

MELIOIDOSIS:
AN INVESTIGATION OF CELLULAR IMMUNE RESPONSES

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

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Other Publications:

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

ABSTRACT

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

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

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

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

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

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

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

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

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

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

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

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LIST OF ABBREVIATIONS

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