result of infection and/or tissue damage is an immediate, initially non-antigen-specific, physiological response, which attempts to repair any damage and works in co-ordination with the adaptive immune response to eliminate foreign bodies from the tissues. The release of inflammatory mediators, such as bradykinin and fibrinopeptides, from injured tissues causes localised vasodilation within minutes (13). The associated increase in blood volume reaching the affected area and increased vascular permeability, brought about by inflammatory mediators, results in localised oedema and an increase in leukocyte extravasation. Activation of the complement system produces anaphylotoxins (C3a, C4a and C5a), which augment the response inducing localised mast cell degranulation and the release of histamine (86,134). Within hours of the initiation of localised inflammation, neutrophils, which migrate along chemotactic gradients produced by the inflammatory response, begin adhering to the vascular endothelium and enter the tissue spaces. Macrophages are also recruited to the site of infection by the secretion of the macrophage inflammatory proteins (MIP-1α and MIP-1β), and together with neutrophils form the first wave of defence against invading bacteria. Paradoxically however, macrophages also provide a niche within which *B. pseudomallei*, a facultative intracellular parasite, can grow and multiply (62,229).

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1.2.1.2 Invasion

The role of neutrophils and macrophages in terms of the innate immune response is to

phagocytose and kill the bacteria in an attempt to prevent the establishment of infection

and spread to other host tissues. However, entry to the intracellular environment is also

an essential requirement for survival in the infective process for *B. pseudomallei*. For

many intracellular bacteria, infection of neutrophils leads to killing of the pathogen due

to the highly phagocytic nature and potent antibacterial activity of these cells

(4,90,115). The significant contribution to reducing bacterial load early in infection

provided by neutrophils has been demonstrated in experimental listeriosis, an acute

disease characterised by extensive neutrophil infiltration at sites of listerial growth (38).

Removal of the infiltrating neutrophils using monoclonal antibodies (mAb) exacerbated

listeriosis in mAb treated mice (172). In addition to their highly potent antibacterial

activity (90) the ability of neutrophils to sequester intracellular bacteria in intracellular

niches (115), limiting bacterial spread, and their short life-span (4,90), providing

insufficient time for an infection to become established, make neutrophils an unsuitable

target. Macrophages and non-phagocytic cells however, provide an environment within

which *B. pseudomallei* can survive and proliferate for prolonged periods (111,162). It

is this ability to persist in the intracellular environment, which is thought to be

responsible for the pathogenesis of *B. pseudomallei* infection and its recrudescence (44)

and may also provide some explanation of why melioidosis is refractory to antibiotic

therapy (198), although the antibiotic resistance of *B. pseudomallei* has also been

demonstrated *in vitro* (222).

Entry into the intracellular environment, whether due to invasion or phagocytosis, is

ultimately a function of the host cell defence mechanisms (115), although in terms of

facultative intracellular pathogens such as *B. pseudomallei* it is often induced through

molecules that first mediate adhesion to the host cell. Adhesion induces uptake through host cell receptor signalling in a process that has been termed the 'zipper mechanism' (40,202). While a specific receptor for this mechanism in terms of *B. pseudomallei* infection has not yet been identified, a cluster of *B. pseudomallei* genes with homology to the type III protein secretion systems of *Salmonella typhimurium* and *Shigella flexneri* has been identified (10,164,199). These secretion systems have been likened to a molecular syringe (198) whereby bacterial proteins are injected directly into the cytosol or plasma membrane of the target, which then interact with host cell receptors and proteins and bring about the uptake of the bacterium. These systems have been demonstrated to induce bacterial uptake in a variety of non-phagocytic cells for both *Salmonella* and *Shigella*. The *Burkholderia* homologues identified (termed the *Burkholderia* secretion apparatus, Bsa) have also been shown to facilitate bacterial invasion of HeLa cells (reviewed in (198)).

Uptake into the intracellular environment of the professional phagocyte should, in terms of a successful immune response, lead to the formation of a phagolysosome. The purpose of the phagolysosome and other associated endocytic vesicles is to effect bacterial killing through a number of mechanisms, such as the production of reactive oxygen and nitrogen intermediates (ROI and RNI), defensins (184) and lysosomal enzymes (115) and also provide immunogenic proteins to enable the establishment of adaptive immunity. Most intracellular bacteria however, interfere with the maturation of the phagosome and alter its composition to facilitate and support their own survival (115).

Transmission electron microscopy has indicated that *B. pseudomallei* can escape from endocytic vesicles as early as fifteen minutes after internalisation (89). It has been

hypothesised that the Bsa is likely to play a role in this, due to observations that dependence on a type III secretion system is a feature of endosomal escape in *Shigella* infection. It has also been demonstrated that *B. pseudomallei* mutants lacking putative components of the Bsa are confined to endosomes with intact membranes in J774.2 macrophages (199). Furthermore the mutant bacteria were found to co-localise with lysosome-associated membrane glycoprotein-1 (LAMP-1), which is a marker of late endosomes, whereas the wild type bacteria did not, suggesting a role for the Bsa in endosomal escape (199).

Following escape from the endosomal compartment, *B. pseudomallei* attempts to establish a successful infection while the host attempts to stifle it. An important factor in the ensuing battle is intracellular iron, which is required by the bacteria and also required for the production of ROI and RNI by the host. To improve iron supply the host utilises transferrin and lactroferrin molecules, which bind iron in the extracellular environment and allow uptake into the cell via transferrin receptors. Iron is then released from the transferrin in the reducing conditions of the early phagosome and transported to the cytosol. Bacteria also utilise a number of mechanisms such as siderophores, transferrin-binding proteins, haem-like proteins and adenosine triphosphate (ATP)-Binding Cassette (ABC) transporters (115) to scavenge iron from the invaded cell for the purposes of infection. The importance of iron in *B. pseudomallei* infection is highlighted by work undertaken by Bancroft and colleagues, who also demonstrated an obligatory role for interferon-gamma (IFN-γ) in host survival in the murine model of *B. pseudomallei* infection (177). When infected, mice developed an acute lethal infection unless a rapid IFN-γ response was mounted within the first 24 hours. Following such a response, a chronic infection was induced that lasted between 2 and 16 months before lethality (177). IFN-γ activated macrophages

down-modulate transferrin receptor expression, resulting in a significant reduction in iron availability within the phagosome and thus ultimately the cell (115). Thus it could be hypothesised that reduced iron, in addition to heightened killing activity, might be an important infection limiting control.

Once the host's defensive mechanisms have been stifled a successful infection can then be established. *B. pseudomallei* is then able to induce the formation of actin-based membrane protrusions (199), an ability shared by other intracellular bacteria such as *Shigella, Listeria* and *Rickettsia* spp., that allows the formation of conduits between cells, enabling the spread of infection. In addition to actin-based motility, *B. pseudomallei* is also capable of inducing cell fusion, an attribute unique to this bacterial pathogen (116). The mechanisms behind the formation of multinucleate giant cells (MNGC) during *B. pseudomallei* infection are yet to be fully elucidated. However the formation of MNGC has been observed both *in vitro* (116) and in human melioidosis patients (228) suggesting it is likely to play a role in the protection of *B. pseudomallei* from the adaptive immune response and to allow recrudescence (115).

1.2.2 The adaptive immune response

In the vast majority of cases an innate immune response alone is insufficient to contain proliferation of the invading pathogen. Thus an adaptive, pathogen-specific, response must be established to aid clearance and protect against future infections with the same pathogen. Central to the establishment of adaptive immunity are dendritic cells (DC).

1.2.2.1 Dendritic cells

Dendritic cells (DC) are a rare, heterogeneous population that reside in most peripheral tissues and organs representing approximately 1-2% of the total cell population

(81,130). They belong to a group of cells called antigen-presenting cells (APCs) defined by their ability to express surface major histocompatibility complex (MHC) and deliver a co-stimulatory signal via CD 80 and CD 86 (the B7 molecules B7-1 and B7-2 respectively) to activate naïve CD 4^+ and CD 8^+ T cells. A variety of cells can function as APCs following induction to express MHC and/or co-stimulatory molecules, although these cells usually only function in this capacity during sustained inflammation and for short periods (81). Of the 'professional' APCs, DC are by far the most potent and the only cells to constitutively express high levels of MHC, CD 80 and CD 86. They are also the only cells capable of efficiently activating naïve T cells without further stimulation from other immune cells (12,45,143,181).

1.2.2.1.1 Haematopoietic development

The development of DC has been described along a number of different pathways that encompass both myeloid and lymphoid lineages (72,157,174). In humans three generalised types of DC have been described: myeloid, plasmacytoid (170) and lymphoid DC. *In vitro* the development of myeloid DC can be achieved from CD 34^+ progenitor cells under the influence of the growth stimulating cytokine granulocyte macrophage-colony stimulating factor (GM-CSF) and the inflammatory cytokine tumour necrosis factor-alpha (TNF-α) (27,126,157). Under this environment CD 34^+ bone marrow progenitors differentiate into CD 14^-, CD $11c^+$, CD $1a^+$ cells that resemble epidermal DC (Langerhans cells) and contain the characteristic Birbeck granules (27,157). The addition of transforming growth factor (TGF)-$\beta 1$ to the culture again results in a DC with a CD 14^-, CD $11c^+$, CD $1a^+$ phenotype, but these cells lack Birbeck granules and are known as dermal or interstitial DC (79). In addition, the development of an intermediate DC stage from CD 34^+ progenitors has been demonstrated under the influence of interlukin (IL) -1β, IL-3, IL-6, erythropoietin (EPO) and stem cell factor

(SCF) (178,180). This cytokine cocktail produces a CD 14^+ CD $1a^-$ cell that has bi-potential for both DC and monocytes and also represents the precursor to plasmacytoid DC (PDC). The differentiation of PDC can thus be induced from these cells under the influence of GM-CSF and IL-4, or directly from progenitor cells in the presence of fms-like tyrosine kinase receptor ligand (Flt-3L). PDC belong to a group of cells designated type I interferon (IFN)-producing cells owing to their capacity to secrete high concentrations of type I IFN after viral challenge (126,157,170,213). They have been described in the peripheral blood and lymph nodes of patients with infectious diseases and recent work suggests their primary role is likely to be in antimicrobial defence (37).

In addition to these myeloid related pathways, a potential link between DC development and lymphocytes has also been demonstrated (74). A subset of CD 34^+, CD 38^+, Lin$^-$, CD 10^+ progenitor cells has been identified in human bone marrow that are capable of giving rise to T, B, Natural Killer and DC but not myeloid cells upon exposure to multiple cytokines (74,181). Lymphoid DC have also been shown to develop from CD 34^+, CD 38^{dim} thymic precursors under the influence of IL-1α, IL-3, IL-7, SCF and GM-CSF (157,181) and from CD 19^+ precursors in the presence of IL-1β, IL-3, IL-7, SCF, Flt-3L and TNF-α in the presence of serum (74,157,181,187). A number of functions have been proposed for lymphoid DC although their capacity for inducing apoptosis and involvement in the elimination of self-reacting T cells has lead to the suggestion that their role is primarily one of regulation rather than stimulating effector functions (181).

In the mouse, DC can be divided into three main subclasses on the basis of their phenotype. While all murine DC express the CD 11c intergrin they can be distinguished by their expression of CD 8α, CD 4, CD 45R (B220) and Gr1. CD $8\alpha^+$

DC, which differentiate along the myeloid lineage (214) (denoted by the expression of the myeloid marker CD 11b), have been described in the thymic cortex and T cell areas of the secondary lymphoid organs (Reviewed in (188)). There is currently no human counterpart to CD $8\alpha^+$ DC that has been identified and this also appears to be the only group of DC specialised in the capture of apoptotic bodies from B cells and tumour cells (104). CD $8\alpha^-$ DC are also of myeloid lineage, differentiating from the same myeloid precursor as CD $8\alpha^+$ DC, and expressing CD 11b. This population can be further divided into CD 4^+ and CD 4^-, both of which are found to reside in the marginal sinus of the spleen, subepithelial dome of Peyer's patches and in the peripheral tissues. The differentiation of DC from monocytes in mice has also been demonstrated (165) and recently a CD $11c^+$ population of DC also positive for CD 45R and Gr1 but with only low expression of CD 11b has been described in the mouse. This population of DC resembles human PDC in their capacity to secrete type I IFN following viral stimulation (37) and are thus now considered the murine equivalent of PDC.

In both humans and mice different DC subsets appear to have specialised functions focused on driving effector and/or regulatory T-cell differentiation (170). A considerable degree of elucidation is still required however, before the mechanisms underlying the plasticity exhibited by DC can be determined. Central to their role in the control of the adaptive immune response however, is their ability to recognise and differentiate the array of pathogens encountered by the immune system.

1.2.2.1.2   Pathogen recognition

Despite the antigen specificity of B and T cells they lack the ability to direct and control immune responses. This complex task is one carried out by DC via germ line-encoded pattern recognition receptors (PRRs) (105,140). Although tremendously diverse,

microorganisms possess common structural features such as, lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid, lipoarabinomannan, unmethylated DNA and bacterial lipoproteins, which are known as pathogen-associated molecular patterns (PAMPs). The role of PRRs in the detection of PAMPs was first hypothesised by Janeway (1992) (105) following the discovery of the gene for the *Drosophila* Toll protein that is involved in the secretion of the anti-fungal peptide drosomycin in response to microbial invasion (reviewed in (113)). In 1997, the first human homologue of Toll was described and later designated Toll-like receptor (TLR) 4, due to the identification of additional murine and human TLR proteins since the discovery of the *Toll* gene in the early 1980s. Mammalian TLR proteins have since been shown to represent a conserved family of innate immune recognition receptors that are found in mammals, insects and plants (139). These receptors are coupled to signalling pathways that enable the activation of immune cells in response to infectious agents in a manner similar to the PRRs envisioned by Janeway (1992) (105).

Research into the functions of PRRs and their ability to recognise PAMPs demonstrated that they offered cells of the innate immune system, such as DC, a means of discriminating between different stimuli. For example, LPS signals through TLR 4, peptidoglycans through TLR 2, CpG bacterial DNA through TLR 7 and 9 and bacterial flagellin signals through TLR 5. Research has also shown that many PAMPs signal through more than one TLR and it has been suggested that it is these combinations of TLR activation that lead to the appropriate expression of cytokines to drive a specific immune response (2,98,139,163,223).

DC have been demonstrated to be the principle cell involved in antigen presentation to naïve T cells (43,85,233) and thus to fulfil this function and initiate adaptive immunity,

DC must engage and activate other immune cells to establish an effective response. Once endocytosed the invading pathogen is digested and processed by the DC to form antigenic peptides. These peptides are then presented, in the context of MHC class I and II molecules, to naïve T cells allow other immune system cells to recognise the invading pathogens and mount a pathogen-specific response (102,145). In addition, the recognition of pathogen-specific immunogenic peptides also enables the production of memory T cells and immunological memory such that future infections can be combated with greater speed and efficacy (39,57,58,64).

1.2.2.1.3   Antigen uptake

Under physiological conditions DC reside in an immature or un-activated state during which they are primed for the uptake and processing of antigenic peptides. In this form DC express relatively low levels of MHC class II and the co-stimulatory molecules CD 80 and CD 86, making them inefficient T cell activators (12,84). They do however, express a plethora of markers involved in the uptake of antigenic peptides and microbes. Immature DC are located at the major portals of microbial entry, such as the skin and mucine membranes, which allows them to perform a sentinel-like function, continuously sampling their external environment via phagocytosis and macropinocytosis (170).

Both phagocytosis and macropinocytosis are actin dependent mechanisms the latter requiring membrane ruffling and resulting in the formation of large intracellular vacuoles (84). While phagocytosis is receptor-mediated, macropinocytosis is a cytoskeletal dependent type of fluid-phase endocytosis, which is constitutive in immature DC and can be transiently induced in macrophages (176). Macropinocytosis represents a major antigen uptake mechanism in immature DC allowing them to rapidly

and non-specifically sample large amounts of the surrounding extracellular fluid. Phagocytosis, in contrast, involves the engagement of specialised receptors, such as C-type lectins like the macrophage-mannose receptor (MMR) (175) or CD 205 (110), or the use of specialised regions of the plasma membrane termed clathrin-coated pits (191,195). This process does not require membrane ruffling and is initiated by a signal in the cytoplasmic tail of an endocytic receptor, which leads to invagination of the membrane and macromolecule internalisation. Thus for both receptor-mediated endocytosis and phagocytosis the first step is the contact between an invading microbe and the DC. During this event, many parallel signalling pathways are simultaneously activated defining the cellular response and regulating internalisation (218). A large number of endocytic receptors are selectively expressed on subpopulations of immature DC including: Fcγ-receptors, C-type lectins, complement receptors, scavenger receptors, lectins, heat shock proteins and integrins such as fibronectin and vitronectin (84,218). Of these, some are involved in transmitting intracellular signals to trigger antigen uptake, while others are primarily involved in binding or increasing the efficiency of internalisation. This latter group of receptors including, chemokine receptor (CCR) 5 (156) and dendritic cell-specific- ICAM 3-grabbing nonintergrin (DC-SIGN) (78), also serves as a target for pathogens, being exploited to facilitate infection (221).

The production of proinflammatory signals and activation of antimicrobial mechanisms usually accompanies microbe internalisation. While certain phagocytic receptors, such as Fc-receptors (166), trigger inflammatory responses directly, others such as complement receptors do not (231,235). In these cases the activation of inflammatory responses during phagocytosis is mediated by additional receptors (not themselves phagocytic) such as TLR's (218). This inflammatory response alone however, is

insufficient to mount a defence against microbial invasion. The ingested pathogen must be processed to form antigenic peptides, which can be used to activate the adaptive immune response.

1.2.2.1.4   Antigen processing and presentation

As previously described, immature DC are poor activators of naïve T cells. Thus pathogen recognition serves two major purposes. First, it triggers effector cells of the innate immune system such as macrophages and neutrophils, which represent an immediate defence at sites of pathogen entry. Second, it spurs DC into an integrated developmental program known as maturation, which transforms them into efficient T cell stimulators (84) allowing the initiation of an adaptive immune response.

The precise coordination between the phenotypical, morphological and functional modifications induced during maturation has yet to be completely elucidated (84). Current opinion is that after an encounter with a pathogen, DC leave the peripheral tissues in an intermediate state of maturation and migrate to the draining lymph nodes where they become fully mature under the control of antigen specific T cells. During this migration, previously internalised microbes and antigens are degraded intracellularly and loaded onto MHC molecules for future presentation to naïve T cells (reviewed in (84)). The DC invokes a strict compartmentalisation on the biogenesis of MHC class I and class II molecules meaning that while both exogenous and endogenous antigens are loaded onto MHC class II molecules, only selected endogenous antigens are loaded onto MHC class I molecules (16).

The loading of peptides from recently processed pathogens onto MHC class II molecules occurs via the endocytic pathway. Shortly after their synthesis in the

endoplasmic reticulum (ER), three α/β MHC class II dimers associate to a trimer of invariant (Ii) chains forming a nonameric molecule (41). Under the influence of transport signals present in the cytoplasmic region of the Ii chain the nonamers then pass through the Golgi apparatus where they are packaged before transport to the endocytic pathway (41). Here they fuse with endosomes and lysosomes, encountering an acidic, protease-rich environment, which leads to the degradation of the Ii chain leaving only a class-II-associated Ii peptide (CLIP) in the peptide binding groove (118,212). Competency to bind antigenic peptides then develops under the control of two nonpolymorphic MHC class II molecules HLA-DM and HLA-DO in humans and H2-M and H2-O in mice, which catalyse the exchange of CLIP for antigenic peptides (118). In parallel to the generation of competent MHC class II molecules, the endocytic pathway also produces antigenic peptides, called epitopes through the proteolysis of ingested pathogen-associated proteins. These bind competent MHC class II molecules forming a MHC class II-epitope complex that is transported to the plasma membrane (159,224).

In their immature form DC exert a tight regulation on the amount of MHC class II-epitope complexes that are expressed on the cell surface through several intracellular mechanisms. First, antigen degradation in immature DC is very inefficient, due to the low efficiency of several of the proteases involved in the endocytic pathway (66). This means that internalised antigen can remain intact for several days in the lysosomal compartments, restricting the availability of antigenic peptides for MHC class II loading (101). Second, low protease activity (specifically cathepsin S) means that some MHC class II haplotypes with a strong affinity for Ii remain associated with a partially degraded form of Ii, called Iip 10, which blocks access to the peptide binding groove (158). Even in cases where Iip 10 is degraded in the lysosomal compartment, MHC

class II-epitope complexes that reach the cell surface are rapidly internalised again either to be associated with new epitopes in recycling endosomes or to be directed to lysosomes where they are degraded (28). In either case this results in a very short-term presentation of MHC class II-epitope complexes on the immature DC surface that is insufficient to stimulate naïve T cells into an effector response.

Maturation signals encountered by DC induce the coordinated modification of all aspects of MHC class II-epitope complex formation and transport (84). A transient increase in MHC class II synthesis occurs (28) accompanied by increased cathepsin S and other protease activity (66) resulting in the increased availability of MHC class II molecules and antigenic peptides with which to form complexes. Once formed, the MHC class II-epitope complexes are rapidly transported to endosomal vesicles where they co-localise with the co-stimulatory molecules CD 80 and CD 86 and MHC class I molecules. The result is a cluster of molecules involved in T cell stimulation that are transported to the cell surface (216). In order to restrict the specificity of T cell stimulation to those antigens encountered in the periphery, maturation is also accompanied by a significant down-modulation of endocytic activity. Most antigen receptors, including Fc receptors, MMR, CD 205 and receptors for heat shock proteins and apoptotic bodies are significantly down-regulated representing the first level of control on antigen uptake (75). The only exceptions to this are clathrin-coated pits and vesicles involved in receptor-mediated endocytosis, which are not down regulated. Secondly, the overall levels of phagocytosis and macropinocytosis are reduced via the GTPase, Cdc42, which plays a central role in the blocking of macropinocytosis and phagocytosis (212). Stabilisation of MHC class II-epitope complexes on the surface is achieved through a significant reduction in the transport of internalised MHC class II molecules to lysosomes for degradation. Later, there is a further focusing of the T cell

response to only those antigens encountered in the periphery. This occurs through down regulation of MHC class II synthesis and the fact that the association of epitopes with newly synthesised MHC class II molecules becomes very inefficient (84).

MHC class I presentation to CD 8^+ T cells is generally limited to those proteins that are actively synthesised within the cell. Epitopes from endogenous antigens are generated in the cytosol by proteosome degradation and then transported to the ER by proteins present in the membrane of rough ER called transporters associated with antigen processing (TAP). Once transported, epitopes are then loaded onto MHC class I molecules, under the control of a loading complex composed of ER resident chaperone proteins such as tapasin, calnexin and calreticulin (42), ready for presentation. Once formed, MHC class I-epitope complexes are rapidly transported through the Golgi and on to the plasma membrane (84). The control exerted on MHC class I biogenesis and loading by the DC varies to that of MHC class II. Studies have shown that the expression of MHC class I-epitope complexes is up-regulated upon the induction of DC maturation (171) although to a lesser extent than MHC class II (28). In contrast however, MHC class I complexes are still efficiently synthesised and transported to the plasma membrane in mature DC indicating a functional difference in the regulation of antigen presentation to CD 4^+ and CD 8^+ T cells (171). While this pathway is suitable for the presentation of viral protein by infected cells and self-proteins, in terms of bacterial, including *B. pseudomallei*, infection cytotoxic immune responses need to be initiated against exogenous antigens not synthesised by the DC.

In most cells the cytosol and ER do not exchange material with the endocytic compartments (80), which means that epitopes from exogenous antigens do not have access to MHC class I molecules. The process of presenting exogenous peptides on

class I molecules was first described by Bevan 1976 (16), who demonstrated that priming of cytotoxic T lymphocyte (CTL) responses *in vivo* could occur after presentation of exogenous antigens by MHC class I molecules, a process he called cross-priming (16). More recent studies have demonstrated the ability of DC to carry out cross-priming of antigens from virally infected cells (189), tumour cells (97), apoptotic and necrotic cells (3,237). Both macropinocytosis (148) and phagocytosis (186) are thought to be major routes of antigen uptake for the cross-presentation of exogenous antigens and dendritic cells are considered especially efficient for generating CTL responses via the cross-presentation pathways (237).

Thus DC act as coordinator of the immune response aiding innate immunity and also bridging the gap between innate and adaptive immunity as well as providing the driving force behind the type of adaptive immune response generated. The ultimate effect of DC maturation is to produce antigenic epitopes from foreign antigens and transform the cell into an efficient APC capable of stimulating naïve T cells to proliferate.

1.2.2.2 T cell stimulation by dendritic cells

Following their exit from the thymus, all $\alpha\beta$ CD 4^+ T cells have a naïve phenotype and require an activation stimulus before they can differentiate into effector cells capable of mediating either type 1 (cellular) or type 2 (humoral) effector responses. The phenotypic similarities of effector T cells mediating type 1 (Th1) and type 2 (Th2) responses means that reliably distinguishing between the two subgroups often relies on an assessment of their functional characteristics (146). Such differentiation is based on the profile of cytokines produced by each subset following activation. Th1 cells can be defined by their production of cytokines such as interferon (IFN)-γ and tumour necrosis factor (TNF)-β while Th2 cells produce interlukin (IL)-4, IL-5, IL-6, IL-10 and IL-13

(146). A third subset of CD 4$^+$ T cells, Th0 cells, producing a mixed cytokine profile

(39,70,151) has also been described. The use of *in situ* mRNA hybridisation (allowing

the detection of cytokines from individual cells) (26) and intracellular protein staining

(150), however, has suggested that the Th0 subset is likely a mixed population of Th1

and Th2 cells (39) rather than a distinct population.

The activation and clonal expansion of CD 4$^+$ T cells is mediated by the engagement of

surface bound receptors on the T cell. The T cell receptor (TCR) is a membrane bound

protein heterodimer composed of either α and β or γ and δ chains. Unlike the B cell

receptor, which directly recognises antigenic peptides derived from invading pathogens,

the TCR must have antigenic peptides presented to it in association with molecules of

the major histocompatibility complex (MHC) family on the surface of antigen

presenting cells (120). This recognition of MHC associated antigen by the TCR is the

central event in T cell activation. The TCR itself consists of a mostly extracellular

heterodimeric ligand binding unit, and an intracellular signalling unit. The signalling

unit consists of three different polypeptide chains collectively known as CD 3, which

associate with the TCR at the T cell membrane and are involved in mediating signal

transduction (120). The signalling process is initiated by the engagement of the

peptide-MHC complex with the TCR, which causes cross-linking and allows the

association of the protein tyrosine kinase, Fyn, with the cytoplasmic tail of the TCR-CD

3 complex (91). This event also causes the translocation of the accessory molecules CD

4 (in class-II restricted T cells) or CD 8 (in class-I restricted T cells), which bring with

them the Lck protein tyrosine kinase, that is bound to their intracellular domains,

allowing association with the cytoplasmic tails of the TCR. Once associated, the

phosphorylation of the immune tyrosine-based activation motif (ITAM) occurs under

the control of the leukocyte common antigen CD 45, which is a transmembrane tyrosine

phosphatase that removes phosphates from the inhibitory sites of Fyn and Lck. This in turn leads to the activation of ZAP-70 (91) that initiates a number of signalling pathways ultimately leading to the activation (by dephosphorylation) of the T-cell-specific nuclear factor, NF-AT, together with NF-κB. These nuclear factors translocate to the nucleus and initiate transcription of the various genes required for activation and clonal expansion (120).

Naïve T cells however, also require a second signal from the co-stimulatory molecule CD 28, which is activated by CD 80 and CD 86 (12). Activation of this molecule initiates a signalling cascade that results in the activation of several other nuclear factors that induce gene transcription strengthening the activation of the T cell (18,120).

Approximately 48 hours after activation, the naïve T cell enlarges into a blast cell and begins a phase of rapid cell division, 2-3 times per day for 4-5 days, which generates a large number of clone progeny cells. These cells then further differentiate into effector T cells, which carry out required immunological functions such as B cell help (CD 4^+ T cells) or cytotoxic killing activity (CD 8^+ cytotoxic T cells), or memory T cells (121). Memory T cells are longer lived than effector cells and can be derived from both naïve and effector cells following an encounter with antigen (46,121). Once differentiated, memory cells reside in the G_0 phase of the cell cycle but possess a lower activation threshold enabling a more rapid activation upon subsequent antigen exposure (121).

Aside from TCR engagement, the outcome of T cell stimulation is influenced by a variety of other factors such as, the subset of DC providing T cell stimulation and the maturation-inducing signals encountered by the DC. Previous work has demonstrated that monocyte-derived human DC matured by CD 40 ligand, IFN-γ, LPS, CpG's or

double-stranded RNA produce high levels of IL-12 and thus a type 1 response. In contrast however, stimulation with prostaglandin E2 does not lead to IL-12 secretion and thus causes a type 2 response (reviewed in (84)). The ability to alter the effector immune response in this way is a concept called polarised maturation. CD 4^+ T cells can be skewed to produce a Th1 or Th2 response through the use of maturation signals or co-stimulatory molecules that favour one response over the other. A similar effect is seen from pathogen-derived signals, which play a critical role in the regulation of cytokine secretion by DC. In the yeast stage, *Candida albicans* induces IL-12 production and a type 1 response; while at the hyphae stage it has been demonstrated to induce IL-4 secretion favouring a type 2 response (84). Tissue specific environmental conditions also play a role as DC from different tissues have been shown to induce different responses to the same stimuli (103). Equally important in determining the outcome of T cell contact is the duration of DC stimulation, since over-stimulated DC have been shown to prime mostly Th2 and non-polarised, or central memory T cells expressing chemokine receptor (CCR) 7 (206).

1.2.3   Humoral immunity

1.2.3.1 B cell stimulation

B cell development begins in the bone marrow as lymphoid stem cells differentiate into progenitor B cells (127,135,173). Maturation of the B cell from this early stage requires not only several local environmental factors such as IL-7 (14,54), but also the rearrangement of the immunoglobulin (Ig) (the B cell receptor) DNA (173). This rearrangement results in the production of a receptor with a unique antigenic specificity and also enables the B cell population to produce receptors to antigens never previously encountered (127,173). As the B cell continues to mature IgM and IgD are expressed

on the cell surface (127) and the population then proceeds through a process of clonal deletion such that B cell receptors reactive against host antigens are removed from the host's repertoire (141,142) before export from the bone marrow.

Once mature, B cells circulate in the blood for a few weeks until they undergo apoptosis (141). Activation within this time is purely a function of encounter with antigen and the result is dependent on whether the antigen is thymus dependent (TD) or thymus independent (TI). An encounter with TI antigens, such as LPS or bacterial flagellin, which does not necessarily lead to an antigen specific response, is dominated by the secretion of IgM and does not lead to the production of memory cells (138).

In contrast to TI antigen activation of B cells, TD antigen activation is dependent on Th cells (94). After binding of antigen by monoclonal Ig on the surface of the B cell, the antigen is internalised and processed through the endocytic pathway into antigenic peptides. Simultaneously, the surface expression of MHC class II and the co-stimulatory molecules CD 80 and CD 86 is up regulated, enhancing the B cells antigen presentation capability (94,141). Antigenic peptides are then loaded onto MHC class II molecules and transported to the cell surface to enable binding to and activation of Th cells. This event causes the secretion of cytokines such as IL-2, IL-4 and IL-5 from the Th cell which bind the B cell leading to clonal expansion and differentiation into antibody secreting plasma cells and memory B cells (94,138).

1.2.3.2 Immunoglobulin isotype and function

Antibodies perform two important activities: the specific binding to an antigen; and the participation in various biological effector functions, which is determined by the isotype of the heavy-chain constant domain. There are five main classes of Ig in mammals;

IgM, IgG, IgA, IgD and IgE, each of which confers class specific structural and functional properties.

IgM mediates opsonisation, which involves the coating of an invading pathogen with antigen specific antibody. Phagocytic cells, recruited to the site of infection, then bind to the opsonising antibody via Fc receptors (specific for the Fc portion of Ig) expressed on the phagocytic cell surface, an event that leads to phagocytosis and destruction of the invading pathogen. IgM is also involved in the activation of the complement pathway, which culminates in the formation of the membrane attack complex that perforates the membrane of Gram positive bacteria and host infected cells causing lysis and death. IgG is divided into 4 subclasses, designated 1 to 4 in humans and 1, 2a, 2b and 3 in mice, each of which has a different biological function. In humans, IgG1 and 3 are the predominant activators of complement and the strongest opsonising isotypes although IgG2 and 4 are also opsonising (81). In mice the IgG2a and 2b subtypes are predominantly involved in the activation of complement and all isotypes are involved in opsonisation (106).

1.2.3.3 Antibody dependent cellular cytotoxicity

In addition to these mechanisms of antibody-assisted killing, a third mechanism, antibody dependent cellular cytotoxicity (ADCC) utilises antibody to facilitate the killing of infected host cells (63,106). IgG secreted during an infection binds to infected host cells through recognition of foreign antigen displayed on the cell surface. This enables non-specific cytotoxic cells, such as natural killer cells, monocytes, macrophages, neutrophils and eosinophils (63), and some evidence suggests, lymphocytes (129), to bind to the target cell, via the Fc region of the antibody, and mediate killing in the absence of complement. Evidence suggests that target cell killing

is mediated through the release of lytic enzymes, perforin, TNF and granzymes (9,15,81) the cumulative effect of which causes lysis and cell death, preventing the spread of infection.

1.2.4 Treatment of *Burkholderia pseudomallei* infection

B. pseudomallei is intrinsically resistant to many antibiotics including aminoglycosides and early β-lactams (48). A complete lack of response to both penicillin and gentamicin, a commonly used combination for the treatment of septicaemia in the tropics, is characteristic of melioidosis (35,109). Despite this, β-lactam drugs such as ceftazidime, co-amoxiclav and imipenem have halved the mortality of melioidosis since the mid 1980's (50). A number of studies have also demonstrated the susceptibility of *B. pseudomallei* to a number of other antibiotics including some third generation cephalosporins (such as cefotaxime and ceftriaxone), ureidopenicillians, tetracyclins, trimethoprim-sulphamethoxazole and fluoroquinolones (reviewed in (33)). Treatment is usually given in two phases, an acute parenteral phase that lasts between 10 – 14 days (50) and 4 weeks (109), followed by an oral eradication phase lasting 3 to 6 months (50,109). Despite many advances in melioidosis treatment, acute mortality, especially in Thailand, still remains high (50).

1.2.4.1 Current Burkholderia vaccines

Increasing recognition of melioidosis as a significant cause of morbidity and mortality together with complications in treatment has led to a number of studies investigating immunoprophylaxis for the disease (229). While presently no vaccine exists to protect against melioidosis, a number of approaches have shown promise, including immunoglobulin therapy (21,24) and conjugate vaccines (reviewed in (22)). Other approaches include the use of recombinant vaccines based both on *B. pseudomallei* (8)

and other bacteria (56), and attenuated mutants of *B. pseudomallei*, which have been used with some success (8).

1.2.5 Pathogenesis of *Burkholderia pseudomallei* disease

The literature suggests that the vast majority of *B. pseudomallei* infections are sub-clinical (7,48,114,229), although this literature does not take into account the degree of seroconversion caused by *B. thailandensis* (20). If, however this is in fact the case, then it suggests that the immune response to *B. pseudomallei* in immunocompetent individuals is usually sufficient to suppress the emergence of disease. However, in a minority of cases of primary infection or during recurrence, an acute, septic infection results, which overwhelms the host immune response and death usually ensues (126,229).

The exact reasons for such a difference in disease presentation still require elucidation. There is evidence that host resistance is strongly influenced by the cell-mediated response. This was first suggested by studies showing an impaired cellular immune state in melioidosis (207), and later through the demonstration that the use of the immunopotentiating agent levamisole as a treatment for melioidosis patients, substantially improved their recovery rate (208). More recent studies have demonstrated a key role for IFN-γ in controlling melioidosis in the mouse model (177), and that natural killer cells and CD 8$^+$ T cells, activated by a cytokine-dependent bystander mechanism, are the most important sources of the rapid production of IFN-γ (124). In contrast, despite the apparent frequency of seroconversion in melioidosis endemic areas, evidence of a role for the humoral immune response in protection is often contradictory. A number of studies into the pathogenesis of and immunity to melioidosis have found no correlation between circulating antibody and protection

against disease (7,114,229). In contrast however, the success of passive immunity studies utilising both immune sera (24) and monoclonal antibodies (112) has also been demonstrated.

These combined data demonstrate that significant further work is required to properly elucidate the immune mechanisms required for protection against melioidosis that would in turn provide a platform from which rational vaccine design could be implemented.

1.3   Immunoprophylaxis

The first accounts of the use of immunoprophylaxis date to about 1000 AD in China (65) where the scabs from smallpox patients that were only mildly affected by disease were used to intranasally inoculate healthy individuals (125). The process was known as variolation and was fairly successful in comparison to the number of victims of natural smallpox infection (65). Vaccine research began during the late 18th century (107,108) and progressed, sometimes using questionable techniques, over the next century leading to the production of vaccines with variable efficacy, against anthrax (154,155), cholera, tuberculosis, typhoid (125), rabies (153) and yellow fever (210,211).

The advent of molecular biology as a scientific discipline radically altered vaccine research and development, with the ability to screen entire proteomes, and even more recently genomes for immuno-stimulatory proteins enabling a much more rapid and rational approach to vaccine development. Together with this improved efficacy, modern day techniques have also enabled improvements in safety, in an increasing number of examples, by removing the need for the use of whole cell killed or attenuated vaccines.

Of equal importance to the identification of immunogenic proteins as vaccine candidates is the formulation in which they are delivered. As discussed, different activation stimuli can have a profound effect on the type of immune response generated highlighting the need to both target the appropriate cell type with the antigen of choice and stimulate the right type of immune response to achieve protection against the infectious agent.

1.3.1 Rational vaccine design

The ultimate goal of a vaccine is to induce long-term immunological memory. Until the early 1990's vaccine programs often failed to effectively prime cellular responses to provide protection against disease, rather focusing on inducing high antibody titres (reviewed in (64)). This strategy had until recently been successful because the pathogens being targeted either did not cause chronic infections or were antigenically stable. Thus the need to understand the mechanisms of cellular immunity has arisen from a requirement to develop vaccines against persistent, chronic and intracellular infections (64).

The primary goal of vaccination is to prime the immune system enabling the host's immune system to respond more rapidly and effectively following infection than would normally be the case after first encountering a pathogen. Thus rational vaccine design requires consideration of the protective immune response, the nature of the immunising antigen, route of exposure, dose and adjuvants to be used. Once the antigen or antigens have been identified consideration then needs to be given to the appropriate route and mechanism of delivery that will induce the optimal immune response (64).

1.4 Dendritic cell immunotherapy and prophylaxis

1.4.1 Dendritic cells as adjuvants for immunisation

The modulation of immunity is an important goal in the treatment and prevention of infectious diseases. Several methods have been employed in attempts to enhance or suppress specific immune responses. These include; the administration of antigens encoded by DNA or viral vectors together with adjuvants, heat shock proteins or cytokines (reviewed in (168)), the adoptive transfer of antigen-specific T cells, or the use of monoclonal antibodies (reviewed in (17)).

DC, as discussed, are initiators and modulators of immune responses against microbial, tumour and self-antigen (12) and the most efficient APC capable of initiating primary immune responses. Following experiments by Inaba *et al* (99), which showed that the injection of *ex vivo* antigen pulsed DC sensitised normal mice to protein antigens, it was suggested that DC might be used directly as a vaccine thus avoiding problems with variable *in vivo* targeting. The immunogenicity of antigens delivered on DC was later demonstrated in human studies involving the single subcutaneous injection of 2-4 x 10^6 antigen-pulsed DC, which led to the rapid expansion of CD 4^+ and CD 8^+ T cell immunity. A subsequent boost several months later led to the expansion of cytotoxic T cells with increased affinity against viral peptide, an observation never made with any other vaccination strategy (57,58).

Several clinical studies have now been published using DC-based immunotherapy although the vast majority of these are in cancer patients (reviewed in (71)). Studies focused on the use of DC-based immunotherapy in chronic infectious diseases such as

human immunodeficiency virus infection are far fewer and have as yet not yielded such encouraging results (71).

In contrast, studies in healthy volunteers have repeatedly demonstrated the immunogenicity of DC. The subcutaneous administration of mature DC pulsed with tetanus toxoid, keyhole limpet haemocyanin and human leukocyte antigen restricted influenza matrix protein, has been shown to prime individuals to each of the pulsing antigens (58). Immune responses were seen to peak one to three months following injection declining between six and nine months. The maintenance of memory was also established following the re-injection of pulsed DC, which resulted in greater, more rapid and higher avidity CD 8^+ T cell responses (57).

In mice, the use of DC as vaccine delivery vehicles has also been demonstrated. Worgall *et al* (2001) (230) showed that immunisation with *Pseudomonas aeruginosa* pulsed DC stimulated a CD 4^+ T cell dependent cell-mediated immune (CMI) response although no antibody production was noted following DC immunisation. They also demonstrated that immunisation with *P. aeruginosa* pulsed DC afforded an increased survival rate in 45% of the population following subsequent intrapulmonary challenge with *P. aeruginosa* (230). Potent type 1, protective responses have also been demonstrated against chlamydial genital tract infection following immunisation with DC pulsed *ex vivo* with killed Chlamydiae (200). In this case immunisation with DC represents the best vaccine available for prevention of disease and achieves levels of protection equivalent to that following infection. In contrast however, Shaw *et al* (2002) (185) later demonstrated a CD 4^+ T cell response following immunisation with DC pulsed with a chlamydial outer membrane protein, which was type 2 in nature and failed to provide protection against disease. This switch in immunity and loss of

protection highlights the need for rational vaccine design and demonstrates the importance of the nature of the antigen used on the final Th1-Th2 balance of the immune response *in vivo* (185).

1.4.2 Potential problems associated with dendritic cell vaccination

Although to date no significant toxicities relating to DC immunisations have been reported (17), a few potential concerns have been highlighted. One such concern is the development of autoimmunity following immunisation with DC pulsed with shared antigens of tumour and self cells. Most examples of this have been low-grade reactions although autoimmune syndromes have been reported (131). In addition, allergic reactions to bovine albumin following administration of foetal bovine serum cultured DC have been documented (131), thus suggesting the need for investigations into serum free culture conditions for DC to be used in immunotherapies. There is also the risk of viral contamination, especially retroviral, of cultured DC, which may then infect the patient rather than having the desired prophylactic effect. Beside clinical problems, another issue is the ability to generate the large numbers of DC needed for large-scale immunisation. Although rapidly advancing, current culture systems are presently inadequate for such a task suggesting the need to develop strategies that can provide protective immune responses with minimum amounts of vaccine and limited boosting (11). Approaches aimed at overcoming this problem include the use of DC-poietins, cytokines that mobilise DC *in vivo*, and 'intelligent missiles', which are generic vaccines containing immunogens, and DC activation molecules that together can be targeted to specific DC subsets (11).

DC are attractive targets for the therapeutic manipulation of the immune system (11). Whilst their plasticity can provide a platform from which host immunity could be

guided to produce the best response for the infectious disease being targeted, their suitability over other vaccination regimens still remains to be proven (17).

1.5   Project objectives

The need for an efficacious vaccine against melioidosis is increasingly highlighted, as complications in treatment become more prevalent (229). A number of approaches to vaccination are currently under investigation, the most successful of which have involved the use of an attenuated mutant of *B. pseudomallei* (8) or passive protection through the use of monoclonal antibodies (112). The effectiveness of passive protection as a technique, whilst initially good, is reduced over time as circulating antibody levels wane. It also fails to stimulate immunological memory meaning it is unsuitable as a vaccination strategy due to the high logistical burden imposed by the requirement for repeated immunisations. The use of live attenuated bacteria is an approach to vaccination used since the beginnings of vaccine research. The success of these studies for *B. pseudomallei* infection suggests that further characterisation of the protective immune response is likely to provide a rational basis for design and aid future vaccine development work on the disease.

The intracellular nature of *B. pseudomallei* suggests the need for the development of long-term cell-mediated immunity in protection against infection. Thus, in this project, DC were selected for investigation as a delivery vector capable of stimulating a strong CMI response. Herein the potential of DC to fulfil this role is evaluated and the appropriateness of their use for *B. pseudomallei* infection considered. The question of rational vaccine design is also addressed through work that begins to elucidate the role of type 1 and type 2 immunity in protection against *B. pseudomallei* infection.

Chapter 2

Materials and Methods

2.1   Isolation and culture of primary cells

2.1.1   Experimental animals

Female BALB/c mice (10-12 weeks old) were obtained from Charles River Ltd, UK. All animals were housed at Dstl, Porton Down under specific pathogen free (SPF) conditions with free access to food and water. All procedures were carried out in accordance with the requirements of the Animal (Scientific Procedures) Act 1986.

2.1.2   Isolation of lymphocyte populations from murine spleen

Spleens were isolated and placed in RPMI-1640 (Sigma, UK) for transport to a class II microbiological safety cabinet. In the cabinet, spleens were gently passed through sterile 70 μm nylon sieves (BD Falcon, UK) to create a single cell suspension and the splenic capsule discarded. After washing by centrifugation at 300g for 10 minutes and discarding the supernatant, the cell pellet was re-suspended in 5 mL sterile complete medium. Complete medium comprised, RPMI-1640 containing 10% heat inactivated foetal bovine serum; HI FBS (Sigma, UK), 1% penicillin / streptomycin / glutamine; PSG (Sigma, UK) and 50 μM 2-mercaptoethanol; 2-ME (Sigma, UK). The cell suspension was layered onto 3 mL lymphocyte separation medium (LSM) (MP Biomedicals, Europe) and centrifuged at 800g for 30 minutes with no braking to prevent mixing during the deceleration phase. After centrifugation, lymphocytes were visible as a band of cells at the interface between the LSM and complete medium. These cells were removed using a plastic pipette, washed by centrifugation at 300g for 10 minutes and then a viable count performed using an ethidium bromide / acridine orange stain, before proceeding to magnetic separation. The counting medium was prepared by adding ethidium bromide and acridine orange to sterile phosphate buffered saline (PBS) at a final concentration of 5 μg mL^{-1} and 3 μg mL^{-1} respectively.

2.1.3   T cell separation from splenocyte cultures

The separation of $CD4^+$ and $CD8^+$ T cells for further use was achieved using the magnet assisted cell separation (MACS) kit specific for each population. Cell isolation kits and all reagents were purchased from Miltenyi Biotech, UK. All procedures were carried out in accordance with the manufacturer's instructions unless otherwise stated.

2.1.4   Isolation and culture of myeloid dendritic cells from bone marrow progenitor cells

The method for the culture of myeloid dendritic cells (DC) was developed from established methods (100,130,183,204). Mice were culled by cervical dislocation and the tibiae and fibulae excised using scissors and forceps. Excess tissue was removed before the bones were sterilised with 70% ethanol and placed in complete medium for transport to a class II microbiological safety cabinet. In the cabinet the bone epiphyses were removed and the bone marrow flushed from the shafts with complete media using a 25-gauge needle and syringe. Gentle pipetting was then used to break up any bone marrow pellets and the cells then washed by centrifugation at 300g for 10 minutes. Following washing, the cell pellet was re-suspended and a viable count was performed with an ethidium bromide / acridine orange stain. The cell concentration was then adjusted to 2×10^6 cells mL^{-1} in complete medium.

Granulocyte-macrophage colony stimulating factor (GM-CSF) (R&D Systems, Europe) at 20 ng mL^{-1} and tumour necrosis factor-alpha (TNF-α) (R&D Systems, Europe) at 10 ng mL^{-1} were routinely added to the cell suspensions. Additional cytokines, interlukin-(IL-) 4 and IL-12 (both obtained from R&D Systems, Europe) were added to DC cultures at a final concentration of 10 ng mL^{-1}during experiments to determine optimum DC culture conditions. The final cell suspension containing the appropriate cytokines

was then plated out into 6-well tissue culture plates (Sterilin, UK) at 2 mL per well and the plates incubated at 37°C in a fully humidified environment in the presence of 5% CO_2 for 4 days.

On day four of the culture the plates were removed from the incubator and the cells gently scraped from the well surface. The suspension was then pooled, centrifuged at 300g for 10 minutes and re-suspended in 5 mL of complete medium per 6-well plate. The suspension was layered in 10 mL aliquots onto 2 mL of 13.7% (w/v) metrizamide (Sigma, UK). Metrizamide was prepared prior to use by adding 7.25g metrizamide to 45 mL RPMI-1640 containing 1% PSG. Once dissolved, 5 mL of HI FBS was added to the metrizamide, which was then separated into 2 mL aliquots and stored at -20°C until use (133). Once layered onto the metrizamide, the cells were centrifuged at 800g for 10 minutes with no braking to prevent any mixing during the deceleration phase. After centrifugation the DC were visible as a band of cells at the interface between the metrizamide and the culture medium that could be easily removed using a plastic pipette. After isolation, the DC were washed twice by centrifugation at 300g to ensure all metrizamide was removed from the cells, counted using an ethidium bromide / acridine orange stain, and re-suspended ready for use.

2.2 Phenotypic analysis of cells

2.2.1 Flow cytometry

Flow cytometric analysis was used to characterise cell populations. Cells to be analysed were washed by centrifugation at 300g for 10 minutes and pellets were re-suspended in buffer (phosphate buffered saline containing 2.5% HI FBS). Cells were then washed again and re-suspended in fresh buffer at a concentration of 1 x 10^7 mL^{-1}. Aliquots of

cells (100 μL) were pipetted into polystyrene flow cytometry tubes (BD Falcon, UK) and Fc block (anti-CD 16 / CD 32) (BD Biosciences, Pharmingen, International) added to each tube at a concentration of 1 μg per 1 x 10^6 cells. Antibodies appropriate to the investigations being undertaken were then added to each tube. Antibodies obtained from BD Biosciences, Pharmingen, International and Serotec, UK were used as described in table 2.1. For staining, cells and antibodies were incubated together for 30 minutes at 2-8°C. Further antibody binding was then halted by the addition of 4% paraformaldehyde to each tube to fix the cells. Cells were fixed for at least 1 hour at 2-8°C and stored for no longer than 72 hours before analysis.

In all flow cytometry studies corresponding isotype controls were used to establish quadrants and / or regions for analysis. Analyses were performed using Cell Quest Pro flow cytometry analysis software.

Supplier	Coupled fluorochrome	Concentration (μg per 1 x 10^6 cells)
BD Biosciences Pharmingen	Fluorescein isothiocyanate (FITC)	1.0
BD Biosciences Pharmingen	R-phycoerythrin (R-PE)	0.2
BD Biosciences Pharmingen	Peridinin chlorophyl-α protein (PerCP)	0.2
BD Biosciences Pharmingen	R-PE-Cychrome (Cy) 5	0.2
BD Biosciences Pharmingen	PerCP-Cy 5.5	0.2
Serotec, UK	FITC	5.0
Serotec, UK	R-PE-Cy 5	2.0

Table 2.1   Flow cytometric fluorochromes

Fluorochromes used for flow cytometry analysis and the concentrations at which they were used.

2.3 Culture of *Burkholderia pseudomallei*

2.3.1 Culture of viable *Burkholderia pseudomallei*

All procedures involving the culture of viable *B. pseudomallei* were performed under ACDP category III conditions. Stock *B. pseudomallei* (NCTC 4845) was previously prepared by Mr Tony Stagg and Mr Richard Beedham (Biomedical Sciences, Dstl, Porton Down) (Stagg, A. J., personal communication) and stored at -80°C on protect beads (Technical Service Consultants Ltd, UK). Cultures were prepared by adding five beads to 10 mL nutrient broth and incubating at 37°C for 24 hours in a static culture. This culture system has been shown to typically yield 1×10^8 cfu mL^{-1} (Stagg, A. J., personal communication) and thus for the purpose of calculating challenge doses such a yield was assumed.

Accurate viable counts were obtained by setting up serial dilutions of the culture and preparing spread plates using 100 µL aliquots of the serial dilutions on nutrient agar (in triplicate). Once spread, plates were incubated at 37°C overnight before manual enumeration of the number of colony forming units (cfu). A dilution was then chosen at which between 20 and 200 cfu were present on the plates and a mean colony count determined from the triplicate. The original stock concentration was then calculated using the equation below:

Number of cfu mL^{-1} = Mean cfu x 10 x dilution factor

2.3.2   Heat inactivation of *Burkholderia pseudomallei*

Heat inactivation was kindly performed by Ms Michelle Nelson (Biomedical Sciences, Dstl, Porton Down).

Bacterial cells were harvested by centrifugation and washed three times in PBS, before re-suspending in one tenth the original volume of PBS. The suspension was then placed in O-ring sealed centrifuge tubes (Sorvall, Connecticut, USA) in a water bath at 80°C for 3 hours, with occasional shaking. Following inactivation, the suspension was checked for viability by inoculating 10 mL volumes of nutrient broth with 0.5 mL aliquots of the suspension to a total volume of 5 mL or 10% of the total volume (whichever was smaller) and incubating at 37°C for seven days. Nutrient agar plates were then inoculated with the total volume of the broth cultures and incubated at 37°C for a further seven days to check for bacterial growth. If no growth occurred on the agar plates the bacterial suspension was considered inactivated.

2.4   Characterisation of the cellular immune response to *Burkholderia pseudomallei*

2.4.1   Maturation of dendritic cells

Dendritic cells were cultured and purified as described in *2.1.4*. The cells at a concentration of 2×10^6 mL^{-1} were transferred to 24-well tissue culture plates in 1 mL aliquots and 'rested' for 24 hours at 37°C in a fully humidified atmosphere in the presence of 5% CO_2. After 'resting', the desired maturation stimuli were added to the DC at the appropriate time points; TNF-α at 50 ng mL^{-1}, lipopolysaccharide (LPS) at 10 μg mL^{-1} or heat killed (HK) *B. pseudomallei* at 1×10^7 cfu mL^{-1}. After co-culture for

the desired length of time, cells were removed from the wells and stained for flow cytometric analysis (*see 2.2.1*) before fixing and storage at 2-8°C.

2.4.2 Primary (naïve) Lymphocyte proliferation assays

Dendritic cells were cultured and purified as described in *2.1.4*. HK *B. pseudomallei* was then added to the culture at the desired concentration and incubated with the DC for 18 hours at 37°C in a fully humidified atmosphere in the presence of 5% CO_2. After incubation, the DC were washed by centrifugation at 300g for 10 minutes. The supernatant was discarded and the pellet re-suspended in fresh complete medium. This was repeated twice to ensure any extracellular antigen had been removed and the DC were finally re-suspended to a concentration of 5 x 10^5 cells mL^{-1} in complete medium without cytokines.

Spleens from naïve mice were removed and gently passed through sterile 70 µm nylon sieves (BD Falcon, UK) into complete medium. The suspension was centrifuged at 300g for 10 minutes and the supernatant discarded. Three mL of sterile 0.85% ammonium chloride (Sigma, UK) was added to the cell suspension, which was then incubated for 5 minutes at room temperature with occasional agitation to ensure thorough mixing. After incubation the cells were washed twice by centrifugation at 300g for 10 minutes to remove any ammonium chloride. After the final wash the cells were counted and re-suspended to a concentration of 5 x 10^6 cells mL^{-1}.

The splenocyte suspension and antigen-pulsed DC were plated out in 100 µL aliquots. Unstimulated DC, HK *B. pseudomallei* alone and complete medium alone were used as negative controls and Concanavalin A (Con A) (Sigma, UK) at a final concentration of 1 µg mL^{-1}, was used as a positive control in all assays.

Plates were incubated at 37°C in a fully humidified environment in the presence of 5% CO_2 for 1 – 6 days depending on the experiment being performed. After the required incubation period 37 MBq (1 μCu) methyl-tritiated thymidine (Amersham Biosciences, UK) was added to each well on the plates, which were then returned to the incubator for a further 24 hours. After this incubation, the cells were harvested onto 96-well GF-C filter microplates (PerkinElmer Life Sciences, UK) using an automated cell harvester (PerkinElmer Life Sciences, UK) and allowed to dry overnight. Once dry, 20 μL of scintillation liquid (Amersham Biosciences, UK) was added to each well of the filter plate and the degree of methyl ^{3}H-thymidine incorporation measured using a microplate scintillation counter (PerkinElmer Life Sciences, UK).

Data were presented as mean counts per minute (CPM), which is calculated from the mean of five replicates from pooled lymphocytes from five separate spleens , ± standard error of the mean (SEM).

2.4.3   Secondary (recall) lymphocyte proliferation assay

2.4.3.1 Unseparated splenocyte proliferation

Splenocytes for memory proliferation assays were prepared in the same manner as those in *2.4.2*. Spleens from immunised mice were used to assess the degree of memory proliferation and splenocytes from naïve animals were used as controls. Once prepared, the lymphocyte suspensions were plated out in 100 μL aliquots. The antigen against which the memory response was being investigated was prepared to twice the optimum concentration in complete medium and then added to the appropriate wells on the assay plate in 100 μL aliquots. Lymphocytes from naïve spleens were used in all assays as a

negative control. Complete medium only and HK *B. pseudomallei* only negative controls and Con A positive controls were also included on all plates. Plates were incubated at 37°C in a fully humidified environment in the presence of 5% CO_2 for 1-6 days as required. The degree of lymphocyte proliferation was then assessed by the incorporation of methyl ^{3}H-thymidine as described in *2.4.2*.

2.4.3.2 Purified T cell proliferation

Antigen-pulsed DC were prepared as described in *2.4.2* and isolated lymphocyte populations were prepared from immunised animals as described in *2.1.2* and *2.1.3*. Once isolated, lymphocyte populations were centrifuged at 300g for 10 minutes to remove the isolation medium and re-suspended to a concentration of 2.5 x 10^6 cells mL^{-1} in complete medium. DC and lymphocytes were then aliquoted into 60-well terasaki plates (Greiner Labortechnik Ltd, UK) in 10 µL volumes. Lymphocyte only, DC only and complete medium only negative controls were included on each plate. Con A at a final concentration of 1 µg mL^{-1} was included as a positive control. Naïve lymphocytes were also incubated with HK *B. pseudomallei* pulsed DC and the data included for comparison. Plates were inverted to allow cells to converge at the bottom of the medium drop in each well, and incubated in a fully humidified atmosphere at 37°C in the presence of 5% CO_2 for 4 days.

After incubation, 37 MBq (1 µCu) methyl ^{3}H-thymidine was added to each well and the plates returned to the incubator for a further 24 hours. Cells were then transferred to the wells of a 96-well tissue culture plate containing 100 µL complete medium, harvested and counted as described in *2.4.2*. Data from these assays was presented as the mean stimulation index (SI), ±SEM. This was due to the fact that the assays required multiple

proliferation plates to be set up on a weekly basis rather than all at the same time. All plates were set up with the appropriate negative control (naïve unstimulated T cells), which was used to calculate the SI:

$$SI = \frac{CPM\ of\ test}{CPM\ of\ negative\ control}$$

Mean SI was derived from four replicates of the pooled lymphocytes of two test subjects.

2.5   Cytokine analysis

2.5.1   Cytometric Bead Array

Cytokine analysis of serum and supernatant samples was carried out using Cytometric Bead Array (CBA) kits and was performed in accordance with the manufacturers' instructions. Kits used were either the mouse inflammation CBA kit (552364, BD Biosciences, UK) or the mouse Th1 / Th2 CBA kit (551287, BD Biosciences, UK).

2.6   Characterisation of the antibody response

2.6.1   Serum antibody quantification using the enzyme linked immunosorbant assay (ELISA)

Assay plates were prepared by coating 96-well ELISA plates (Immulon 2, Dynex Technologies, UK) with the HK *B. pseudomallei*. Fifty μL PBS containing HK *B. pseudomallei* at a concentration of 1×10^6 cfu mL^{-1} was added to each well of the desired number of 96-well plates. Wells to be used for the generation of a standard curve were coated with Fab specific mouse IgG at 5 μg mL^{-1}. The plates were covered

and incubated overnight at 2-8°C. After incubation the plates were washed three times with PBS containing 0.02% Tween 20 (Sigma, UK) using a SKATRON-SkanWasher 400 automated plate washer.

Blotto (2% (w/v) skimmed milk powder in PBS) was added to each well in 200 µL aliquots and the plates blocked for 1 hour at 37°C. After the blocking step plates were again washed three times.

Test samples were diluted 1/100 in 2% Blotto and mouse total IgG standard (Sigma, UK) was diluted to the optimum starting concentration (100 ng mL^{-1}), also in 2% Blotto. After washing, 100 µL aliquots of the standard (in triplicate) and test samples (in duplicate) were added to the appropriate top wells of the plate and 50 µl of 2% Blotto added to all other wells on the plates. The standard and samples were then double diluted down the plate. Plates were covered and incubated at 37°C for 2 hours. After incubation the plates were washed and 50 µL anti-mouse total IgG-horse radish peroxidase (HRP) conjugate (Immunologicals direct, UK) (diluted 1/2000 in 2% Blotto) added to each well. Plates were incubated for a further 2 hours at 37°C. After incubation the plates were washed for the final time and 100 µL ABTS substrate - 3 ABTS tablets (Sigma, UK) in 50 mL citrate buffer + 5 µl hydrogen peroxide (Sigma UK) - added to each well. The plates were left for 20 minutes for the colour to develop and read at 414nm.

Optical density readings were converted to antibody concentrations using the standard curves set up on each ELISA plate. Analysis was performed using 'Ascent'

spectrophotometer analysis software. Results were displayed as mean concentration, of five test subjects each assayed in duplicate ± SEM.

2.7   *In vivo* experimentation

All procedures involving the use of *B. pseudomallei* were carried out under ACDP containment level III conditions unless the bacteria had been previously demonstrated to be dead. Under such circumstances, procedures were carried out under ACDP containment level II conditions.

2.7.1   Dendritic cell immunisation

HK *B. pseudomallei* pulsed DC were prepared as described in *2.4.2*. Following incubation the DC were removed from the incubator and washed by centrifugation at 300g for 10 minutes, discarding the supernatant and re-suspending the cell pellet in sterile PBS. This was repeated twice to ensure any extracellular antigen was removed. After the final wash, DC were re-suspended to 1×10^6 cells per 100 µL in sterile PBS for intra-dermal (ID) immunisation or 2×10^6 cells per 100 µL in sterile PBS for intra-nasal (IN) immunisation.

DC administered via the ID route were given in 100 µL aliquots (50 µL into each hind leg), while IN immunisation was carried out using 50 µL aliquots into the nostrils of animals under Halothane (RMB Animal Health Ltd, UK) sedation.

2.7.2   Immunisation with heat killed *Burkholderia pseudomallei*

HK *B. pseudomallei* were prepared to a concentration of 5×10^4 cfu mL^{-1} in sterile PBS. A vial of MPL + TDM adjuvant system (Sigma, UK) was warmed to 37°C and then

reconstituted with 2 mL of the HK *B. pseudomallei* in PBS preparation. In accordance with the manufacturers' instructions the vial was then vortexed for 2 to 3 minutes, inverted and vortexed for a further minute before use. Immunisation was performed with 100 µL aliquots of the emulsion (50 µL into each hind leg), which had cooled to room temperature, via the intra-muscular (IM) route.

2.7.3 Determination of LD$_{50}$ for *Burkholderia pseudomallei* (NCTC 4845)

Viable *B. pseudomallei* were prepared as described in *2.3.1* and serially diluted to produce a dose range between 10 cfu mL^{-1} and 1 x 10^5 cfu mL^{-1}. Aliquots (100 µL) of each of the challenge inocula were then plated out onto nutrient agar (in triplicate) and incubated at 37°C overnight to provide accurate counts for the final dose calculation (*see 2.3.1*).

Groups of 5 female BALB/c mice were challenged with 100 µL viable *B. pseudomallei* via the intra-peritoneal (IP) route with a challenge dose from the dose range described. Animals were closely observed for 35 days following challenge, at which point the experiment was terminated and any survivors humanely culled. Time to death and / or number of survivors was recorded for each group and the LD$_{50}$ then calculated using the method of Reed and Muench (169).

2.7.4 *Burkholderia pseudomallei* challenge studies

Viable *B. pseudomallei* were prepared as described in *2.3.1* and diluted to give an approximate challenge dose of 1 x 10^5 cfu mL^{-1}. Challenge was performed with 100 µL aliquots of the challenge inoculum via the IP route. After challenge, mice were closely observed for 35 days, at which point experiments were terminated and any survivors humanely culled.

2.7.5   Assessment of post-challenge bacterial load

Tissue samples from survivors were collected for bacteriology at day 35 post-challenge. Brains, spleens, livers, lungs and blood were taken to provide samples for analysis. Organs were passed through 70 μm nylon sieves into sterile PBS to create a cell suspension and blood diluted 1 in 2 in sterile PBS. Aliquots of 100 μL of each of the tissue samples were then spread onto nutrient agar plates and incubated at 37°C for 24 hours. After incubation the plates were assessed for bacterial growth and the number of colonies manually enumerated.

2.8   Statistical analysis

Statistical analyses were performed, with the kind assistance of Mr Robert Gwyther (Dstl, Biomedical Sciences). Analysis of the mature population of DC following culture (*section 3.1.2.1*) was performed using general linear model Analysis Of Variance (ANOVA) while all remaining *in vitro* experiments were analysed using two-way ANOVA's. The only exception was analysis of the naïve *in vitro* proliferation assays (*section 3.2.2*), which were analysed using a multivariate ANOVA. Analysis of the challenge and LD_{50} data was performed using PRISM graph pad survival analysis software, and p-values calculated using the log rank test for trend.

Chapter 3

Results

3.1 Growth, manipulation and characterisation of *ex vivo* isolated cells

3.1.1 Culture of dendritic cells from bone marrow progenitor cells

DC were initially cultured for 7-10 days in the presence of GM-CSF with media changes on days 4 and 8 as described in the literature (183,203). Whilst these systems succeeded in generating DC, yields were often not sufficient and removing the DC from the culture plates, by dislodging proliferating aggregates, caused a high degree of non-specific maturation. Using magnetic sorting methods to further purify the cells compounded this problem and also induced maturation.

The method was further refined and TNF-α was added to cultures in an attempt to improve yields. The use of metrizamide as a density gradient on which to purify the DC was also investigated. The addition of low concentrations (1 ng mL^{-1}) of TNF-α to the culture also significantly improved the culture rate such that proliferating aggregates of DC were abundant by day four of the culture (Figure 3.1). Flow cytometric analysis of DC at day 4 of the culture following metrizamide purification revealed a cell population with a high forward scatter / side scatter profile, which typically constituted greater than 80% of the total population (Figure 3.2). Further flow cytometric analysis of these cells with a panel of antibodies revealed a phenotype typically expressed by myeloid DC; MHC class II^{++}, CD 11c$^+$, CD 80$^+$, CD 86^{++}, CD 205$^+$, CD 11b$^+$, CD 14$^+$, CD 34$^+$, CD 8α^+, CD 54$^+$ and CD 40$^+$ (Figure 3.3) (this phenotypic pattern was consistently noted in five separate experiments). It also demonstrated that the cell population contained a low percentage (typically less than 20%) of contaminating cell populations, assessed on the basis of the T cell marker CD 3, the B cell marker CD 19,

the macrophage / monocyte marker F4/80 and the granulocyte marker Ly.6G (Table 3.1).

Cell Marker	Cell Lineage association	Percentage expression in DC population
CD 3	T cells	4.76% (±0.38)
CD 19	B cells	1.14% (±0.2)
F4/80	Macrophages / Monocytes	7.82% (±0.35)
Ly.6G	Granulocytes	7.3% (±0.59)

Table 3.1   Contaminating cell populations present in a dendritic cell culture

Percentage expression of non-dendritic cell lineage cell markers within the metrizamide purified dendritic cell population.   Contamination by other leukocyte populations was typically approximately 20%. Number in parentheses equals standard error of the mean (SEM), calculated from expression observed in five separate cultures.

The effect of adding either IL-4 or IL-12 to the culture was also investigated. The addition of either of these cytokines resulted in no notable phenotypic differences to the DC population (data not shown). However, while typical cultures with GM-CSF and TNF-α (GT), and GM-CSF, TNF-α and IL-4 (GT4) produced yields of between 7 and 10 x 10^6 DC per mouse, a pronounced reduction in yield (2 to 3 fold) was noted in all cultures including IL-12 (GT12).

3.1.2   Dendritic cell responsiveness to maturation stimuli

3.1.2.1 Effect on phenotype

The proportion of mature to immature DC present in purified DC populations affects their ability to process and present antigen. If too high a proportion of the DC were already mature before exposure to antigen, their ability to generate an appropriate immune response would be hindered as a result of the reduced uptake and processing faculties of mature DC. A mature DC phenotype is characterised by high MHC class II expression. The mature proportion of cells observed for the GT and GT12 cultures was 24.45% and 23.91% respectively, while the GT4 culture produced a mature population of 31.05% (Figure 3.4) although this was not found to be significantly greater ($p>0.05$). This experiment was repeated five times and no significant variation was noted between experiments ($p>0.05$).

The responsiveness of the DC population to maturation stimuli was investigated, which allowed the establishment of their suitability for further experimentation. DC cultured under the three cytokine conditions, GT, GT4 and GT12, were exposed to either the inflammatory cytokine, TNF-α, or the bacterial cell wall component, lipopolysaccharide (LPS) from *Salmonella typhimurium*. Cell maturation status was assessed on the basis of high MHC class II expression in addition to high expression of the co-stimulatory molecules CD 80 and CD 86 and the cell adhesion molecule CD 54, at 6, 24 and 48 hours post-exposure. Increased expression of MHC class II, CD 80, CD 86 and CD 54 is associated with DC maturation and is essential for the interaction with, and stimulation of naïve T cells.   Figures 3.5 to 3.7 illustrate the effect of each maturation stimulus on DC cultured in each of the cytokine environments compared to control unstimulated DC.

For DC cultured in the GT cytokine environment (Figure 3.5), exposure to TNF-α induced an increase in the percentage of MHC class II and CD 86 bright cells after 6 hours, which continued increasing to 48 hours post exposure. An increase in the percentage of CD 54 bright cells was noted 24 hours after exposure, which increased further after 48 hours. No increase in the percentage of CD 80 bright cells was observed. A similar pattern of expression was noted for MHC class II, CD 86 and CD 54 following exposure to LPS. In addition, an increase in the percentage of CD 80 bright cells was observed, which was at its greatest 48 hours post exposure.

This pattern of marker expression over the 48 hours post exposure was also observed for DC cultured in the GT4 and GT12 cytokine environments (Figures 3.6 and 3.7 respectively), although the final percentages of expression varied. The only exception to this was exposure of the GT12 cultured DC to TNF-α. This population initially showed an increase in the percentage of MHC class II, CD 86 and CD 54 bright cells up to 24 hours post-exposure, which was followed by a reduction in the percentage of bright cells for each marker by 48 hours.

3.1.2.2 Effect on cytokine secretion

At each of the flow cytometric analysis time points, supernatant samples were taken and analysed for the cytokines IFN-γ, IL-5, IL-2, IL-10 and IL-6. Concentrations observed at 6, 24 and 48 hours after exposure were normalised against that observed from control unstimulated DC. No increase in IL-6 secretion was noted for any of the DC groups (GT, GT4 or GT12) and although assessed in later experiments, IL-4 and IL-12p70 concentrations were not measured due to the inability to distinguish between cytokine added to the culture and cytokine secreted by the DC. For the GT cultured DC (Figure

3.8), stimulation with TNF-α resulted in a significant increase in IL-10 (p<0.05) by 24 hours post-exposure. An increase in IL-2, IL-5 and IFN-γ secretion was also observed by 24 hours although these were not found to be significant (p>0.05). Exposure of the GT cultured DC to LPS produced a similar pattern of cytokine secretion although there was an approximately two-fold increase in IL-10 secretion (p<0.05). Again, increases in IL-2, IL-5 and IFN-γ secretion were not found to be statistically significant.

Exposure of the GT4 cultured DC to TNF-α induced an initial increase in IL-10 secretion (p<0.05), which fell by 24 hours post-exposure. Increases in IFN-γ, IL-2 and IL-5 were not significant. Exposure of this group to LPS led to a significant increase in IFN-γ and IL-10 secretion by 24 hours post-exposure (p<0.05) compared to control unstimulated DC. IL-10 secretion was also noted to increase in the GT12 group following exposure to LPS, which had the greatest level of IL-10 secretion (p<0.01) 48 hours post-exposure. Increases in the other cytokines following LPS exposure and all cytokines following TNF-α exposure were not found to be significant (p>0.05).

3.2   *In vitro* immune responses to *Burkholderia pseudomallei*

3.2.1   Dendritic cell maturation following exposure to heat killed *Burkholderia pseudomallei*

3.2.1.1 Effect on morphology and phenotype

Following successful establishment of DC responsiveness to maturation stimuli, their responsiveness to *B. pseudomallei* was assessed. An important step in the ability of DC to 'deliver' *B. pseudomallei* associated antigens to naïve T cells and generate a

protective immune response, is their ability to respond to the bacteria and undergo maturation. Maturation status was determined by the cell surface expression of MHC class II, CD 80, CD 86 and CD 205 at 0, 12, 24, 48, 72 and 96 hour time points. The maturation response to HK *B. pseudomallei* was more rapid than that observed to TNF-α and LPS, with significantly increased cell surface expression of all four markers noted by 12 hours post exposure (p<0.01) (Figures 3.11 and 3.12). Continued culture in the presence of HK *B. pseudomallei* over the next 84 hours demonstrated that the mature population of DC, identified by high expression (bight fluorescence) of the four markers, persisted throughout the culture (figure 3.13).

In addition to flow cytometric analysis, morphological responses to *B. pseudomallei* exposure were observed using phase contrast microscopy over the first 24 hours of the culture. Figure 3.14 demonstrates the resulting morphological changes of the DC following exposure to HK *B. pseudomallei*. Elongation of the DC can be seen by 3 hours post exposure. A mature DC morphology with the presence of long dendrite like projections was apparent by 18 hours post exposure, and a gradual increase in mature morphological phenotype can be seen over the remainder of the 24-hour observation period.

3.2.1.2 Effect on cytokine secretion

Supernatants from DC exposed to HK *B. pseudomallei* were assayed for the presence of IFN-γ, IL-5, IL-4, IL-2, IL-12p70, IL-10 and IL-6. Figures 3.15 and 3.16 show the cytokine secretion profile of DC 12 hours (the time at which phenotypic maturation was observed) after exposure to HK *B. pseudomallei* compared to control unstimulated DC. IL-6 is depicted in a separate graph due to the much larger scale required for this cytokine. An increase in all cytokines assayed was noted except IL-4, which did not

increase above control levels. Significant increases in IL-10 (p<0.05), IL-6 (p<0.01) and IL-12p70 (p<0.05) were noted compared to control unstimulated DC.

3.2.2 *In vitro* lymphocyte proliferation to heat killed *Burkholderia pseudomallei*

The ability of the DC population to present *B. pseudomallei* associated epitopes to naïve T cells was next assessed. Proliferation assays were optimised by varying both the concentration of HK *B. pseudomallei* to which the DC were exposed and the ratio of DC to lymphocytes. Figure 3.17 demonstrates the proliferative responses of naïve lymphocytes to a range of stimulation conditions. For all concentrations of HK *B. pseudomallei* and DC to lymphocyte ratios investigated, the proliferative response was significantly greater than that of the negative control (p<0.001). For all concentrations of HK *B. pseudomallei*, except 1×10^6 cfu mL^{-1}, the greatest proliferative response was seen with a DC to lymphocyte ratio of 1 to 10. Multivariate analysis of variance revealed that this ratio produced significantly greater proliferation (Mean Log CPM = 3.703, 95% CI = 3.685 to 3.721) as compared to the 1 to 100 (Mean Log CPM = 3.605, 95% CI = 3.589 to 3.622) and 1 to 1000 (Mean Log CPM = 3.340, 95% CI = 3.323 to 3.358) ratios. Analysis of the different concentrations investigated revealed that the greatest level of proliferation observed was for the concentration 1×10^4 cfu mL^{-1} ($\equiv 5 \times 10^3$ cfu per 1×10^6 DC) (Mean Log CPM = 3.668, 95% CI = 3.647 to 3.689). This concentration was observed to elicit significantly greater proliferative responses (p<0.05) compared to the other concentrations investigated except 1×10^7 cfu mL^{-1} (Mean Log CPM = 3.632, 95% CI = 3.611 to 3.653), which was not found to be statistically different. Analysis of the proliferation observed over each of the six days of the proliferation assays, demonstrated that optimum proliferation was observed after four days of co-culture (Mean Log CPM = 3.697, 95% CI = 3.673 to 3.721), and this

proliferation was found to be significantly different from that measured on other days of the assay ($p<0.05$).

On the basis of these data a pulsing concentration of 1×10^4 cfu mL^{-1} was selected and used for all future studies.

3.3 Characterisation of immune responses to dendritic cell immunisation

Having successfully established the ability of the DC to generate primary immune responses to *B. pseudomallei* associated antigens *in vitro*, the immune responses generated following immunisation with HK *B. pseudomallei* pulsed DC were characterised. Review of the literature had suggested that successful *in vivo* immune responses to DC immunisation took four to five weeks to become established (17,119). Thus, an immunisation regimen of prime at day 0 and boost at day 28 was chosen to allow for such a lag phase in the establishment of an adaptive immune response. Intradermal (ID) and intranasal (IN) administration routes were investigated.

3.3.1 Investigation of cellular memory responses

Groups of five mice were immunised with either DC pulsed with HK *B. pseudomallei*, via the ID route, or HK *B. pseudomallei* delivered in MPL + TDM, via the intramuscular (IM) route. Immune memory status was assessed four weeks following the priming dose (Figure 3.18) and then five weeks after the booster dose (Figure 3.19), which gave an indication of the immune status at the point of challenge. Memory responses following priming with HK *B. pseudomallei* in MPL + TDM peaked after six days of culture at which point responses were significantly greater ($p<0.05$) than the level of proliferation seen for the naïve control group. Immunisation with a priming

dose of HK *B. pseudomallei* pulsed DC stimulated a significantly greater memory response (p<0.001) than that observed following immunisation with HK *B. pseudomallei* in MPL + TDM, which was also much more rapid, peaking after four days of culture. Memory responses following the booster dose were more rapid for both the test groups. Proliferation for the group primed and boosted with HK *B. pseudomallei* in MPL + TDM was greater (p<0.05) than that seen after the priming dose and also peaked a day earlier at five days after culture. While no significant difference was observed in the level of memory proliferation seen in the group primed and boosted with HK *B. pseudomallei* pulsed DC, proliferation was noted to peak a day earlier after the booster dose at three days of culture demonstrating a faster recall response. These initial data therefore suggested that the regimen chosen was suitable for the generation of a rapid recall response.

3.3.2 Investigation of humoral responses

In addition to cellular responses, humoral responses were also investigated following immunisation (Figure 3.20). Immunisation with HK *B. pseudomallei* delivered in MPL + TDM (IM) induced a strong anti-*B. pseudomallei* antibody response, which was significantly greater than both the DC immunised (ID) and naïve groups (p<0.05) after a single immunisation. Boost immunisation with HK *B. pseudomallei* in MPL + TDM (IM) at day 28 augmented the response to approximately two-fold the levels achieved after the priming dose. No antibody production was observed following immunisation with HK *B. pseudomallei* pulsed DC above that seen in the naïve group at either time point.

3.3.3   Attempt to establish a balanced immune response and investigation of the intradermal and intranasal delivery routes.

Despite the successful establishment of a strong cellular recall response following immunisation with pulsed DC, protection against virulent *B. pseudomallei* challenge has been demonstrated using HK *B. pseudomallei* delivered in MPL + TDM (8,112), which suggested that antibody may play a role.   Thus the immunisation regimen was altered such that an additional group of mice was primed with HK *B. pseudomallei* pulsed DC at day 0 and then boosted with HK *B. pseudomallei* delivered in MPL + TDM at day 28. The aim of this modification was to establish a balanced immune response following immunisation, which would generate both humoral and cellular responses upon challenge.   The effect of delivering the DC via the IN route was also investigated to compare any differences observed.

Figures 3.21 and 3.22 show a comparison of the cell mediated and humoral immunity at day 63 (five weeks after the booster dose) for each of the immunisation groups following either ID or IN delivery of the DC respectively.   HK *B. pseudomallei* delivered in MPL + TDM was administered via the IM route only and the group that received two doses of this formulation is included on both the ID (Figure 3.21) and IN (Figure 3.22) graphs for comparison purposes.   Following priming with HK *B. pseudomallei* pulsed DC via either the ID or IN, a significantly greater memory recall response (p<0.001) was observed compared to IM immunisation with HK *B. pseudomallei* delivered in MPL + TDM.   Antibody responses for the groups immunised with two doses of HK *B. pseudomallei* in MPL + TDM (IM) were significantly greater than those observed in the groups that received HK *B. pseudomallei* pulsed DC (p<0.05) via either the ID or IN route.

For the group primed with HK *B. pseudomallei* pulsed DC via the IN route, substitution of the day 28 DC dose with a dose of HK *B. pseudomallei* delivered in MPL + TDM afforded no significant changes in either the cellular or antibody responses observed ($p > 0.05$). For the group primed with HK *B. pseudomallei* pulsed DC via the ID route however, a significant increase in antibody production was observed ($p < 0.05$) when the day 28 DC dose was substituted with a dose of HK *B. pseudomallei* delivered in MPL + TDM (compared to the group that received two doses of HK *B. pseudomallei* pulsed DC). No significant change in the cellular recall response was observed as a result of this substitution ($p > 0.05$).

3.4 Protective efficacy of dendritic cells as a vaccine delivery vector

3.4.1 Determination of the LD_{50} for virulent *Burkholderia pseudomallei* (NCTC 4845)

Before proceeding to challenge experiments to test the efficacy of DC immunisation, a naïve challenge experiment to determine the LD_{50} via the IP route for the strain of *B. pseudomallei* being used was performed. Figure 3.23 shows the survival curve for each of the challenge groups, together with the mortality data table constructed to allow calculation of the LD_{50}.

Calculation of the LD_{50} was performed as follows in accordance with the method of Reed and Muench (169).

Interpolated value = $\dfrac{50\% - \% \text{ mortality below } 50\%}{\% \text{ mortality above } 50\% - \% \text{ mortality below } 50\%}$

$\therefore$ Interpolated value = $\dfrac{50 - 0}{80 - 0}$ = 0.625

Log LD_{50} = Log dose below 50% mortality + interpolated value

$\therefore LD_{50} = 10^{(1.886 + 0.625)}$ = 324.7 cfu

Following calculation of the LD_{50} a target challenge dose of approximately 1×10^4 cfu (≈ 30 LD_{50} doses) per mouse was chosen to ensure that an acute infection was induced following challenge.

3.4.2 Challenge with virulent *Burkholderia pseudomallei*

3.4.2.1 Intradermal dosed groups

Following successful establishment of an LD_{50} and a standardised challenge dose, the protective efficacy of DC immunisation was next tested. Mice immunised with either HK *B. pseudomallei* pulsed DC via the ID route, HK *B. pseudomallei* delivered in MPL + TDM via the IM route or a combination of the two formulations were challenged with virulent *B. pseudomallei* (NCTC 4845) injected into the peritoneal cavity (IP route) (Figure 3.24). Due to the inability to quantify challenge doses prior to challenge, mice were challenged with 5.3×10^4 cfu (163 LD_{50}) rather than the target challenge dose of 1 $\times 10^4$ cfu. Animals were closely observed for 35 days following challenge, during which time any animals showing signs of severe illness were humanely culled.

All naïve mice died on day 10 post challenge. All mice primed and boosted with HK *B. pseudomallei* pulsed DC died by day 16 post challenge. The group primed and boosted with HK *B. pseudomallei* in MPL + TDM displayed a significantly delayed time to death and 20% survival rate (p<0.005) compared to the naïve controls. The final group primed with HK *B. pseudomallei* pulsed DC and then boosted with HK *B. pseudomallei* in MPL + TDM displayed a significant delay in time to death and a 60% survival rate (p<0.005) compared to the naïve controls. In addition, a significant increase in time to death and survival rate was noted (p<0.005) for this last group when compared to the group primed and boosted with HK *B. pseudomallei* pulsed DC (Figure 3.24).

Assessment of the remaining bacterial burden (Table 3.3) demonstrated evidence of bacterial clearance. One mouse from each of the groups with survivors had no *B. pseudomallei* present in any of the tissues cultured (brain, liver, spleen and blood). While the remaining survivors in the group that was primed with HK *B. pseudomallei* pulsed DC and boosted with HK *B. pseudomallei* in MPL + TDM did have evidence of *B. pseudomallei* in the spleen samples, this was limited (<10 cfu). Taken with the inability to culture organisms from one of the survivors and the fact that no hepatosplenomegaly was present or abscesses apparent on any organs suggests effective bacterial clearance was in progress and had the experiment been extended, complete clearance may have been achieved.

Immunisation group	Number of colony forming units counted			
	Brain	Liver	Spleen	Blood
Pulsed DC[1] / B.ps[2]	0	0	<10	0
Pulsed DC / B.ps	0	0	0	0
Pulsed DC / B.ps	0	0	<10	0
B.ps / B.ps	0	0	0	0

[1] DC pulsed with HK *B. pseudomallei*

[2] HK *B. pseudomallei* in MPL + TDM

Table 3.2   Assessment of remaining bacterial burden in challenge survivors

Challenge survivors were assessed for *B. pseudomallei* present in tissues (brain, liver, spleen and blood). One mouse from each of the groups with survivors had no *B. pseudomallei* present in any of the tissues cultured. While the remaining survivors in the group primed with HK *B. pseudomallei* pulsed DC and boosted with HK *B. pseudomallei* in MPL + TDM did have evidence of *B. pseudomallei* in the spleen samples, this was limited.

3.4.2.2 Intranasal dosed groups

Mice immunised with either HK *B. pseudomallei* pulsed DC via the IN route, HK *B. pseudomallei* delivered in MPL + TDM via the IM route or a combination of the two formulations were challenged with virulent *B. pseudomallei* (NCTC 4845) via the intra-peritoneal (IP) route (Figure 3.25). Again due to the inability to quantify challenge doses prior to challenge, mice were challenged with 8.8×10^4 cfu (271 LD_{50}) rather than the target challenge dose of 1×10^4 cfu. Animals were closely observed for 35 days following challenge, during which time any animals showing signs of severe illness were humanely culled.

All the naïve mice and those primed and boosted with HK *B. pseudomallei* pulsed DC died by day 9 post challenge. No delayed time to death or survival was observed in the group that had been primed with HK *B. pseudomallei* pulsed DC and boosted with HK *B. pseudomallei* delivered in MPL + TDM. The group primed and boosted with HK *B. pseudomallei* in MPL + TDM again demonstrated a 20% survival rate, which was found to be statistically significant (p<0.05) when compared to the naïve controls.

3.5   Elucidation of the protective immune response

Data so far had indicated a possible role for antibody in protection against *B. pseudomallei*, which due to its intracellular nature, was unexpected. Thus the protective immune response (following immunisation with HK *B. pseudomallei* pulsed DC and HK *B. pseudomallei* in MPL + TDM) demonstrated by the challenge experiments was further investigated. Groups of 18 mice were immunised with each of the different immunisation combinations, HK *B. pseudomallei* pulsed DC were delivered via the ID route and HK *B. pseudomallei* in MPL + TDM via the IM route as previous. At weekly intervals following the day 0 immunisation two mice from each group were cardiac punctured while under terminal anaesthesia (halothane) and their spleens excised. CD 4^+ and CD 8^+ T cells were then purified and assayed for memory phenotype as described in *2.4.3.2*. Sera were assayed for anti-*B. pseudomallei* antibodies, which were also isotyped.

3.5.1   Magnet assisted isolation of T cells from murine spleen

Magnetic separation of CD4$^+$and CD8$^+$ T cells from murine spleen was performed in accordance with the manufacturer's instructions. Isolation was performed on a single column, which afforded around 91% (± 4.7%) purity in the isolated cell type, as

determined by flow cytometry analysis (data not shown as consistent with manufacturer reported purity).

3.5.2 CD 4$^+$ / CD 8$^+$ memory T cell responses to immunisation

Figure 3.26 shows the CD 4$^+$ T cell memory response at weekly intervals during the immunisation regimen. Immunisation with a priming dose (Day 0) of either HK *B. pseudomallei* pulsed DC or HK *B. pseudomallei* in MPL + TDM produced a significant increase in the CD 4$^+$ memory T cell recall response ($p < 0.05$) compared to the naïve controls. Prime immunisation with pulsed DC produced a significantly greater ($p < 0.01$) recall response than that produced following immunisation with HK *B. pseudomallei* in MPL + TDM. In both cases the response peaked 2 to 3 weeks following the priming dose and then returned to naïve levels by day 30 of the immunisation regimen. After administration of the booster doses (Day 28) the group primed and boosted with HK *B. pseudomallei* in MPL + TDM failed to respond to the boost dose, while the group primed and boosted with HK *B. pseudomallei* pulsed DC did respond. Although this response was significantly greater ($p < 0.05$) than that of the naïve controls, it was not as pronounced as the recall response following the priming dose and had returned to naïve levels by day 44. The group primed with HK *B. pseudomallei* pulsed DC and boosted with HK *B. pseudomallei* in MPL + TDM exhibited a lag phase for the first week after the booster immunisation during which time the recall response continued to drop. By day 44 the recall response had increased to approximately two-fold the levels achieved after the priming dose. This response was significantly greater than all the other test groups ($p < 0.01$) and was also more prolonged, not returning to naïve levels until day 65.

CD 8$^+$ T cell recall responses (Figure 3.27) also demonstrated a biphasic response to immunisation. Priming with HK *B. pseudomallei* pulsed DC resulted in a peak recall response by day 16, which was greater than that of the other test groups (p<0.05), and had returned to naïve levels by day 30. The group primed with HK *B. pseudomallei* in MPL + TDM did not produce a recall response greater than that observed for the naïve controls (p>0.05). Following the booster dose, recall responses greater than the naïve controls were observed from the group primed and boosted with HK *B. pseudomallei* pulsed DC (p<0.05). No statistically significant increase was noted for the group primed and boosted with HK *B. pseudomallei* in MPL + TDM (p>0.05). The group primed and boosted with HK *B. pseudomallei* pulsed DC also showed an increased CD 8$^+$ T cell recall response out to day 44 of the immunisation regimen (p<0.05). The group primed with HK *B. pseudomallei* pulsed DC and boosted with HK *B. pseudomallei* in MPL + TDM again exhibited a lag phase for the first week following booster immunisation. This lag phase, gave way to a significantly increased recall response (p<0.05), which continued to increase out to day 58, the increase between days 58 and 65 was not found to be significant (p>0.05).

3.5.3   Antibody responses to immunisation

3.5.3.1 Total IgG responses

Total IgG responses were determined at weekly intervals during the immunisation regimen and are shown in Figure 3.28. Priming doses for all groups did not stimulate any significant antibody production above that of naïve levels (p>0.05). Whilst this was expected for DC immunisation, it was unusual following immunisation with HK *B. pseudomallei* in MPL + TDM, which previous experiments had shown was very effective at stimulating antibody production (figure 3.20). Following booster

immunisation, the group primed and boosted with HK *B. pseudomallei* pulsed DC, as expected, produced no antibody above naïve levels. The other two immunisation groups showed a steady increase in total IgG over the next 5 weeks. Both these responses were found to be significantly greater compared to the naïve controls ($p<0.05$) although the group primed and boosted with HK *B. pseudomallei* in MPL + TDM had significantly less total IgG by week five post-booster immunisation than in previous experiments.

3.5.3.2 IgG subclass responses

In the naïve group very little anti-*B. pseudomallei* antibody was detected for any of the isotypes (<200 ng mL^{-1}), especially IgG1 where concentrations were below detection limits (figure 3.29). Mice primed and boosted with HK *B. pseudomallei* pulsed DC also failed to mount an anti-*B. pseudomallei* antibody response for any of the isotypes tested ($p>0.05$), compared to the naïve controls. Mice primed and boosted with HK *B. pseudomallei* in MPL + TDM showed significantly greater concentrations of IgG2a, 2b and 3 than the naïve controls ($p<0.05$) (figure 3.29). Greater concentrations of IgG2a and 2b were also noted compared to the group primed with HK *B. pseudomallei* pulsed DC and boosted with HK *B. pseudomallei* in MPL + TDM ($p<0.05$). This latter group was however noted as having significantly greater IgG1 concentrations compared to all other test groups ($p<0.001$) (figure 3.29).
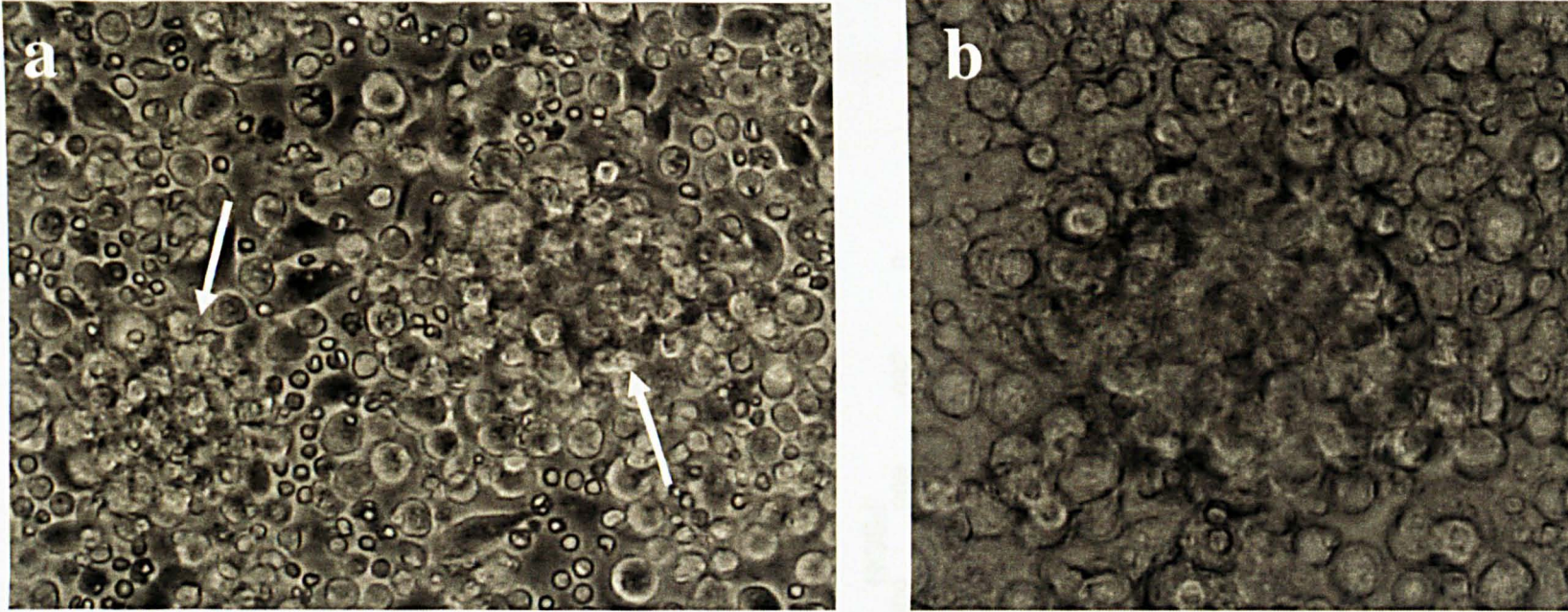
Figure 3.1   Phase contrast images of dendritic cell culture

a)   Phase contrast image of proliferating balls of dendritic cells (x20 magnification) indicated by the white arrows. Balls of proliferating cells

were observed after four days of the culture of bone marrow progenitor cells in the presence of GM-CSF and TNF-α.

b)   Phase contrast image (x40 magnification) of a proliferating ball of dendritic cells.
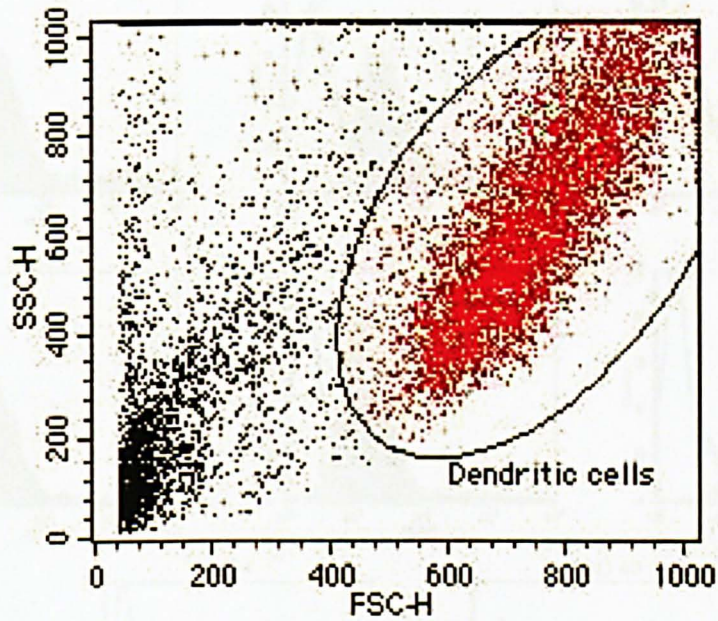
Figure 3.2   Forward and side scatter profile of purified dendritic cells

Flow cytometry forward scatter (FSC-H) / side scatter (SSC-H) profile of dendritic cells

following purification from culture using metrizamide density centrifugation gradient.

Dendritic cells (gated in red) exhibit a typical high FSC-H / SSC-H profile and typically

constituted greater than 80% of the culture population.
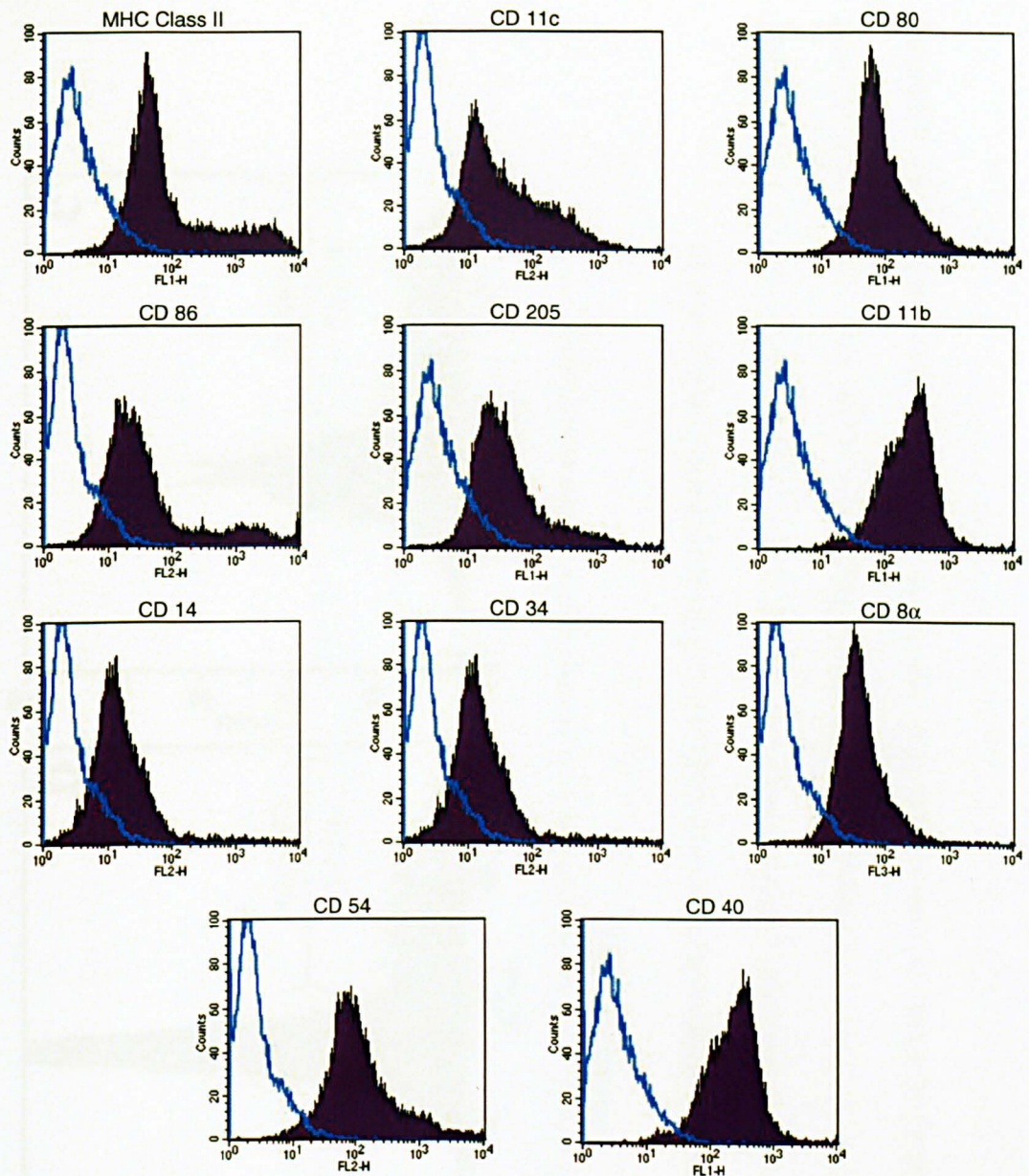
Figure 3.3   Immature dendritic cell phenotype

Phenotype of immature dendritic cells obtained after four days of culture in the presence of GM-CSF and TNF-α, and purified on a metrizamide density gradient: MHC class II^{++}, CD 11c^{+}, CD 80^{+}, CD 86^{++}, CD 205^{+}, CD 11b^{+}, CD 14^{+}, CD 34^{+}, CD 8α^{+}, CD 54^{+} and CD 40^{+}. Blue open curve shows negative control, purple closed curve the stained cell population. Expression pattern was consistently observed in five separate experiments.

MHC Class II
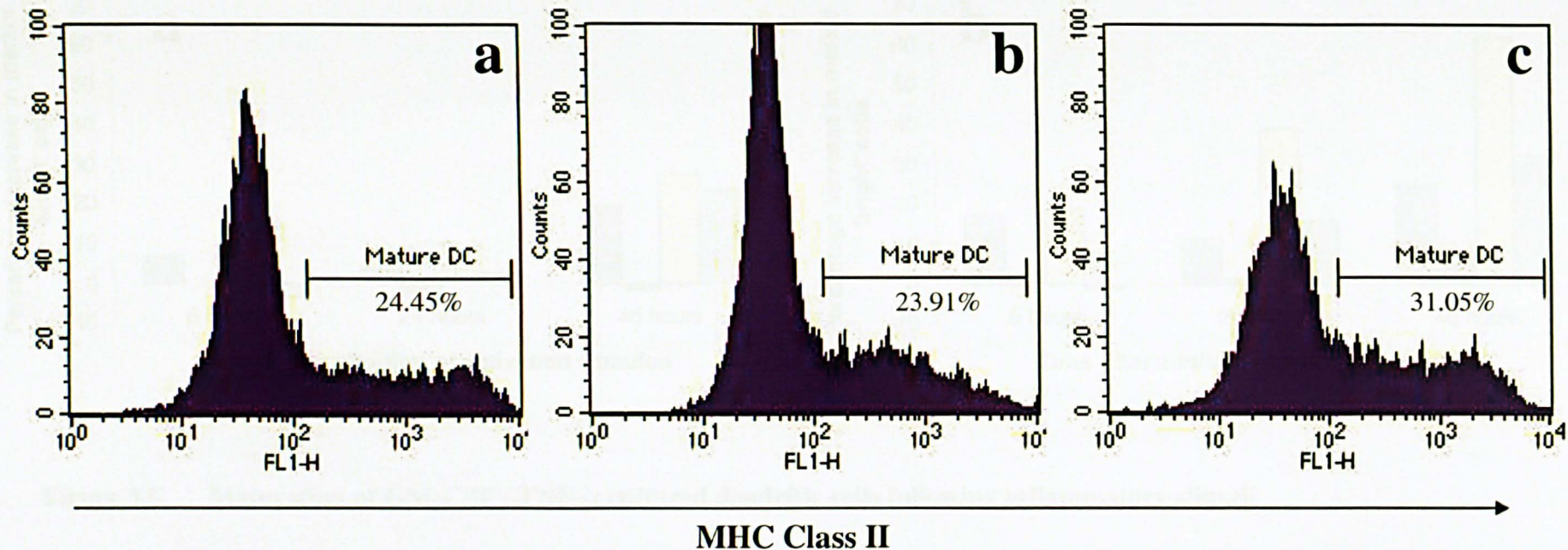
Figure 3.4   Comparison of the proportion of mature dendritic cells following culture in different cytokine environments

Percentage of mature dendritic cells occurring under each of the three cytokine culture conditions: a) GM-CSF & TNF-α (24.45%); b) GMCSF, TNF-α & IL-12 (23.91%); c) GM-CSF, TNF-α & IL-4 (31.05%). The mature population was determined on the basis of high MHC class II expression, which is considered characteristic of a mature dendritic cell phenotype. This experiment was repeated five times and no significant variation was noted between experiments ($p>0.05$).
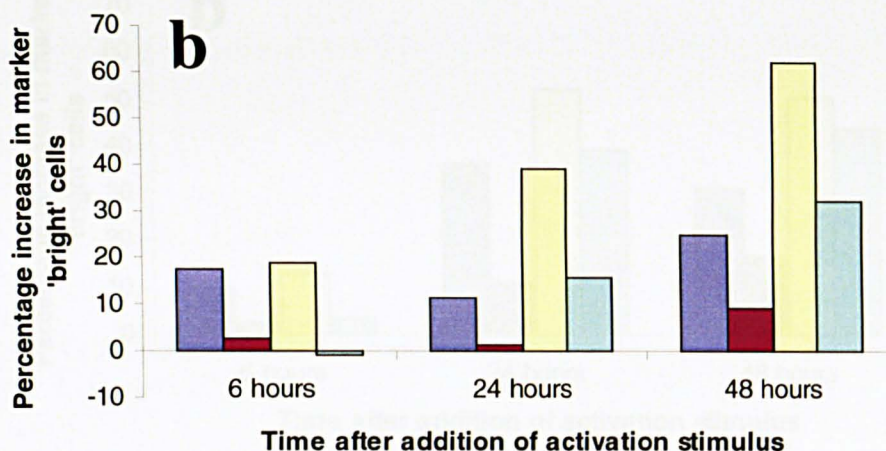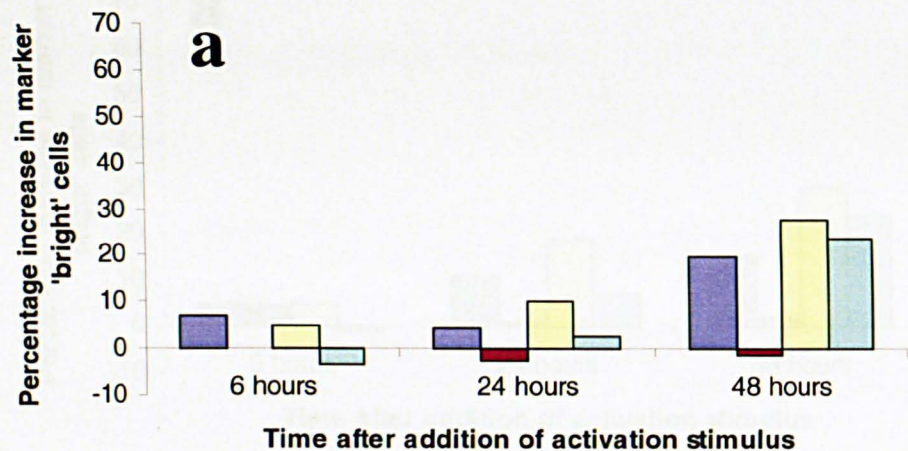
Figure 3.5   Maturation of GM-CSF / TNF-α cultured dendritic cells following inflammatory stimuli

Maturation was assessed in terms of the increase in percentage of mature cells, depicted by high expression of MHC class II (blue), CD 80 (red), CD 86 (yellow) and CD 54 (turquoise), as compared to control unstimulated dendritic cells.

a)   Maturation following exposure to 50 ng mL^{-1} TNF-α resulted in a marked increase in all markers, except CD 80, with the greatest expression observed 48 hours after exposure.

b)   Maturation following exposure to 10 ng mL^{-1} LPS stimulated a marked increase in all markers. Maturation resulting from LPS exposure occurred more rapidly and to a greater extent than that stimulated by exposure to TNF-α.

Figure 3.6   Maturation of GM-CSF / TNF-α / IL-4 cultured dendritic cells following inflammatory stimuli

Maturation was assessed in terms of the increase in percentage of mature cells, depicted by high expression of MHC class II (blue), CD 80 (red), CD 86 (yellow) and CD 54 (turquoise), as compared to control unstimulated dendritic cells.

a)   Maturation following exposure to 50 ng mL^{-1} TNF-α resulted in a marked increase in MHC class II, CD 86 and CD 54, with the greatest expression observed 48 hours after exposure.

b)   Maturation following exposure to 10 ng mL^{-1} LPS stimulated a marked increase in all markers. Maturation resulting from LPS exposure occurred more rapidly and to a greater extent than that stimulated by exposure to TNF-α, peaking after 24 hours.
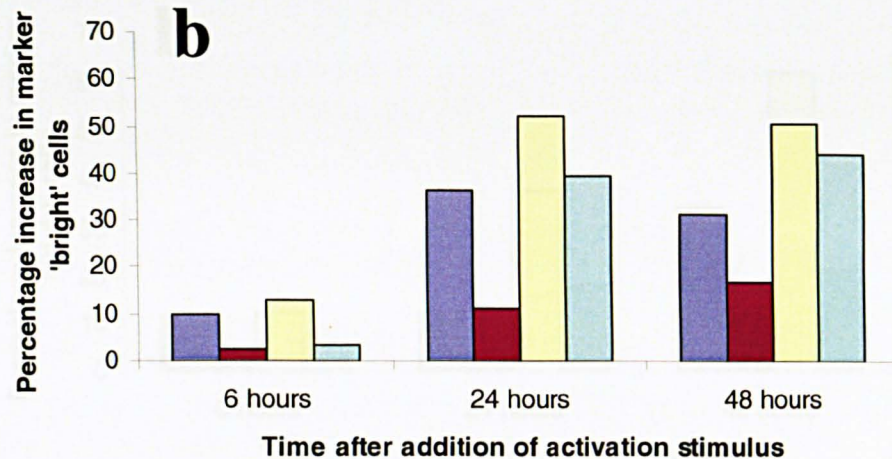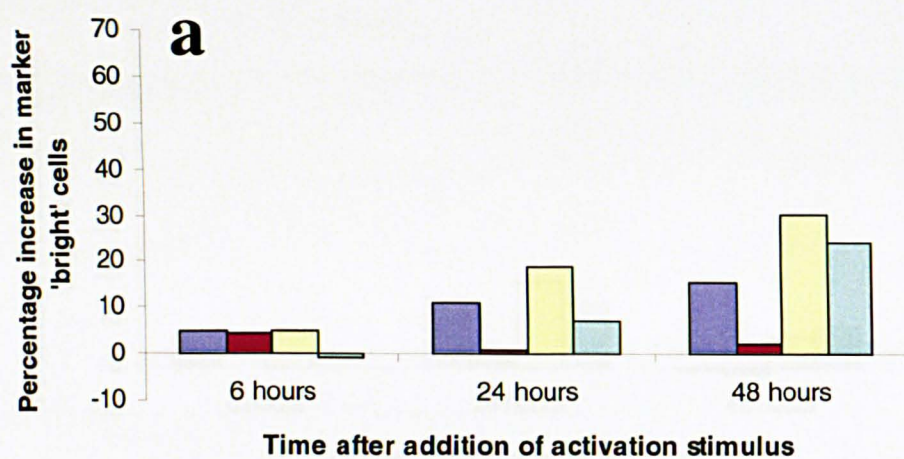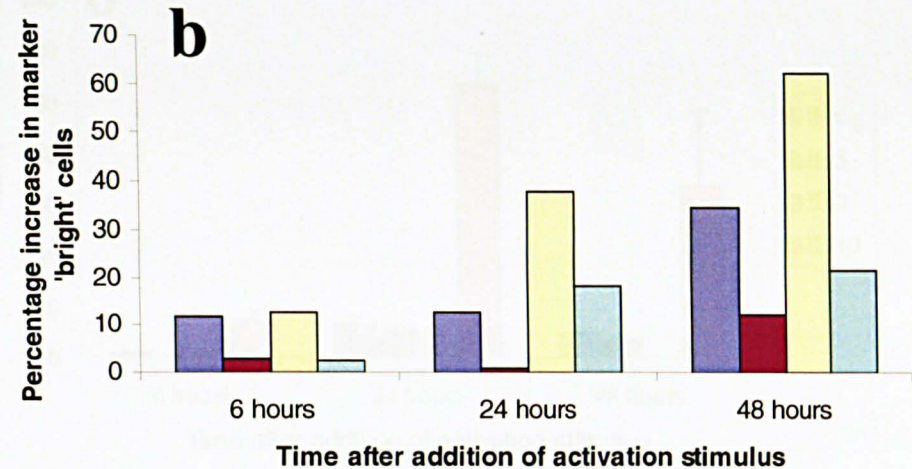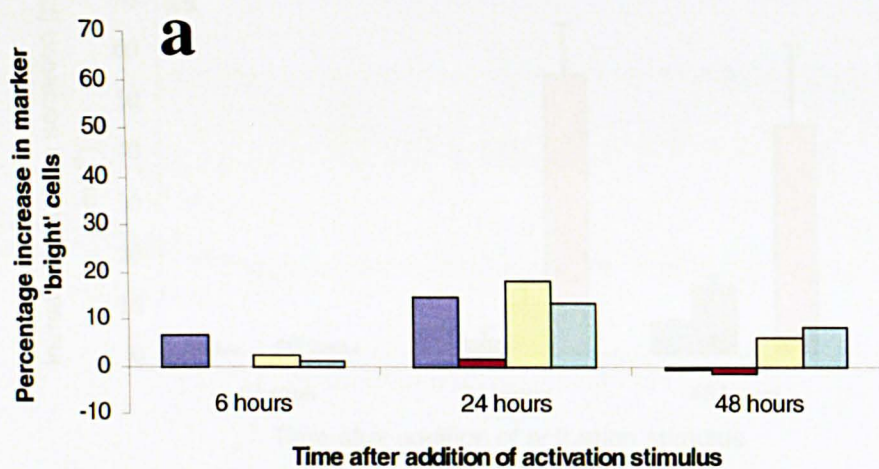
Figure 3.7   Maturation of GM-CSF / TNF-α / IL-12 cultured dendritic cells following inflammatory stimuli

Maturation was assessed in terms of the increase in percentage of mature cells, depicted by high expression of MHC class II (blue), CD 80 (red), CD 86 (yellow) and CD 54 (turquoise), as compared to control unstimulated dendritic cells.

a)   Maturation following exposure to 50 ng mL^{-1} TNF-α resulted in an increase in MHC class II, CD 86 and CD 54 by 24 hours. Further culture, however, was associated with decreased expression by 48 hours.

b)   Maturation following exposure to 10 ng mL^{-1} LPS stimulated a marked increase in all markers. Maturation resulting from LPS exposure occurred more rapidly and to a greater extent than that stimulated by exposure to TNF-α, peaking after 48 hours.
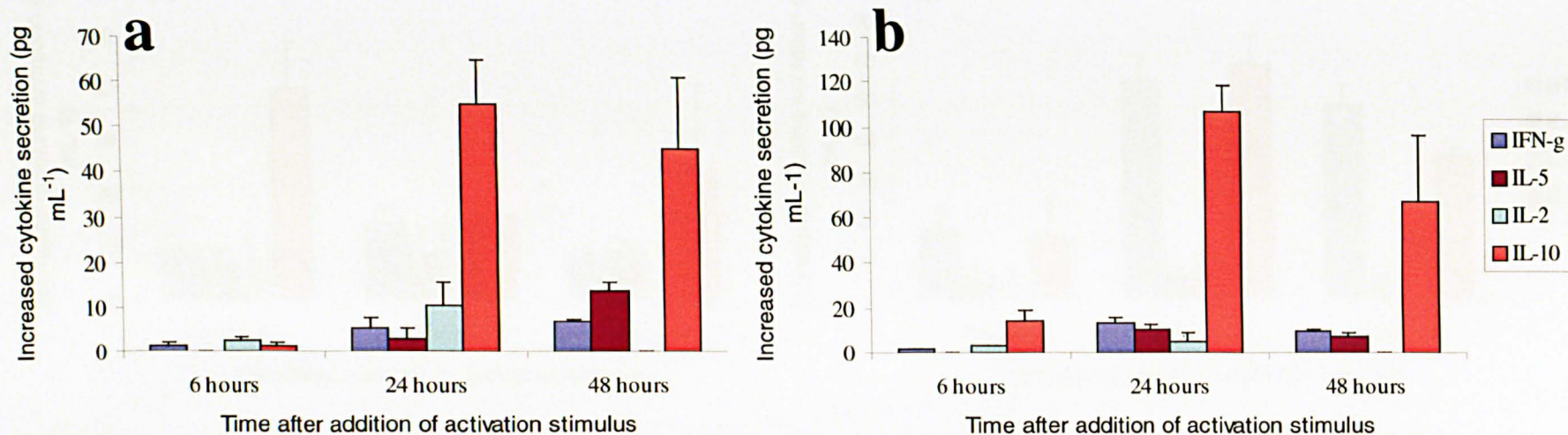
Figure 3.8   **Cytokine secretion by GM-CSF / TNF-α cultured dendritic cells following inflammatory stimuli**

Cytokine secretion from DC cultured in the presence of GM-CSF and TNF-α. Cytokines assayed were IFN-γ (blue), IL-5 (red), IL-2 (turquoise) and IL-10 (orange bar).   IL-4, IL-12p70 and IL-6 were also assayed but no increase in secretion was noted.   All concentrations are normalised against control unstimulated DC. Error bars signify +SEM, which is calculated from the mean of three replicates.

a)   Cytokine secretion following exposure to 50 ng mL^{-1} TNF-α was seen to increase for all cytokines assayed by 24 hours, of which the increase in IL-10 was found to be significant ($p<0.05$). IL-2 was not detectable by 48 hours post-exosure.

b)   Cytokine secretion following exposure to 10 μg mL^{-1} LPS demonstrated the same pattern as for TNF-α exposure with approximately a two-fold increase in concentration. Again IL-10 was found to be significantly greater ($p<0.05$) compared to control secretion levels.
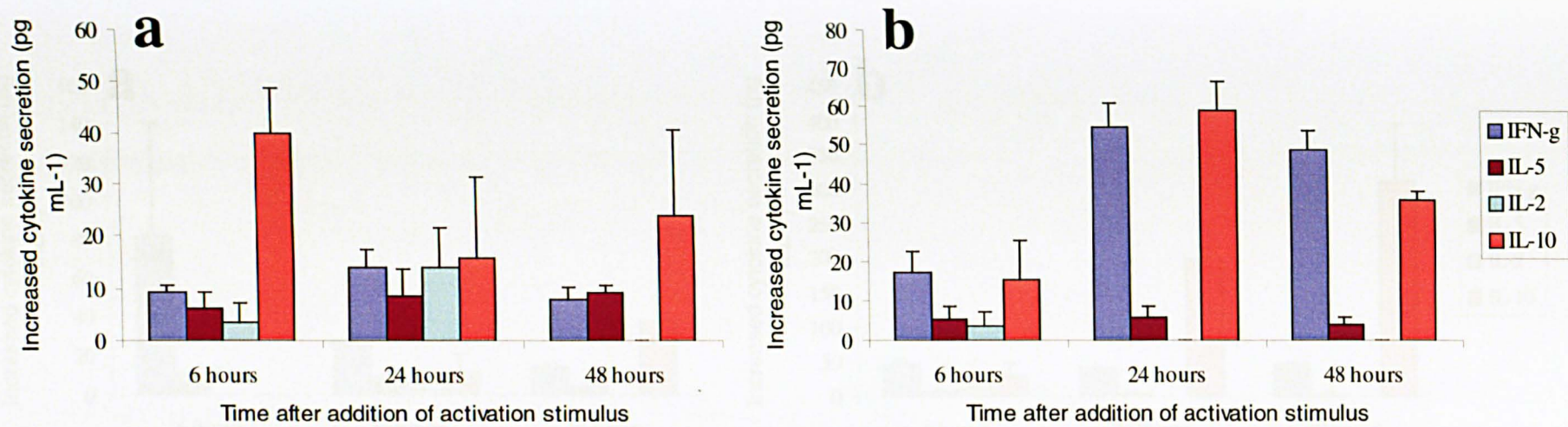
Figure 3.9   **Cytokine secretion by GM-CSF / TNF-α / IL-4 cultured dendritic cells following inflammatory stimuli**

Cytokine secretion from DC cultured in the presence of GM-CSF, TNF-α and IL-4.   Cytokines assayed were IFN-γ (blue), IL-5 (red), IL-2 (turquoise) and IL-10 (orange bar). IL-4, IL-12p70 and IL-6 were also assayed but no increase in secretion was noted.   All concentrations are normalised against control unstimulated DC. Error bars signify +SEM, which is calculated from the mean of three replicates.

a)   Cytokine secretion following exposure to 50 ng mL^{-1} TNF-α was too variable to be able to discern any significant patterns.

b)   Cytokine secretion following exposure to 10 μg mL^{-1} LPS was seen to significantly increase for both IFN-γ (p<0.05) and IL-10 (p<0.05) compared to control unstimulated DC.
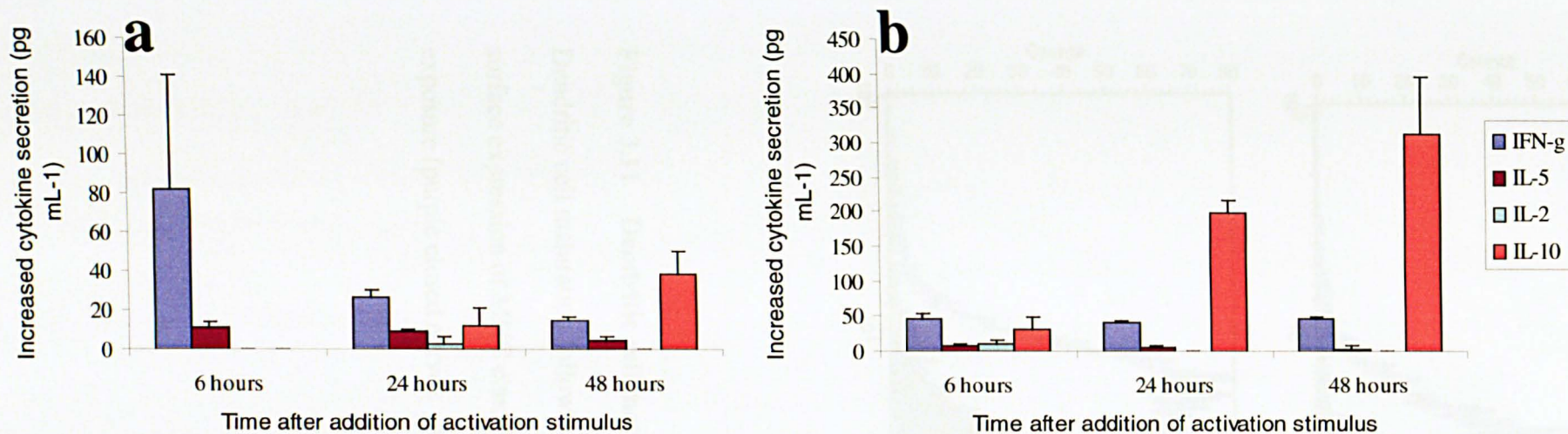
Figure 3.10   Cytokine secretion by GM-CSF / TNF-α / IL-12 cultured dendritic cells following inflammatory stimuli

Cytokine secretion from DC cultured in the presence of GM-CSF, TNF-α and IL-12. Cytokines assayed were IFN-γ (blue), IL-5 (red), IL-2 (turquoise) and IL-10 (orange bar). IL-4, IL-12p70 and IL-6 were also assayed but no increase in secretion was noted. All concentrations are normalised against control unstimulated DC. Error bars signify +SEM, which is calculated from the mean of three replicates.

a)   Cytokine secretion following exposure to 50 ng mL^{-1} TNF-α was too variable to be able to discern any significant patterns.

b)   Cytokine secretion following exposure to 10 μg mL^{-1} LPS was seen to significantly increase for both IFN-γ ($p<0.05$) and IL-10 ($p<0.01$) compared to control unstimulated DC.
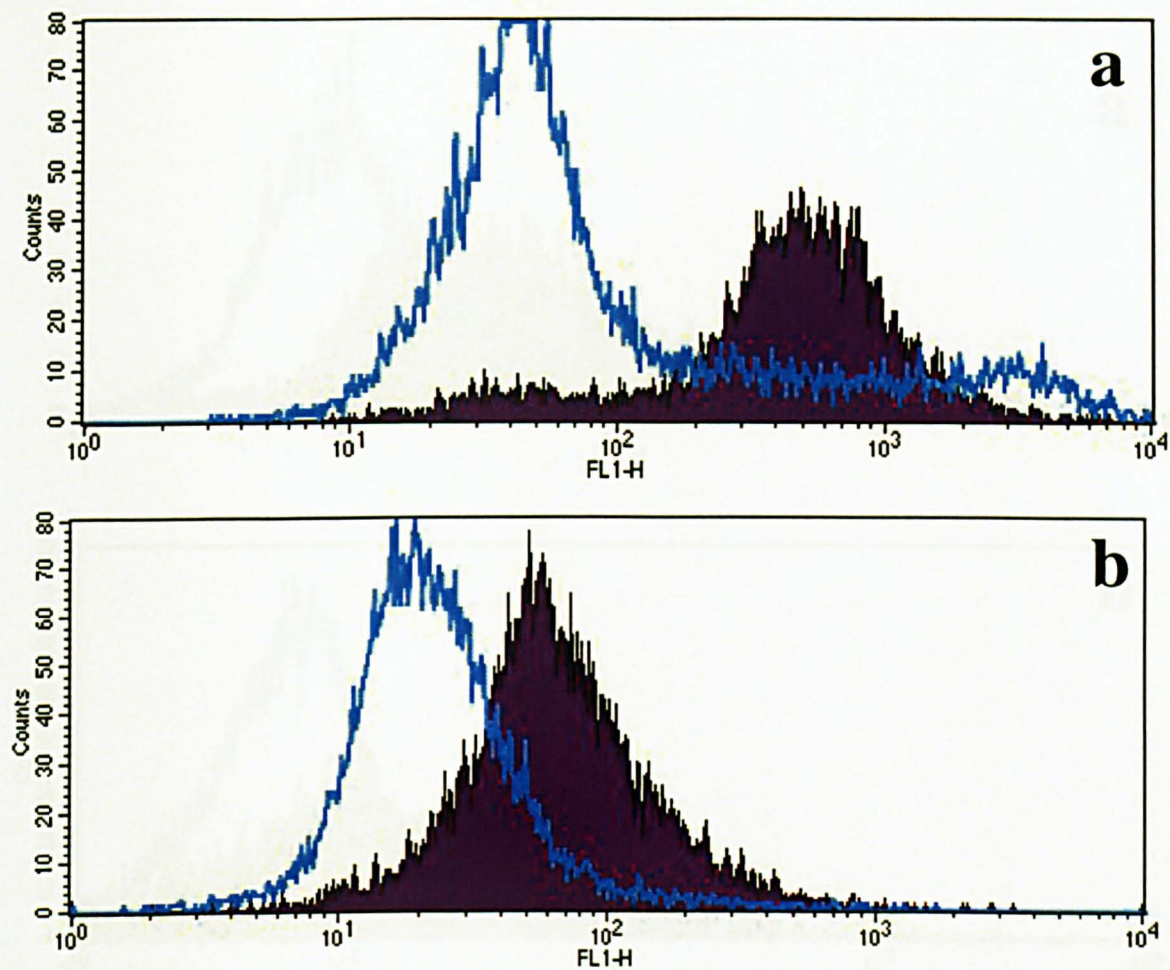
Figure 3.11   Dendritic cell maturation following exposure to HK *B. pseudomallei*

Dendritic cell maturation following exposure to heat killed *B. pseudomallei*. Cell

surface expression of MHC class II (a) and CD 80 (b) was noted to peak 12 hours post

exposure (purple closed curve). Blue open curve depicts expression at 0 hours.
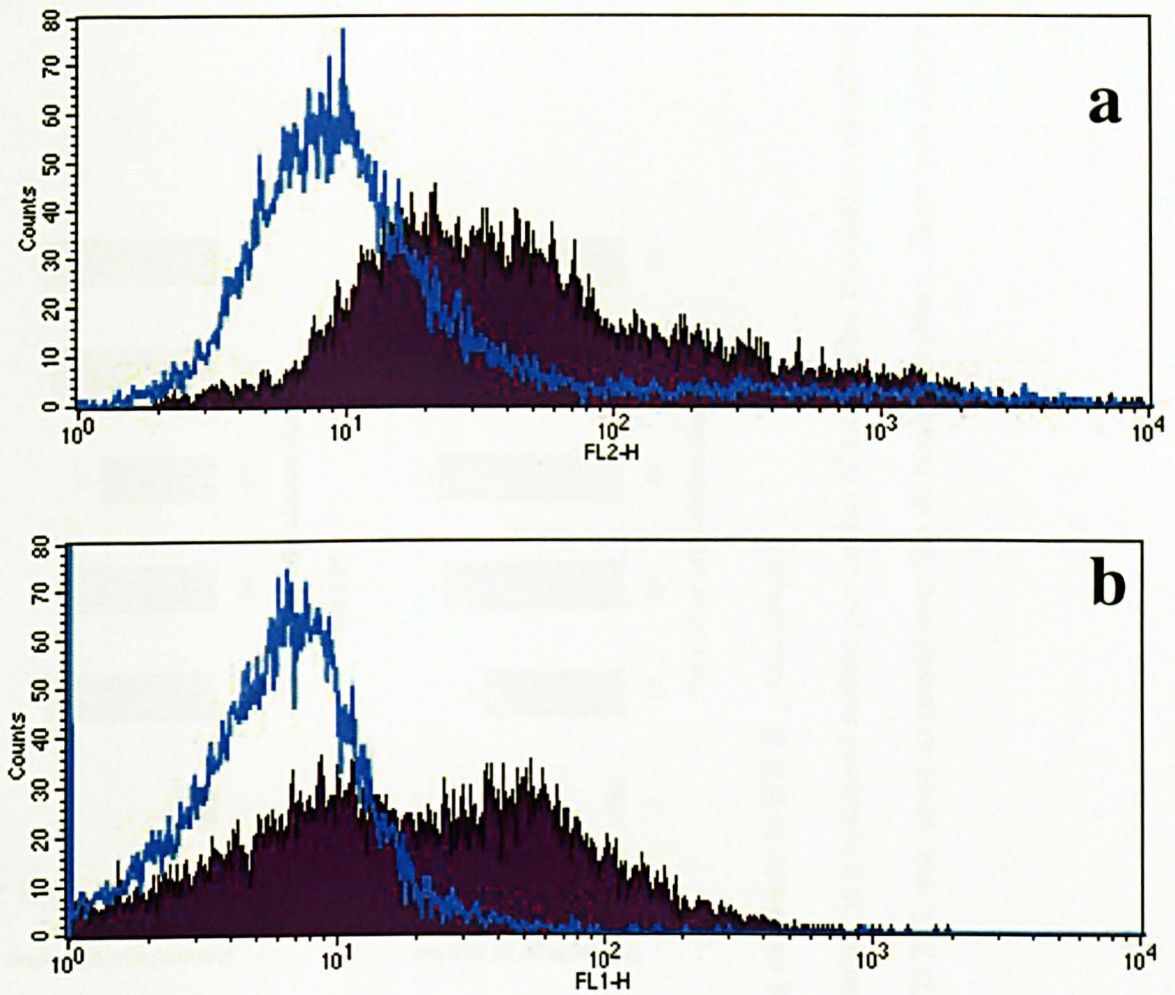
Figure 3.12   Dendritic cell maturation following exposure to HK *B. pseudomallei*

Dendritic cell maturation following exposure to heat killed *B. pseudomallei*. Cell

surface expression of CD 86 (a) and CD 205 (b) was noted to peak 12 hours post

exposure (purple closed curve). Blue open curve depicts expression at 0 hours.
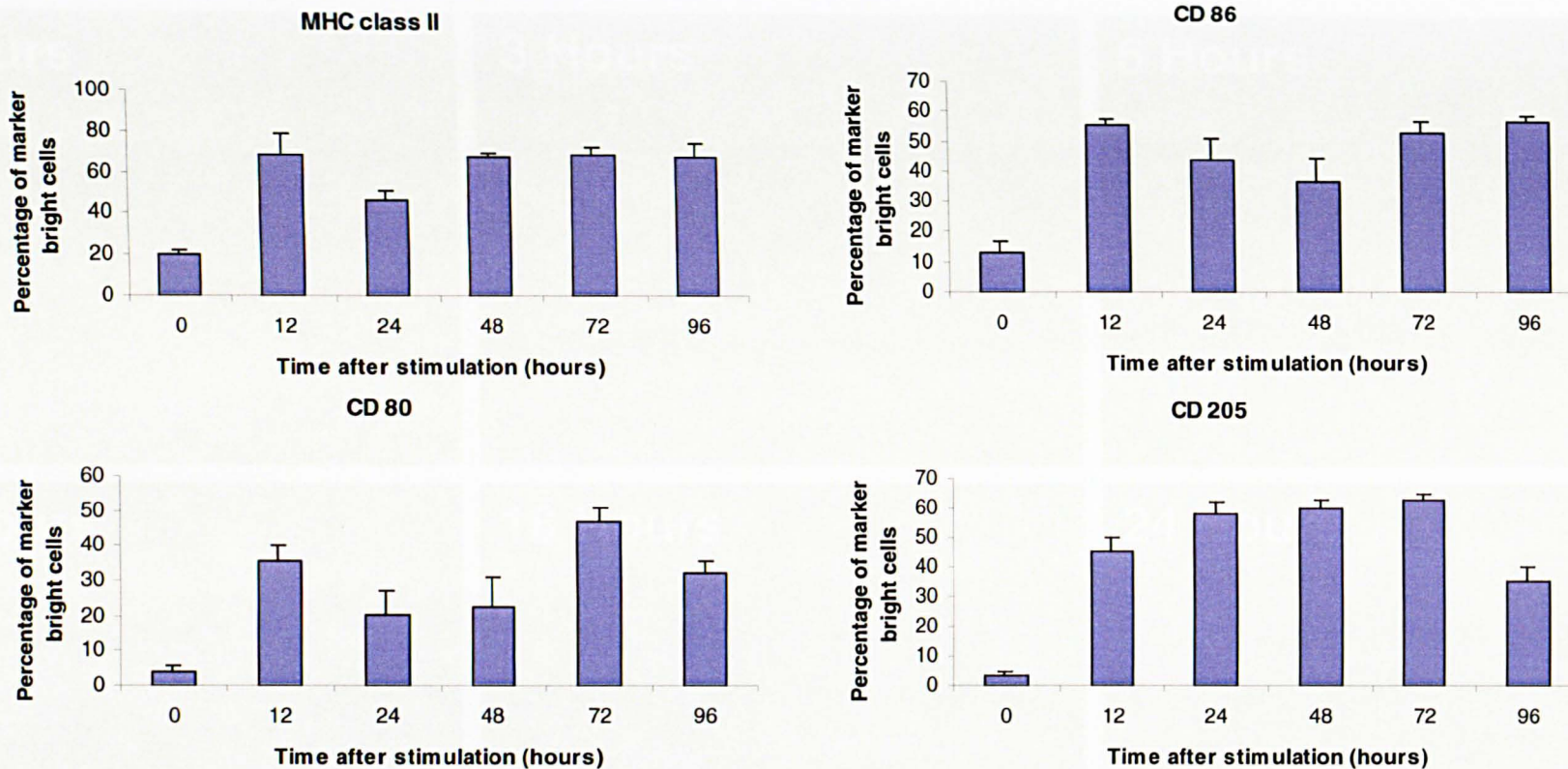
Figure 3.13 Percentage of marker bright dendritic cells following exposure to HK *B. pseudomallei*

Continued culture of the DC in the presence of HK *B. pseudomallei* resulted in a sustained mature population of cells. This population, identified

by the expression of high levels of MHC class II, CD 80, CD 86 and CD 205, was noted to persist over the 96 hours of culture. Error bars signify

+SEM, calculated from the mean of five experiments
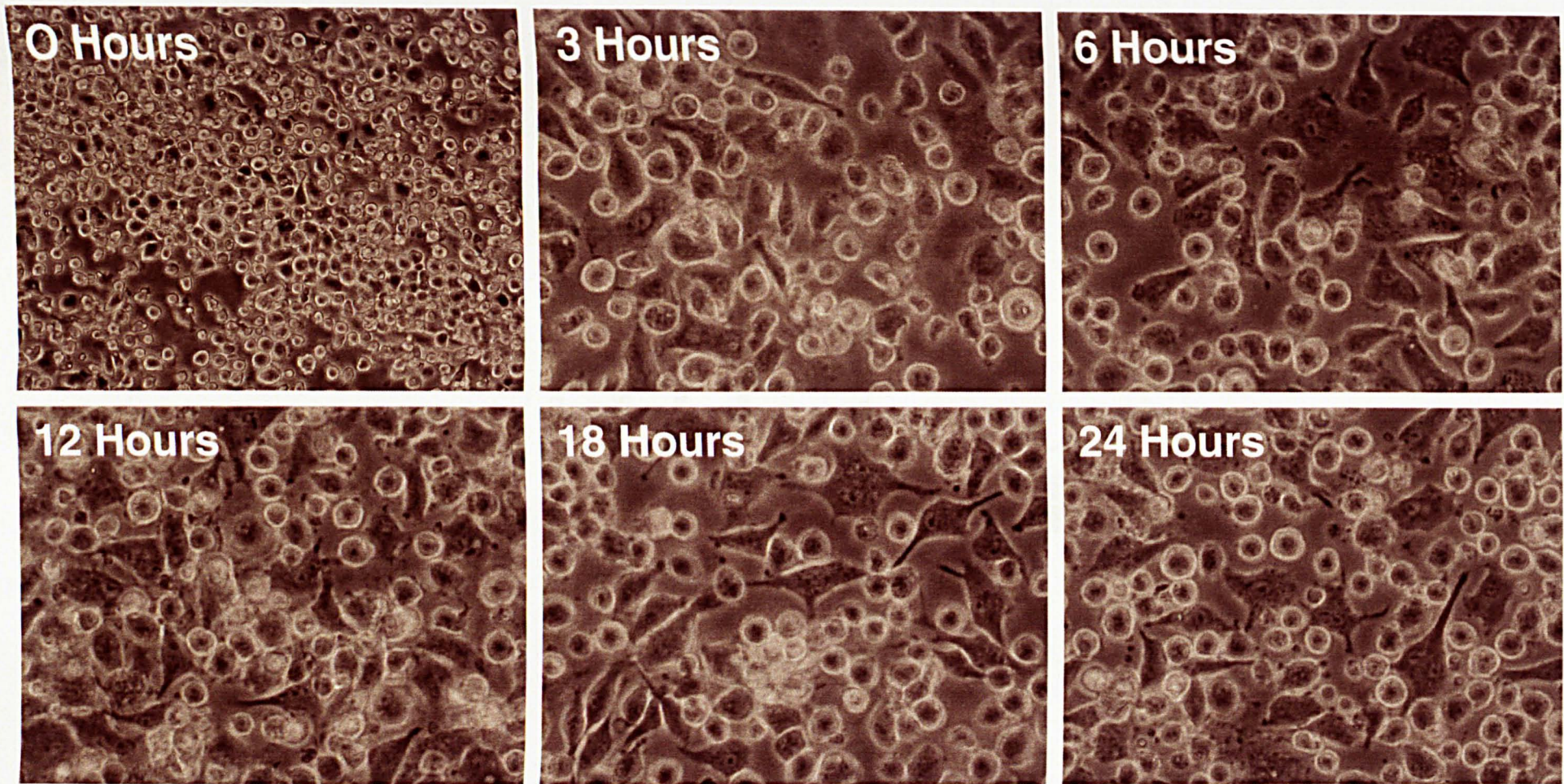
Figure 3.14   Dendritic cell morphological changes following exposure to HK *B. pseudomallei*

Changes in DC morphology following exposure to 1×10^4 cfu HK *B. pseudomallei* per 2×10^6 cells. Elongation of the DC is apparent by 3 hours with long dendritic like projections becoming clearly visible by 18 hours.
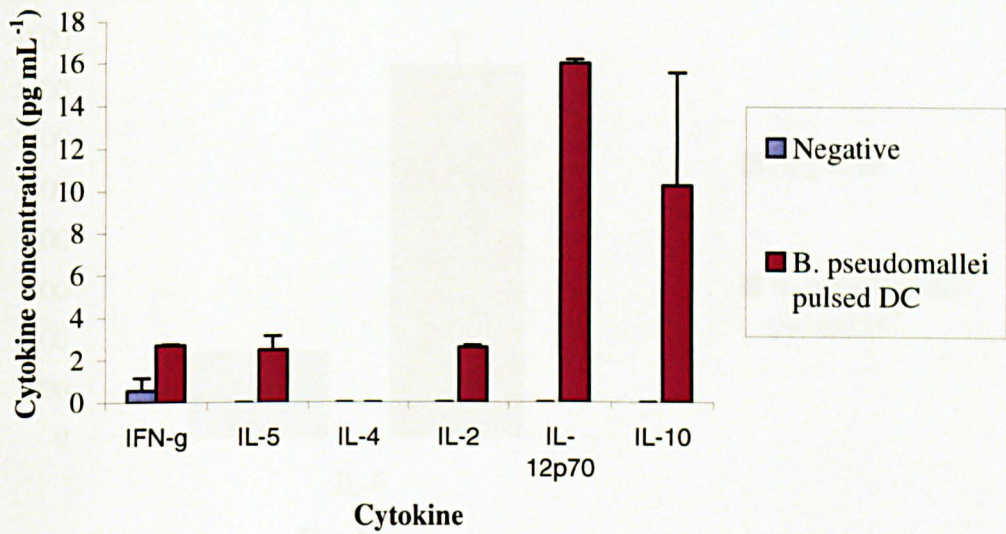
Figure 3.15 **Dendritic cell cytokine secretion following exposure to HK *B.***

pseudomallei

Dendritic cell cytokine secretion, 12 hours after exposure to HK *B. pseudomallei*.

Increases in IFN-γ, IL-5 and IL-2 secretion were noted together with significant

increases in IL-12p70 ($p<0.05$) and IL-10 ($p<0.05$) secretion. Error bars signify +SEM,

which is calculated from the mean of three replicates.
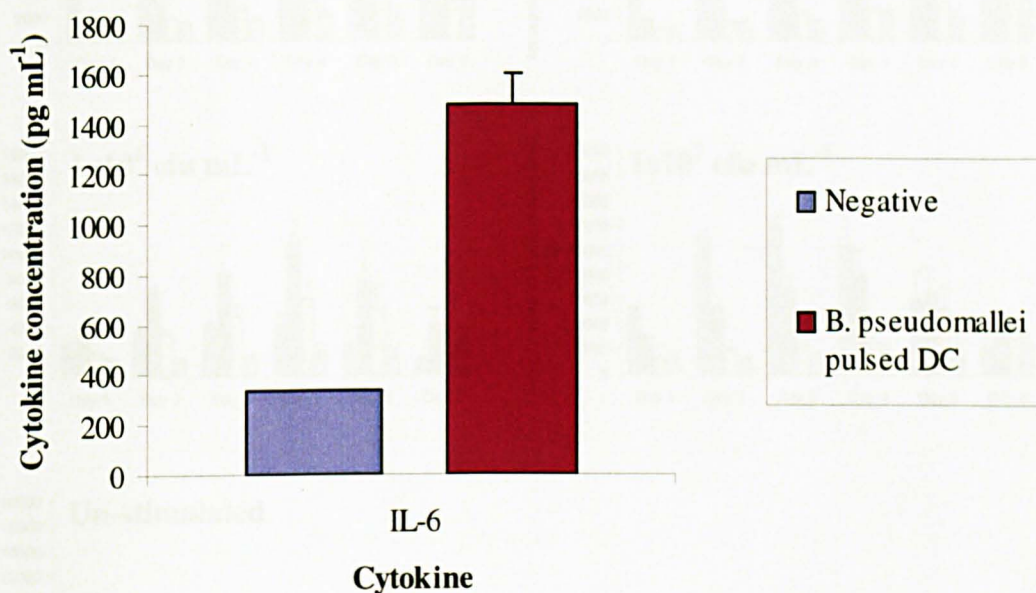
Figure 3.16   Dendritic cell IL-6 secretion following exposure to HK *B. pseudomallei*

Dendritic cell secretion of IL-6, 12 hours after exposure to HK *B. pseudomallei*. A significant increase in IL-6 ($p<0.01$) secretion was observed compared to control unstimulated DC. Error bars signify +SEM, which is calculated from the mean of three replicates.
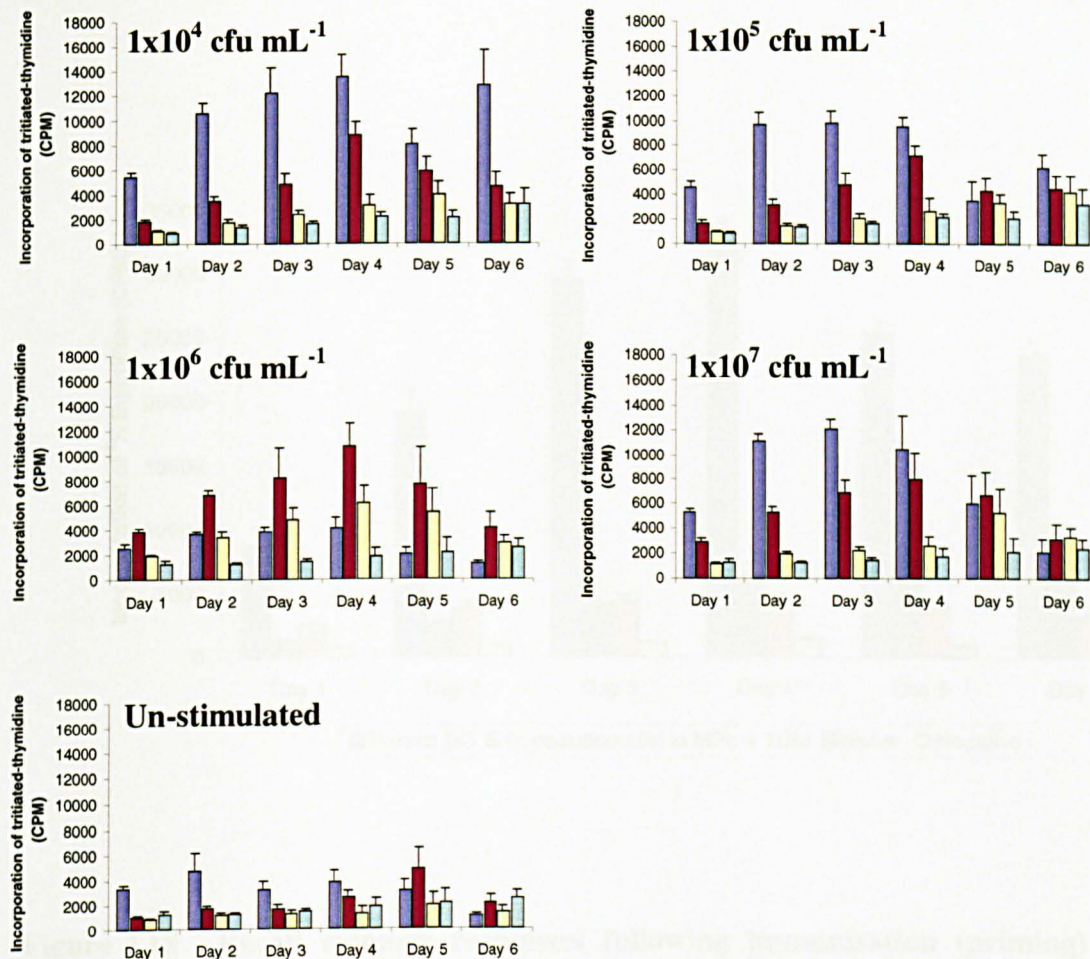
Figure 3.17   Naïve lymphocyte proliferation to HK *B. pseudomallei* pulsed

dendritic cells

For all concentrations and ratios investigated, proliferation was significantly greater than that of the negative control ($p<0.001$). A DC to lymphocyte ratio of 1 to 10 resulted in the greatest level of proliferation ($p<0.05$). The optimum concentration was determined as 1×10^4 cfu mL^{-1} ($p<0.05$) and the greatest level of proliferation occurred between two and four days of co-culture ($p<0.05$). Blue bars represent the DC to lymphocyte ratio 1 to 10, red bars 1 to 100 and yellow bars 1 to 1000. Turquoise bars represent the lymphocyte only control. Error bars signify +SEM, which is calculated from the mean of five replicates from pooled lymphocytes from five separate spleens.
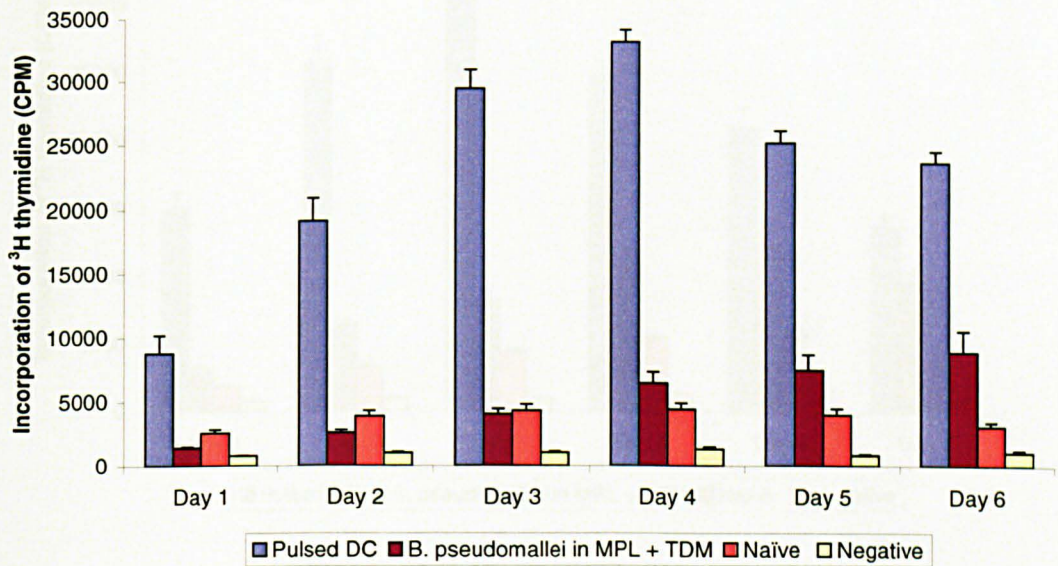
Figure 3.18   **Recall memory responses following immunisation (priming) with HK *B. pseudomallei* pulsed dendritic cells or HK *B. pseudomallei* delivered in adjuvant**

Memory responses four weeks after the priming dose with HK *B. pseudomallei* in MPL + TDM peaked after six days of culture at which point responses were significantly greater (p<0.05) than the level of proliferation seen for the naïve controls. Immunisation with a priming dose of HK *B. pseudomallei* pulsed DC however, stimulated a significantly greater memory response (p<0.001) than that observed following immunisation with HK *B. pseudomallei* in MPL + TDM, which was also much more rapid peaking after four days of culture. Error bars signify +SEM, which is calculated from the mean of five replicates from pooled lymphocytes from five separate spleens.

Chart legend: ▨ Pulsed DC ▪ B. pseudomallei in MPL + TDM ▪ Naïve ▫ Negative

Y-axis: Incorporation of ³H thymidine (CPM), scale 0 to 35000

X-axis: Day 1, Day 2, Day 3, Day 4, Day 5, Day 6

Figure 3.19 **Recall memory responses following immunisation (booster) with HK**
B. pseudomallei pulsed dendritic cells or HK B. pseudomallei
delivered in adjuvant

Memory responses five weeks after the booster dose were noted to be more rapid for both the test groups. Proliferation for the group primed and boosted with HK *B. pseudomallei* in MPL + TDM was greater than that seen after the priming dose and also peaked a day earlier after five days of culture. While no significant difference was observed in the level of memory proliferation seen in the group primed and boosted with HK *B. pseudomallei* pulsed DC, proliferation was noted to peak a day earlier after the booster dose after three days of culture demonstrating a faster recall response. Error bars signify +SEM, which is calculated from the mean of five replicates from pooled lymphocytes from five separate spleens.
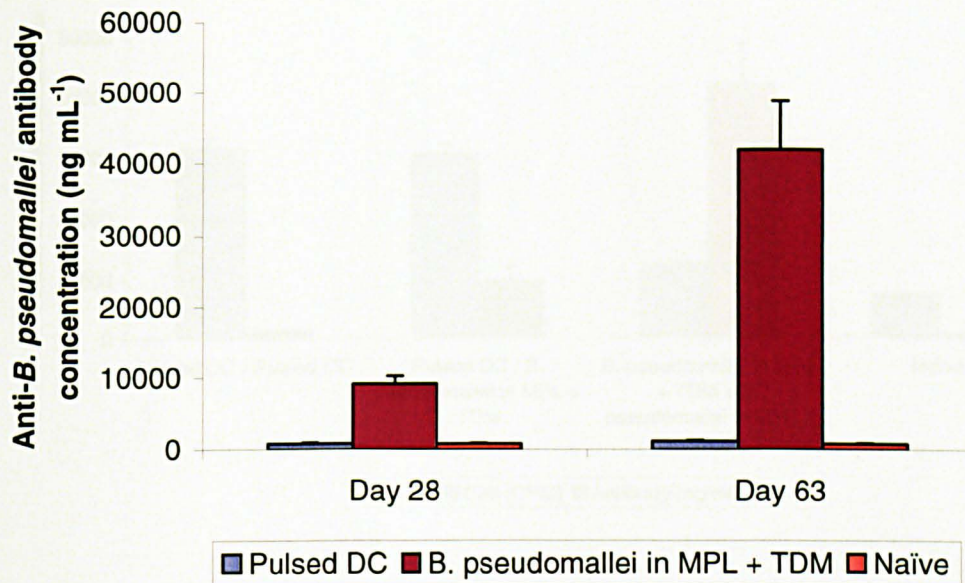
Figure 3.20   Humoral responses following immunisation with HK *B. pseudomallei* pulsed dendritic cells or HK *B. pseudomallei* delivered in adjuvant

Immunisation with HK *B. pseudomallei* delivered in MPL + TDM (IM) induced a strong anti-*B. pseudomallei* antibody response, which was significantly greater than both the HK *B. pseudomallei* pulsed DC immunised (ID) and naïve groups (p<0.05) after a single immunisation. Booster immunisation at day 28 augmented the response to approximately four-fold the levels achieved after the priming dose. No antibody production was observed following immunisation with HK *B. pseudomallei* pulsed DC above that seen in the naïve group at either time point. Error bars signify +SEM, which is calculated from the mean of four replicates from sera from five test subjects.
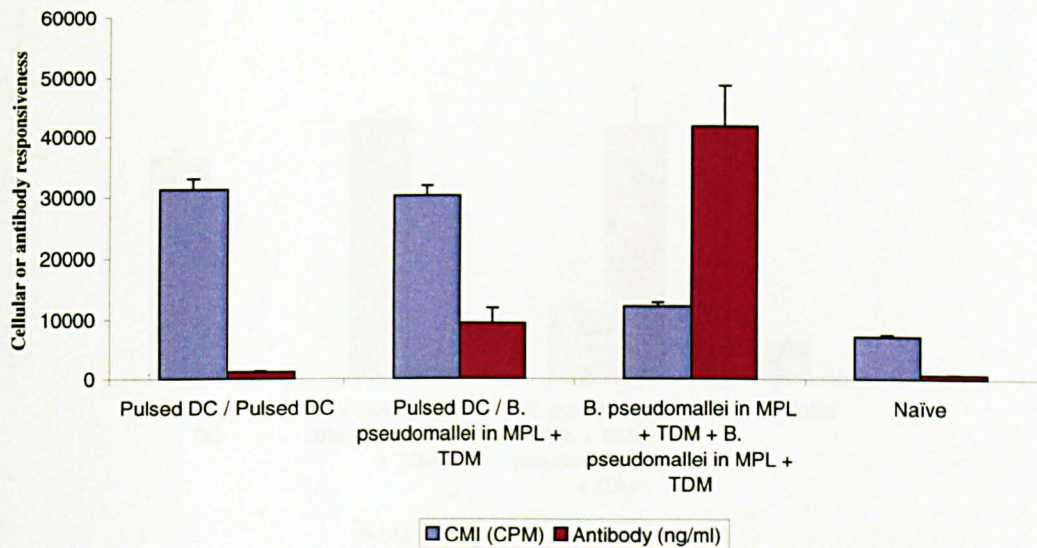
Figure 3.21   Cellular and humoral responses following intradermal immunisation
Following ID immunisation, groups primed with HK *B. pseudomallei* pulsed DC displayed a significantly greater cellular recall response ($p < 0.001$) than immunisation with HK *B. pseudomallei* in MPL + TDM (IM). Antibody responses however, for the group primed and boosted with HK *B. pseudomallei* in MPL + TDM were significantly greater than those observed in the groups that received HK *B. pseudomallei* pulsed DC ($p < 0.05$) as one or both immunisation doses. Substitution of the booster DC dose with HK *B. pseudomallei* in MPL + TDM resulted in a significant increase in antibody production ($p < 0.05$) as compared to the group that received two doses of HK *B. pseudomallei* pulsed DC. No significant change in the cellular recall response was observed as a result of this substitution. Error bars signify +SEM, which is calculated from the mean of five replicates from pooled lymphocytes from five separate spleens.
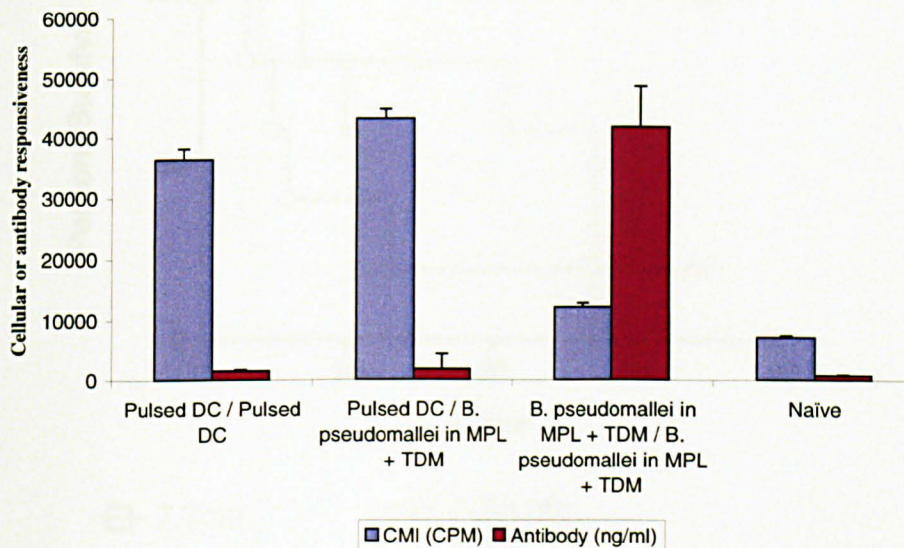
Figure 3.22   Cellular and humoral responses following intranasal immunisation

Following IN immunisation, groups primed with HK *B. pseudomallei* pulsed DC displayed a significantly greater cellular recall response (p<0.001) than immunisation with HK *B. pseudomallei* in MPL + TDM (IM). Antibody responses however, for the group primed and boosted with HK *B. pseudomallei* in MPL + TDM were significantly greater than those observed in the groups that received HK *B. pseudomallei* pulsed DC (p<0.05) as one or both immunisation doses.

For the IN dosed groups, substitution of the day 28 HK *B. pseudomallei* pulsed DC dose with a dose of HK *B. pseudomallei* delivered in MPL + TDM afforded no significant changes in either the cellular or antibody responses observed. Error bars signify +SEM, which is calculated from the mean of five replicates from pooled lymphocytes from five separate spleens.
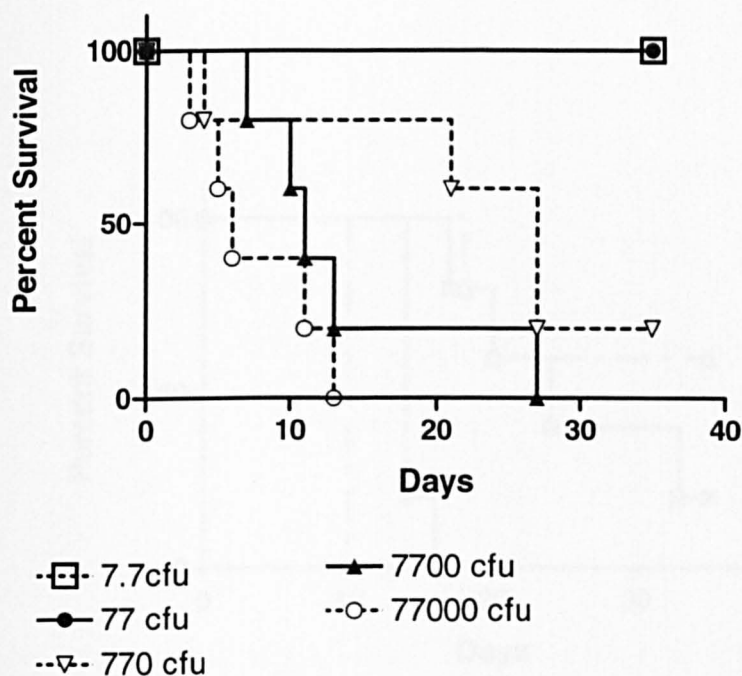
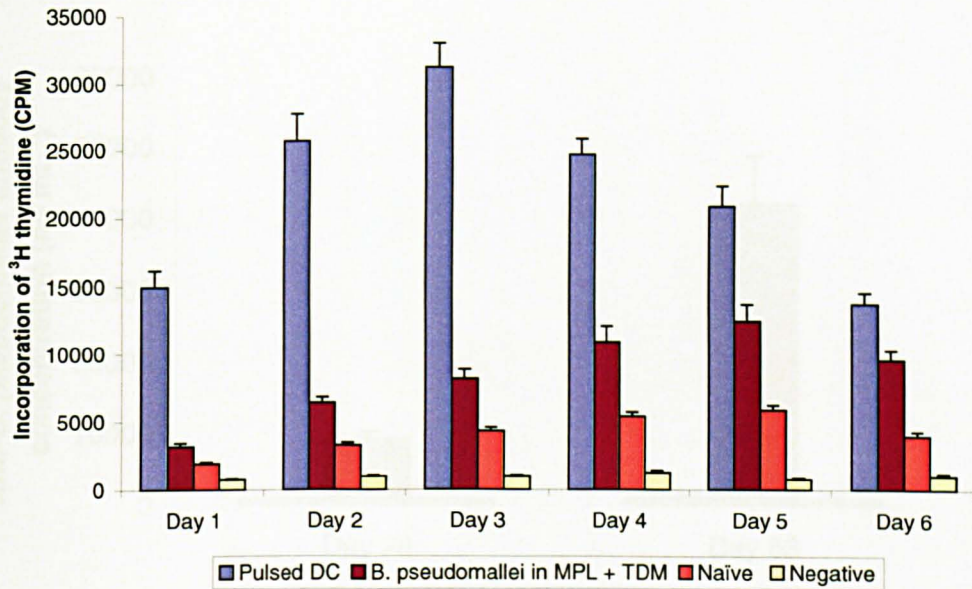| Dose (cfu)	Dilution	Died	Survived	Mortality Ratio	Percentage
77000	1×10^{-3}	5	0	5/5	100
7700	1×10^{-4}	5	0	5/5	100
770	1×10^{-5}	4	1	4/5	80
77	1×10^{-6}	0	5	0/5	0
7.7	1×10^{-7}	0	5	0/5	0

Figure 3.23   LD$_{50}$ experiment survival curve and mortality data table

All test subjects from the groups challenged with 7.7×10^4 cfu and 7.7×10^3 cfu *B.*

pseudomallei (NCTC 4845) died by days 12 and 28 respectively. The 7.7×10^2 cfu

challenged group exhibited a 20 % survival rate while the remaining two groups

challenged with 77 or 7.7 cfu both showed 100% survival. The mortality data table

shows the doses and dilutions for each test group together with the mortality data

allowing calculation of the LD$_{50}$ using the method of Reed and Muench (169).

- —▲— HK *B. pseudomallei* Pulsed DC / HK *B. pseudomallei* Pulsed DC
- --△-- HK *B. pseudomallei* Pulsed DC/ B. pseudomallei in MPL+TDM
- —■— B. pseudomallei in MPL+TDM / B. pseudomallei in MPL+TDM
- --O-- Naive
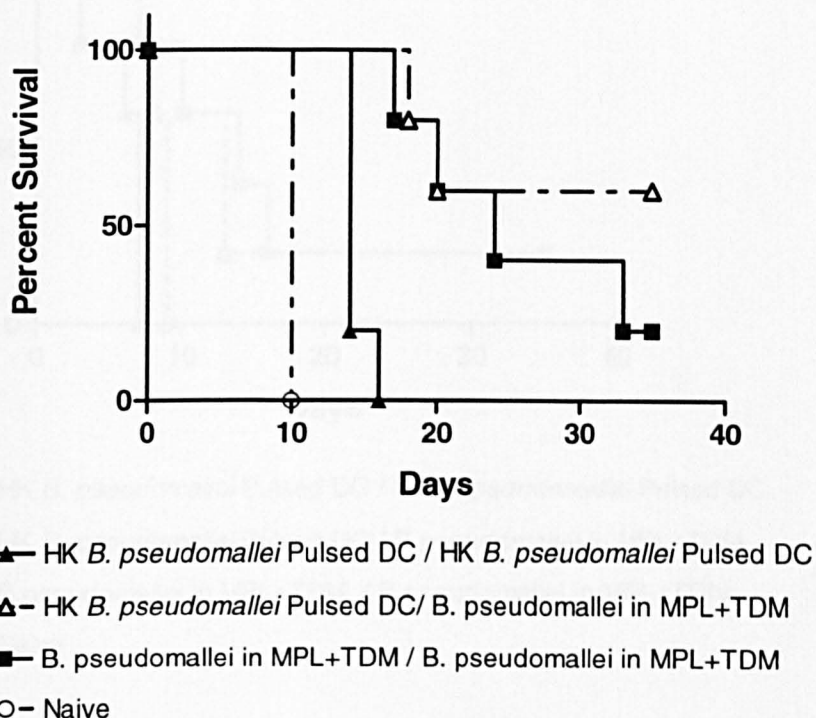
Figure 3.24   Survival curve for intradermal immunised groups

All naïve mice died by day 10 post challenge. All mice primed and boosted with HK *B.*
pseudomallei pulsed DC died by day 16 post challenge. The group primed and boosted
with HK *B. pseudomallei* in MPL + TDM displayed a significantly delayed time to
death and 20% survival rate (p<0.005) compared to the naïve controls. The final group
primed with HK *B. pseudomallei* pulsed DC and then boosted with HK *B. pseudomallei*
in MPL + TDM displayed a significant delay in time to death and a 60% survival rate
(p<0.005) compared to the naïve controls. In addition, a significant increase in time to
death and survival rate was noted (p<0.005) for this last group when compared to the
group that was primed and boosted with HK *B. pseudomallei* pulsed DC.
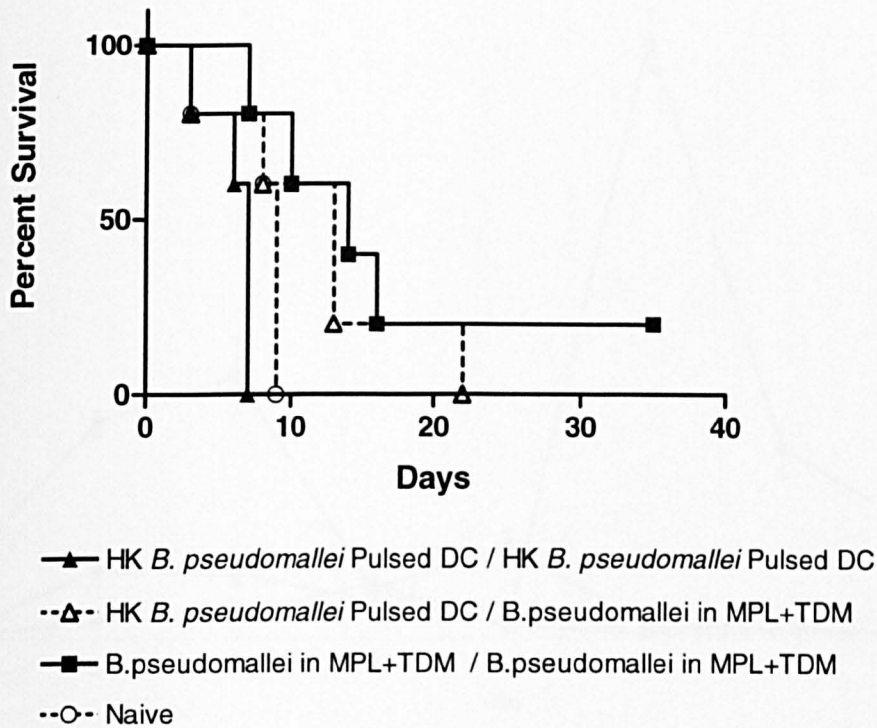
Figure 3.25   Survival curve for intranasal immunised groups

All the naïve mice and those primed and boosted with HK *B. pseudomallei* pulsed DC died by day 9 post challenge. No delayed time to death or survival was observed in the group that had been primed with HK *B. pseudomallei* pulsed DC and boosted with HK *B. pseudomallei* delivered in MPL + TDM. The group primed and boosted with HK *B. pseudomallei* in MPL + TDM again demonstrated a 20% survival rate, which was found to be statistically significant ($p<0.05$) when compared to the naïve controls.
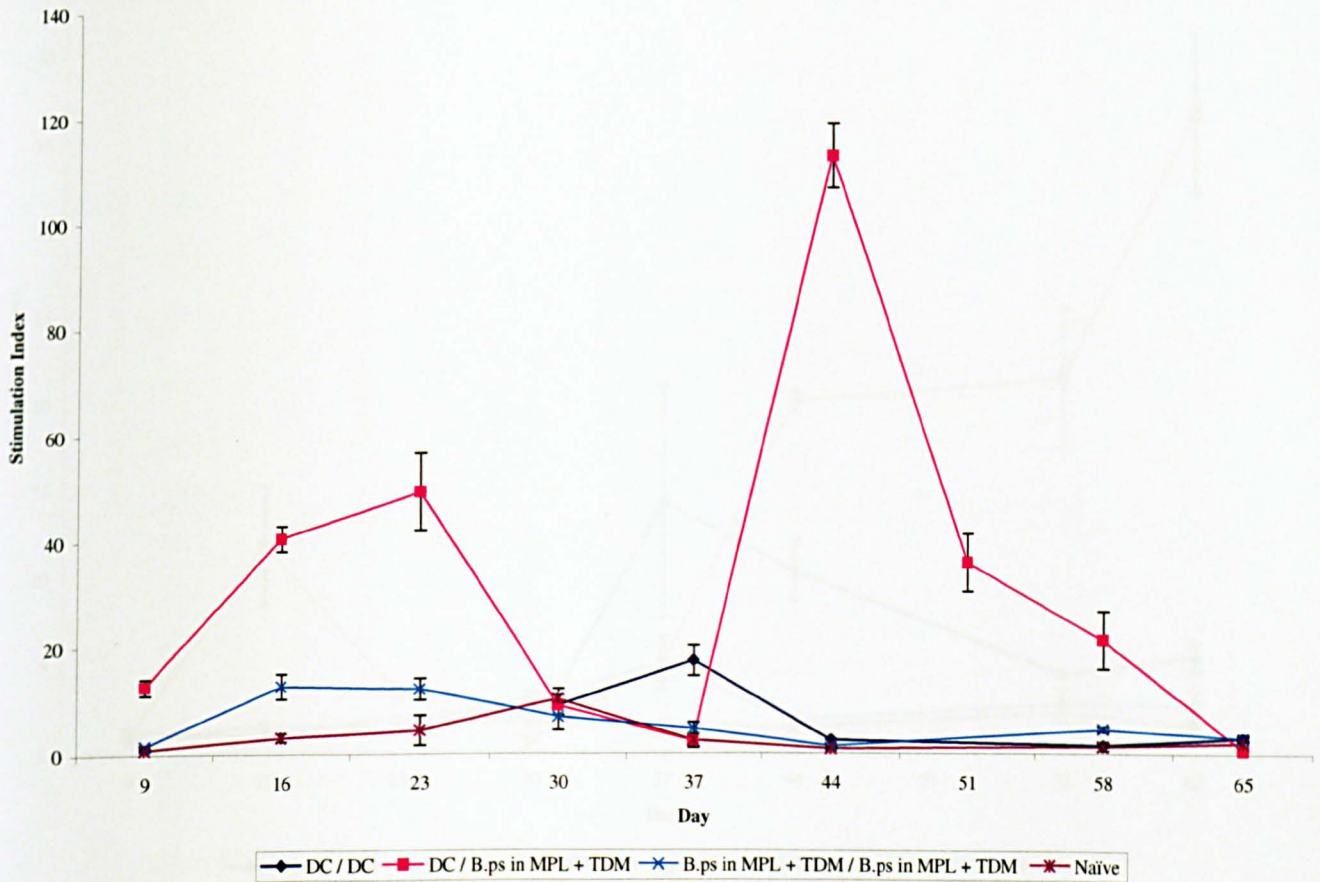
Figure 3.26   Splenic CD 4⁺ T cell recall responses following prime and boost immunisations

Priming with either HK *B. pseudomallei* pulsed DC or HK *B. pseudomallei* in MPL + TDM produced a significant increase in the CD 4⁺ memory T cell recall response (p<0.05) compared to the naïve controls.   Immunisation with HK *B. pseudomallei* pulsed DC produced a significantly greater (p<0.01) recall response than immunisation with HK *B. pseudomallei* in MPL + TDM.   After booster immunisation the greatest recall response was noted for the group primed with HK *B. pseudomallei* pulsed DC and boosted with HK *B. pseudomallei* in MPL + TDM (p<0.01).   Error bars signify ±SEM, which is calculated from the mean of four replicates from the pooled T cells from two test subjects.
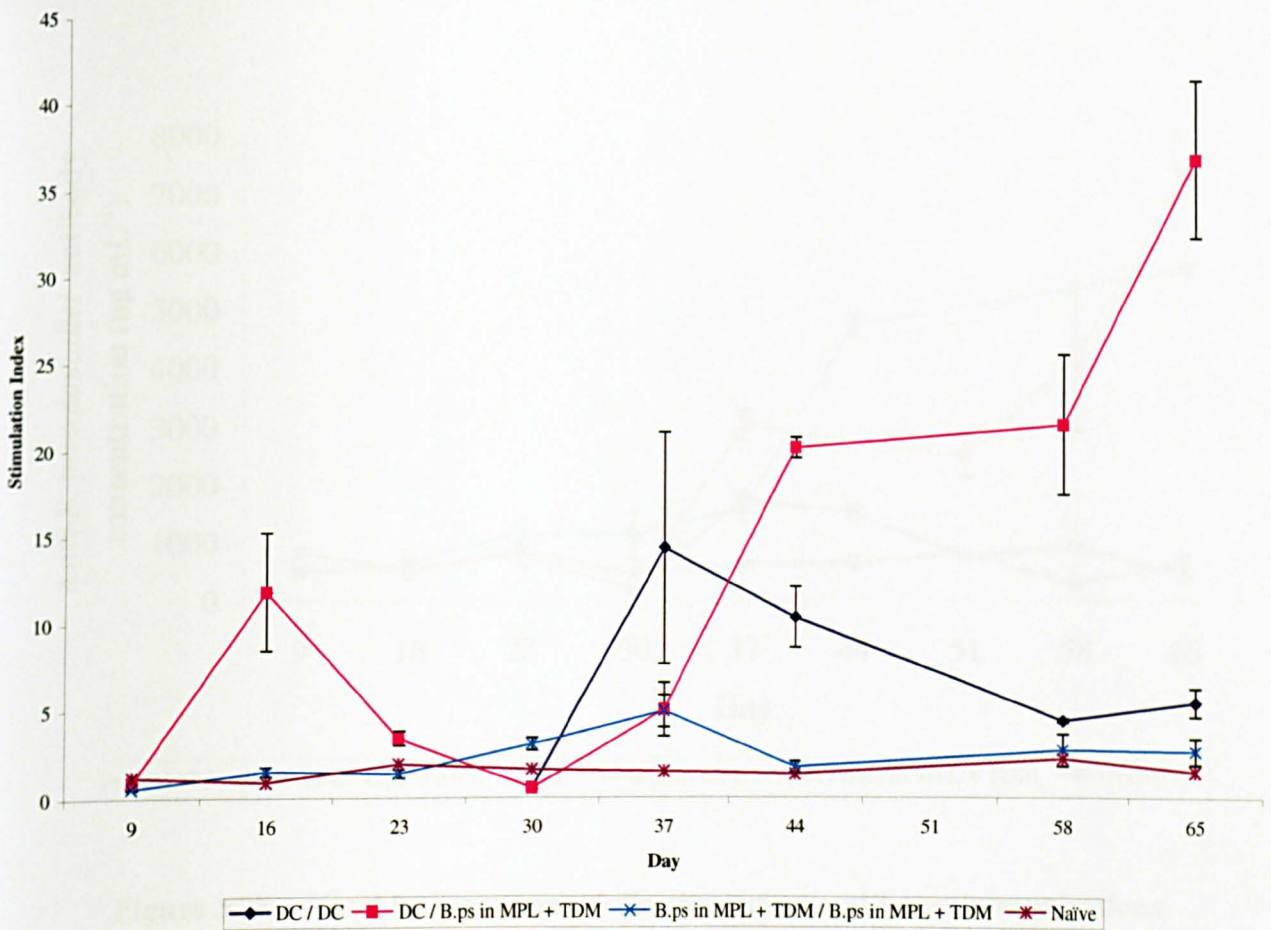
Figure 3.27   Splenic CD 8⁺ T cell recall responses following prime and boost

   immunisations

Immunisation with HK *B. pseudomallei* pulsed DC resulted in the greatest recall

response following priming (p<0.05).   Following booster immunisation, recall

responses greater than the naïve controls were observed from the group primed and

boosted with HK *B. pseudomallei* pulsed DC (p<0.05).   This immunisation group also

showed a trend of increased CD 8⁺ T cell recall responses out to day 44 (p<0.05).   The

group primed with HK *B. pseudomallei* pulsed DC and boosted with HK *B.*

pseudomallei in MPL + TDM showed a significantly greater response than all other

groups (p<0.05).   Error bars signify ±SEM, which is calculated from the mean of four

replicates from the pooled T cells from two test subjects.
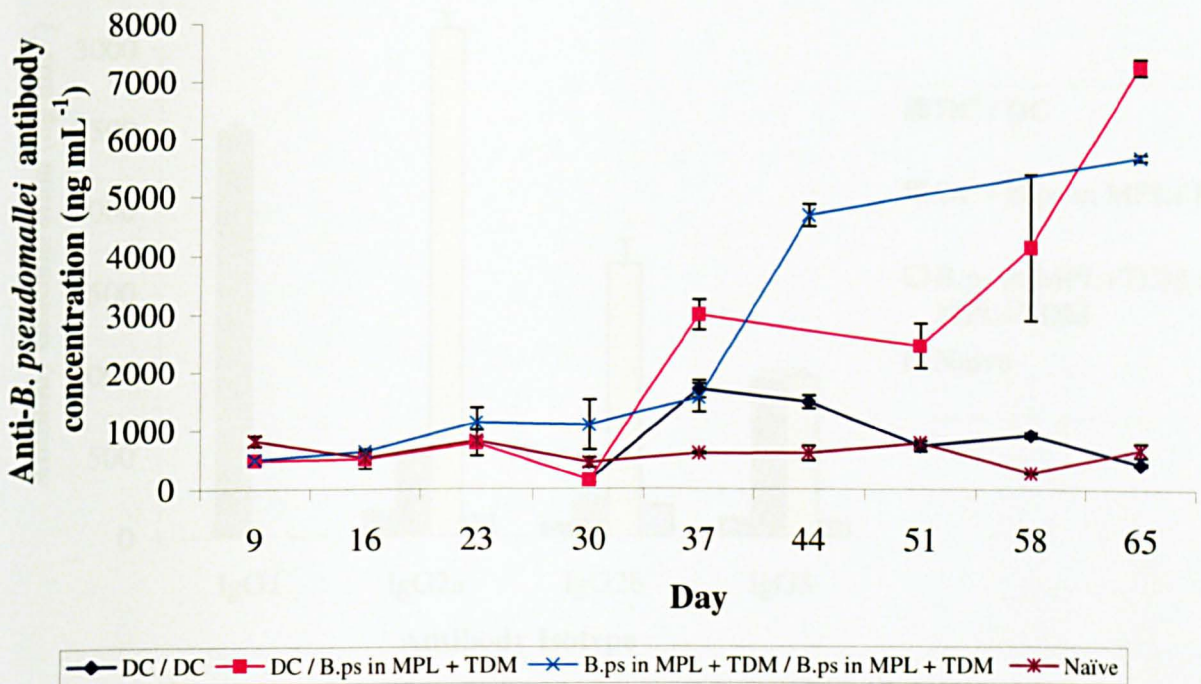
Figure 3.28   Total IgG responses following prime and boost immunisations

Prime doses for all groups did not stimulate any significant antibody production above that of naïve levels. Whilst this was expected for DC immunisation, it was unusual following immunisation with HK *B. pseudomallei* in adjuvant, which previous experiments had shown was very effective at stimulating antibody production. Following boost immunisation the group receiving two doses of pulsed DC, as expected, produced no antibody above naïve levels. The other two immunisation groups, show a steady increase in total IgG over the next 5 weeks. Both these responses were found to be significantly greater compared to the naïve controls although the group administered two doses of HK *B. pseudomallei* in adjuvant had significantly less total IgG than in previous experiments. Error bars signify ±SEM, which is calculated from the mean of four replicates from the sera from two test subjects.
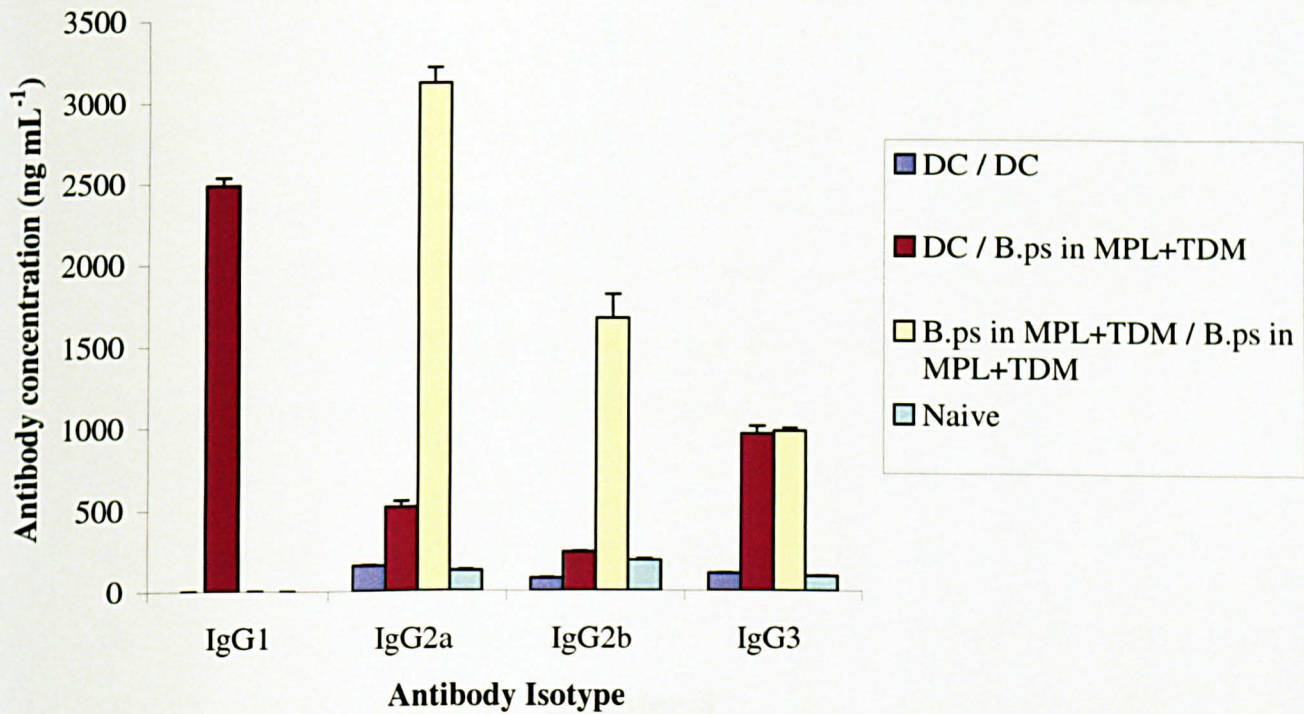
Figure 3.29   IgG subclasses following different immunisation regimen

Mice primed and boosted with HK *B. pseudomallei* pulsed DC failed to mount an antibody response greater than that seen for the naïve controls.   Mice primed and boosted with HK *B. pseudomallei* in MPL + TDM showed significantly greater concentrations of IgG2a, 2b and 3 than the naïve controls ($p < 0.05$).   Greater concentrations of IgG2a and 2b were also noted compared to the group primed with HK *B. pseudomallei* pulsed DC and boosted with HK *B. pseudomallei* in MPL + TDM ($p < 0.05$).   This latter group was however noted as having significantly greater IgG1 concentrations compared to all other groups ($p < 0.001$).   Error bars signify +SEM, which is calculated from the mean of four replicates from the sera from two test subjects.

Chapter 4

Discussion and Conclusions

4.1 Introduction

The need for an efficacious vaccine against *B. pseudomallei* is clear, particularly in the context of the increasing antibiotic resistance of the organism (35,48,190). This study addresses a particular approach to vaccination, utilising dendritic cells as a vaccine delivery vehicle, and has also partially elucidated the immune response required to achieve protection against *B. pseudomallei* infection.

4.2 Dendritic cell culture, phenotype and maturation

The isolation and culture of a wide range of DC subtypes has been reported, which provides evidence of the heterogeneity of the DC population. The data from this study describes the generation of high yield, high purity cultures of DC in a relatively short time scale. DC produced in this culture system, when the culture medium was supplemented with GM-CSF and TNF-α, were phenotypically myeloid. The addition of either IL-4 or IL-12 to the culture, which has previously been shown to have a polarising effect on DC (92), did not result in any notable phenotypic changes to the DC produced from this culture system. Cytokine secretion patterns did vary however, under the different cytokine environments. The addition of IL-4 to the culture was associated with an increase in IFN-γ secretion following LPS stimulation, compared to the GT cultured DC ($p<0.05$), suggesting a homeostatic response from the DC population in response to the high levels of IL-4, which would have produced a Th2 cytokine bias. The presence of IL-12 in the culture however, did not stimulate an opposite homeostatic response, which might have been expected. The most notable difference from the group cultured in the presence of IL-12 was a significant increase in IL-10 secretion following LPS stimulation, compared to the GT cultured DC ($p<0.01$). IL-10 is a potent anti-inflammatory factor that has been shown to inhibit macrophage activation resulting in

the reduced expression of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-12

and GM-CSF (52,69). Thus the presence of high concentrations of IL-12 in the culture

might have stimulated the production of IL-10 in response to the artificial inflammatory

environment created by IL-12 in the culture medium. The presence of TNF-α in the

culture would also likely have compounded this effect, and may account for the IL-10

secretion seen in all three culture environments.

Maturation responses, assessed through increases in the cell surface markers MHC class

II, CD 80, CD 86 and CD 54, revealed similar patterns of expression during the 48

hours following exposure to inflammatory stimuli. However the varying effect of

adding either IL-4 or IL-12 to the culture meant that only GM-CSF and TNF-α were

used as a supplement to the culture for further experimentation.

4.3   *In vitro* responses

Further work then assessed the response of the dendritic cells following exposure to HK

B. pseudomallei.   The time-to-maturation response, compared to LPS or TNF-α

exposure, was quicker following exposure to HK *B. pseudomallei*. There are a number

of potential explanations for this; the most likely of which is concentration. Although

the number of bacteria added to the DC culture was standardised, molar comparisons

were not made between LPS concentrations from the HK *B. pseudomallei* and that of

the purified LPS, so that the concentration of LPS added when using HK *B.*

pseudomallei may have been optimum for inducing maturation.   In addition, the

presence of other inflammatory and/or maturation-inducing bacterial products, when

HK *B. pseudomallei* was added to the culture, are also likely to have had a stimulatory

effect on maturation.   In addition to quicker maturation, the high expression of MHC

class II, CD 80, CD 86 and CD 205 was maintained over 96-hours of *in vitro* culture with no further stimulation (Figure 3.13).

Cytokine secretion from the DC following exposure to HK *B. pseudomallei* demonstrated a mixed cytokine profile (Figures 3.15 and 3.16). No IL-4 secretion was observed (consistent with the literature, which suggests that professional APC's do not secrete IL-4 (59)) and changes in IL-2, IL-5 and IFN-γ secretion were not statistically significant. Significant increases in IL-12p70 ($p < 0.05$), IL-10 ($p < 0.05$) and IL-6 ($p < 0.01$) were however noted.

These three cytokines play contrasting roles in the generation of immune responses. IL-12 has been shown to play a central role in the generation of type 1 T helper cell responses and cell-mediated immunity (77,161); stimulating the proliferation of activated T and NK cells (76,117,197,227), enhancing the lytic activity of NK cells and cytotoxic T lymphocytes (36,117,227) and inducing the production of IFN-γ by both T and NK cells (31,117,197,227).

In contrast, IL-6 is more often associated with Th2 T cell development and the stimulation of antibody mediated immunity (149), inducing the expression of the IL-4 gene during T cell activation (59). Produced by a number of cell types including DC (59) in response to inflammatory stimuli, IL-6 also induces the synthesis of acute phase proteins in heptocytes, the terminal differentiation of B cells to plasma cells, differentiation of monocytes to macrophages and the growth of haematopoietic stem cells (93).

IL-10 is a pleiotropic cytokine that has an important role in regulating the immune response (226) and is produced by a range of cells including DC (68,144). The main biological functions of IL-10 are to limit and terminate inflammatory responses, block pro-inflammatory cytokine secretion and regulate the differentiation of T cells, B cells, NK cells and mast cells (226).

The conflicting roles of these cytokines meant that few conclusions could be drawn regarding likely *in vivo* immune responses on the basis of the *in vitro* data alone. Of initial concern however, in terms of attempting to generate primary immune responses *in vivo* was the high level of IL-10 secretion noted from the DC cultures. IL-10 has been shown to inhibit the production of IL-2, IFN-γ, IL-4 and IL-5 (53,182,205) by CD 4^+ T cells, stifling T cell responses. It has also been shown to suppress APC activation and antigen presentation by inhibiting the expression of MHC class II, CD 80 and CD 86 on macrophages and dendritic cells (32,60). Although the maturation data (Figures 3.11 to 3.13) had suggested that such an effect on the maturation of the DC was not present, it was important to assess the ability of HK *B. pseudomallei* pulsed DC to generate primary immune responses.

To achieve this aim DC, pulsed with varying concentrations of HK *B. pseudomallei*, were incubated with T lymphocytes in different ratios. This enabled not only an assessment of their ability to generate primary immune responses, but also an opportunity to optimise conditions for later *in vivo* work. Statistically, optimum *in vitro* proliferation was observed at the relatively low pulsing concentration of 1×10^4 cfu mL^{-1} of HK *B. pseudomallei*. However, actual differences between the peak proliferation observed at each of the concentrations investigated were relatively small, suggesting that the observed statistical difference might not correlate with an *in vivo* biological

difference. Repeat experiments and the investigation of pulsing doses below 10^4 cfu mL^{-1}, which were unable to be carried out due to project time constraints, may have better clarified any such effect. What was evident from this data was that the DC were generating primary immune responses. This was inferred from the fact that the unpulsed DC failed to generate equivalent responses to the pulsed DC, demonstrating the lack of a non-specific proliferative response from the DC alone. Later experiments (e.g. Figure 3.18) also showed that the addition of HK *B. pseudomallei* to naïve lymphocytes, although stimulatory, was not as effective at inducing lymphocyte proliferation as HK *B. pseudomallei* pulsed DC. Thus, taken together these data indicate that the primary responses seen were *B. pseudomallei* specific. This was of particular importance in the context of *B. pseudomallei* infection, as an intracellular pathogen, and also when considering the suitability of DC as a vaccine system, which could demonstrably produce an effective CMI response.

4.4 *In vivo* responses

Investigation of the *in vivo* immune responses following immunisation with HK *B. pseudomallei* pulsed DC or *B. pseudomallei* in MPL + TDM, demonstrated a superior T cell recall response ($p<0.001$) after immunisation with HK *B. pseudomallei* pulsed DC following both prime and booster immunisations. Investigation of the humoral responses to immunisation demonstrated a significantly lesser anti-*B. pseudomallei* antibody response from the group immunised with HK *B. pseudomallei* pulsed DC, which failed to generate any antibody above naïve levels, compared to the group immunised with HK *B pseudomallei* in MPL + TDM ($p<0.05$). Due to the lack of available literature on the protective immune response requirements for *B. pseudomallei* infection, the immunisation regimen was further altered in an attempt to generate a more balanced immune response for better comparison. Hence the two vaccine

formulations were combined to create a regimen of a priming (day 0) dose of HK *B. pseudomallei* pulsed DC followed by a booster (day 28) dose of HK *B. pseudomallei* in MPL + TDM. The effect of delivering the HK *B. pseudomallei* pulsed DC via the IN instead of the ID route was also investigated. This immunisation group was originally intended for an aerosol challenge experiment, but laboratory equipment problems at the time of challenge prevented the experiment going ahead. As a result the immunised mice were challenged via the IP route to allow a comparison of the different routes of immunisation. Following ID immunisation of the HK *B. pseudomallei* pulsed DC and a booster immunisation with HK *B. pseudomallei* in MPL + TDM (IM), an increase in the concentration of circulating anti-*B. pseudomallei* antibody ($p < 0.05$) compared to immunisation with two doses of HK *B. pseudomallei* pulsed DC was noted. The same combination of formulations, but utilising the IN route for delivery of the HK *B. pseudomallei* pulsed DC, resulted in no such increase in humoral response ($p > 0.05$), despite a CMI recall response that was equivalent to that noted following ID administration of the HK *B. pseudomallei* pulsed DC. The reasons for this difference have not been investigated here due to project monetary constraints, but are possibly due to the very different routes of administration between the prime and booster immunisations when the IN route was used. *In vitro* proliferation data demonstrated that both routes of immunisation (ID and IN) successfully primed cellular systemic immunity, but it is possible that IN administration of the HK *B. pseudomallei* pulsed DC may not have primed for systemic humoral immunity. It is difficult to speculate as to the exact reasons and mechanisms for the lack of humoral immunity following the booster dose of HK *B. pseudomallei* in MPL + TDM. Especially since exposure of APC to antigen in the lungs generally favours a humoral over a cellular response, due to the potential consequences to the host of damage to pulmonary tissues resulting from a strong cytotoxic response (128). It does however, highlight the importance of the route

of immunisation on the type of immune response that ensues and also the need for rational vaccine design.

Challenge investigations using the different immunisation regimens suggested that antibody might play an important role in protection against *B. pseudomallei* infection. Following ID immunisation, the group receiving two doses of HK *B. pseudomallei* pulsed DC (ID) all died, despite a superior cell-mediated immune response (p<0.001). Whilst those receiving two doses of HK *B. pseudomallei* in MPL + TDM (IM) or the combined regimen, which both had increased antibody levels (p<0.05), exhibited 20% (p<0.005) and 60% (p<0.005) survival rates respectively. In addition, a significant increase in time to death and survival rate (p<0.005) was noted for the combined regimen when compared to the group that received two doses of HK *B. pseudomallei* pulsed DC only. Data from the IN administered groups also corroborated these results, since the group receiving two doses of HK *B. pseudomallei* in MPL + TDM again exhibited a 20% survival rate (p<0.05) while the group primed and boosted with HK *B. pseudomallei* pulsed DC via the IN route all died. In addition, the group primed with HK *B. pseudomallei* pulsed DC (IN) and boosted with HK *B. pseudomallei* in MPL + TDM, which failed to mount a significant humoral response, also all died. This data therefore supported passive protection studies (21,24,111), which have previously suggested that antibody might play a role in protection against *B. pseudomallei*, with high circulating antibody concentrations being correlated with survival in mice and rats.

Assessment of the survivor's bacterial burden at day 35-post challenge demonstrated that bacteria could not be cultured from any of the tissue samples from one subject of both groups with survivors. While the remaining survivors did have evidence of a

bacterial burden, this was limited and only present in splenic tissues, suggesting the infection might have been completely cleared had the experiment been extended.

4.5   Elucidation of the protective immune response

Taken together, these data provided strong evidence of a role for both humoral and cell-mediated immunity in protection against *B. pseudomallei* infection since although the presence of a significant anti-*B. pseudomallei* antibody response (compared to naive controls) was correlated with survival, the presence of a superior CMI response improved survival rates from 20% to 60% ($p<0.05$). Thus the immune responses generated by the different immunisation regimens were further characterised in an attempt to determine the protective immune response. IN administration of the DC was not considered here since no protection was afforded using this delivery route.

Characterisation of the CD 4^+ and CD 8^+ T cell recall responses over the nine weeks of the immunisation regimen demonstrated a stark difference in the immune response generated. Significantly greater T cell recall responses (both CD 4^+ and CD 8^+) were noted following priming with HK *B. pseudomallei* pulsed DC (ID) and boosting with HK *B. pseudomallei* in MPL + TDM (IM). In addition, the CD 8^+ T cell recall response was observed to improve over the nine weeks of the regimen unlike the other groups in which it had diminished to naïve levels by two weeks post-boosting. This continued increase following this immunisation regimen resulted in a significantly greater CD 8^+ T cell response at the point of challenge (day 65 of the immunisation regimen). It is particularly noteworthy that such an increase in CD 8^+ T cell recall response was not observed in the group primed and boosted with HK *B. pseudomallei* pulsed DC. This suggested that the combination of priming with HK *B. pseudomallei* pulsed DC and

boosting with HK *B. pseudomallei* in MPL + TDM was required to generate such a response.

Recent work by Bancroft and colleagues (88) has suggested that CD 8$^+$ T cells may play little or no role in protection against *B. pseudomallei* infection in the mouse model of melioidosis. This is based on the observation that mice immunised with the 2D2 mutant of *B. pseudomallei* (8) and then challenged following ablation of CD 8$^+$ T cells with monoclonal antibodies, showed no observable increase in morbidity or mortality. CD 8$^+$ T cells however, have been shown to contribute to rapid IFN-γ production following *B. pseudomallei* infection (124), which would serve to improve innate host cell killing mechanisms, augmenting the cytotoxic effect. The rapid induction of IFN-γ has also been shown to prevent acute *B. pseudomallei* infection (177).

In contrast to the CD 8$^+$ data, ablation of CD 4$^+$ T cells during the 2D2 work showed that they were required for protection (88) as their removal significantly reduced the time-to-death in mice challenged with fully virulent *B. pseudomallei*. This suggests that the significantly improved CD 4$^+$ T cell response, observed following immunisation with a combination of HK *B. pseudomallei* pulsed DC and HK *B. pseudomallei* in MPL + TDM, might have contributed to the improved survival in this group.

In addition to the role of CMI, the antibody data also provided some insight into the role of humoral immunity in protection against *B. pseudomallei* infection. Total IgG antibody responses increased as expected following booster immunisation in the groups receiving either two doses of HK *B. pseudomallei* in MPL + TDM or the group primed with HK *B. pseudomallei* pulsed DC and boosted with HK *B. pseudomallei* in MPL + TDM. Although increased anti-*B. pseudomallei* IgG titres were correlated with survival

in the challenge experiments (Figure 3.24), there was a disparity between the concentration of antibody observed and survival, in that the greatest survival was not observed from the group with the highest antibody concentration. The significantly improved cell-mediated response (particularly CD 4$^+$ T cells) in the group immunised with a combination of HK *B. pseudomallei* pulsed DC and HK *B. pseudomallei* in MPL + TDM (Figure 3.21) may provide an explanation for this observation. However, further clarification of the role of antibody might have been gleaned from investigation of the IgG subclasses produced by immunisation and was therefore carried out.

Analysis of the IgG subclasses revealed another stark difference between the group immunised with the combination regimen and the group immunised with two doses of HK *B. pseudomallei* in MPL + TDM. Despite the typically greater antibody response of the group receiving two doses of HK *B. pseudomallei* in MPL + TDM, it was found to be predominantly an IgG2a response with no detectable IgG1. In contrast the group primed with HK *B. pseudomallei* pulsed DC and boosted with HK *B. pseudomallei* in MPL + TDM developed a predominantly IgG1 response ($p<0.05$) although all the isotypes were present. In the mouse, the IgG2a and 2b isotypes are the only isotypes to activate complement although this is unlikely to be the mechanism of bacterial killing in the group immunised with two doses of HK *B. pseudomallei* in MPL + TDM, because of the resistance of *B. pseudomallei* to complement lysis (196). Opsonisation is however, likely to play a role and may facilitate improved uptake and killing by phagocytes, although this has not been formally demonstrated for *B. pseudomallei*. The reasons for the low survival rate observed following immunisation with only HK *B. pseudomallei* in MPL + TDM, are likely several-fold. Efficient opsonisation would require high antibody concentrations and the intracellular nature of *B. pseudomallei* infection would only provide a small window of opportunity for such a mechanism of

bacterial clearance. Once intracellular, the lack of a significant cell-mediated response to augment the humoral response would also mean that effective bacterial killing and clearance would be stifled.

IgG1, generated only following immunisation with a combination of HK *B. pseudomallei* pulsed DC and HK *B. pseudomallei* in MPL + TDM, while also opsonising, is also the only isotype in mice to bind to the low affinity Fcγ RII (CD 32). This receptor is the most widely distributed of the Fcγ receptors (219) and is significantly more effective at inducing phagocytosis (61) than Fcγ RI (CD 64) and RIII (CD 16). Of particular importance with regard to Fcγ RII, is its role in antibody dependent cellular cytotoxicity (ADCC). This process enables the killing of target cells in the absence of complement activation and CD 8$^+$ cytotoxic T lymphocyte activity. During ADCC, lysis occurs following specific contact between the Fcγ RII on the effector cells and IgG1 located on the target cells (63,167). Further more, ADCC occurs at antibody concentrations well below that required for complement lysis or efficient killing mediated by phagocyte uptake of opsonised bacteria (63).

As discussed in section *1.2.2.2*, cytokines secreted during T cell stimulation play a pivotal role in the type of immune response that ensues. Differences in the cytokine environment during T cell stimulation are also likely to have had an influencing role on the different IgG subclass profiles observed from each of the immunisation regimens. This could be either from the DC used as a delivery vector or from host DC, which have been shown to be the primary cell involved in antigen presentation following immunisation with protein in adjuvant (85). *In vitro*, DC pulsed with HK *B. pseudomallei* demonstrated a mixed cytokine profile that did not appear to favour a particular type of immune response. By considering the *in vivo* responses to

immunisation however, it is possible to better understand how each of these cytokines potentially influenced the ensuing immune response.

Following immunisation with two doses of HK *B. pseudomallei* in MPL + TDM, significant levels of anti-*B. pseudomallei* IgG antibody were noted, predominantly composed of the IgG2a subclass and with no detectable IgG1. This suggests a dominant role for IL-12 (secreted following pulsing of the DC with HK *B. pseudomallei*, Figure 3.15) during immunisation with this regimen, which has been shown to decrease IgG1 production and enhance the production of IgG2a (215). The high concentration of antibody produced however, also indicates the influence of IL-6 and IL-10 (also secreted following pulsing of the DC with HK *B. pseudomallei*, Figures 3.16 and 3.15 respectively). IL-6 is involved in the terminal differentiation of B cells to plasma cells (93) and IL-10 enhances B cell survival and is a co-factor for the proliferation of B cell precursors and mature B cells (226). Indeed IL-6 and IL-10 have been shown to be essential for the production of IgG and other isotypes by B cells (25).

Subclass switching to IgG1, as observed following immunisation with a combination of HK *B. pseudomallei* pulsed DC and HK *B. pseudomallei* in MPL + TDM, occurs predominantly under the control of IL-4 (149). Thus IL-6 is again essential since APC do not produce IL-4, but rather IL-6, which then stimulates the secretion of IL-4 by activated CD 4$^+$ T cells (59). IL-10 has also been shown to have an effect on isotype switching (25) and in addition increases the expression of Fcγ RII, a function that has been correlated with improved ADCC (209). In terms of the T cell response observed from immunisation with the combination regimen, IL-10 has been shown to have stimulatory effects on CD 8$^+$ T cells, inducing their recruitment, cytotoxic activity and proliferation (82,179). These data suggest this cytokine might have been central to the

in vivo response that developed from this regimen, allowing the induction of a strong

CD 8$^+$ T cell proliferative response and isotype switching to IgG1.

These functions of IL-10 do not however, negate a role for IL-12, which as previously mentioned is also pivotal in stimulating the proliferation of activated T cells (76,117,197,227) and in enhancing the lytic activity of cytotoxic T lymphocytes (36,117,227). Both these functions also suggest a possible role for this cytokine in the immune response generated by immunisation with the combination regimen.

Consideration of immune regulatory functions of each of these cytokines suggests that they are each equally likely to have played a role in the developing immune response. It is unclear from this study, the precise effects of the cytokines secreted by the DC *in vitro* and further *in vivo* work is required to properly elucidate the role of the cytokine milieu post immunisation and post challenge.

4.6 Conclusions

The complexities of *B. pseudomallei* infection, coupled with its plethora of immune evasion strategies have so far eluded successful prophylaxis. Recent work is now beginning to shed light on the type of immune response required for protection against infection (8,88,124).

The data presented here suggest that the immune response needed for protection against *B. pseudomallei* infection is likely to require strong humoral responses of the appropriate isotype and possibly subclass to facilitate both the innate and adaptive cellular responses. Although further work is required to verify its role in protection, ADCC provides a plausible mechanism for the role of antibody in *B. pseudomallei*

infection, enabling the killing of bacteria during the extracellular phase of infection and targeting of infected cells once the infection becomes intracellular. It also offers an explanation for the previously demonstrated protection in passive transfer studies, in which the greatest levels of protection against *B. pseudomallei* were achieved when using IgG1 monoclonal antibodies to the exopolysaccharide (EPS), as compared to other monoclonal antibodies against EPS (IgG2a and IgG2b), LPS (IgG2a) and protein (IgG2a and IgG3) (112). In addition, data presented in this study highlights a potential relationship between immunoglobulin isotype and subclass and protection. This suggests that further work to ascertain whether such a correlation is also apparent in survivors of *B. pseudomallei* infection might be appropriate to aid future therapeutic and diagnostic efforts.

Although mechanisms for the intracellular survival of *B. pseudomallei* are yet to be elucidated (62,111), these data, together with other studies (88,177), demonstrate the potential importance of an effective CMI response both in controlling infection and achieving bacterial clearance following survival of the primary infective phase. Further work is still required to fully elucidate the role of T cells in protection, although data form this study does support the work of Bancroft and colleagues (88) who suggests a role for CD 4$^+$ T cells. In addition a potential role for CD 8$^+$ T cells is suggested by the data, most likely mediated through cytotoxic effector function or the secretion of IFN-γ. However the data from this study does not answer the question as to whether this role is essential for survival following *B. pseudomallei* infection.

Finally, DC are a heterogeneous population consisting of distinct subsets with common as well as unique functions (152). This plasticity of function and unique capacity to induce and regulate immune responses makes them an attractive target not only as

vectors for the delivery of immunogenic antigens, but also as *in vivo* targets for immune modulation (152). The data presented demonstrates the suitability of DC as a delivery vector capable of generating effective CMI responses against intracellular bacteria. While this is not the first time this has been demonstrated, it does show the effective application of this technology to a pathogen capable of causing significant morbidity and mortality to which there is currently no licensed vaccine.

4.7   Future work

This work has demonstrated the considerable potential of DC as candidates for the induction of cellular immune responses, but further work is required to optimise them as a delivery vehicle. There are many avenues of investigation such as alternative culture methods, different DC subtypes and the type and amount of antigen used for priming, which could potentially enable the required immune response to be generated using DC alone.

The drawbacks associated with using DC as vaccine delivery vectors are well published (11,17,45,57,58,67,71,87), but there is also significant potential for the use of DC as a mechanism for the evaluation of potential vaccine candidates. Further investigation of the protective immune response to *B. pseudomallei* infection is also perhaps warranted especially with regard to the different roles of humoral and cell-mediated immunity. In particular, the role and functionality of CD 4^{+} T cells and of different immunoglobulin isotypes and subclasses in protection against infection requires further elucidation. Such information would likely provide an effective platform for future rational vaccine design against *B. pseudomallei.*

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Appendix