# MELIOIDOSIS:

# AN INVESTIGATION OF CELLULAR IMMUNE RESPONSES

**Thesis submitted by**

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**James Cook University**

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

## *DECLARATION*

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## *PUBLICATIONS*

### Publications resulting from this Thesis:

1. **Barnes J L**, Ulett G C, Ketheesan N and Hirst R G. 2001. Induction of multiple chemokine and colony stimulating factor genes in experimental *Burkholderia pseudomallei* infection. *Immunology and Cell Biology* 79: 490-501.
2. Ketheesan N, **Barnes J L**, Ulett G C, VanGessel H J, Norton R E, Hirst R G and LaBrooy J T. 2002. Demonstration of a cell-mediated immune response in melioidosis. *Journal of Infectious Diseases* 186(2): 286-289.
3. **Barnes J L** and Ketheesan N. 2004. Melioidosis: An emerging infection. *Australasian Science* 25(2): 29-31.
4. **Barnes J L**, Warner J, Melrose W, Durrheim D, Speare R, Reeder J and Ketheesan N. 2004. Adaptive immunity in individuals exposed to *Burkholderia pseudomallei* in Papua New Guinea: a possible role for T cells in determining outcome of infection. *Clinical Immunology (submitted)*.
5. **Barnes J L** and Ketheesan N. 2003. Melioidosis: the route of infection influences disease outcome. *Acta Tropica (in preparation)*.
6. **Barnes J L** and Ketheesan N. 2003. Demonstration of cell-mediated immune responses in a murine model of melioidosis. *Infection and Immunity (in preparation)*.

### Other Publications:

7. Ulett G C, Currie B, Clair T, Mayo M, Ketheesan N, LaBrooy J L, Gal D, Norton R, Ashhurst-Smith C, **Barnes J L**, Warner J and Hirst R G. 2001. *Burkholderia pseudomallei* virulence: definition, stability and association with clonality. *Microbes and Infection*: 3(8): 621-31.
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 $IgG_1$ ($P<0.01$) 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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AIDS – acquired immunodeficiency syndrome
ANOVA – analysis of variance
APC – antigen presenting cell
$Ara^-$- non arabinose assimilating
$Ara^+$ - arabinose assimilating
ARDS – acute respiratory distress syndrome
BCG – bacillus Calmette-Guérin
Bp – base pairs
BP-LPS – *B. pseudomallei* LPS
BpLy1 – *B. pseudomallei* lysate (NCTC 13179)
cDNA – complementary DNA
CepLy – *B. cepacia* lysate
CF – cystic fibrosis
cfu – colony forming units
CLT – cytolethal toxin
CMI – cell-mediated immunity
ConA – concanavalin A
CP- capsular polysaccharide
cpm – counts per minute
CSF – colony stimulating factor
DNA – deoxyribonucleic acid
dNTP – deoxynucleoside triphosphate
EIA – ezyme immunoassay
ELISA – enzyme linked immunosorbent assay
FCS – foetal calf serum
G-CSF – granulocyte CSF
GM-CSF – granulocyte macrophage CSF
H&E – haematoxylin and eosin
HIFCS – heat-inactivated FCS
HIV – human immunodeficiency virus
HLA – human leucocyte antigen
HV – highly virulent
IFA- indirect fluorescent antibody
IFN-γ – gamma interferon
Ig – immunoglobulin
IHA – indirect haemagglutination
IL – interleukin
*in* – intranasal
*ip* - intraperitoneal
IP-10 – interferon-γ-inducible protein 10
*iv* – intravenous
KC - cytokine-induced neutrophil chemoattractant
$LD_{50}$ – fifty percent lethal dose
LIX – LPS-induced C-X-C chemokine
LPS – lipopolysaccharide
LV – less virulent
M – 123 bp DNA size marker
MAC – membrane attack complex
MCP-1 – monocyte chemoattractant protein-1
M-CSF – macrophage CSF
MHC – major histocompatibility complex
Mig – monocyte interferon-γ-inducible protein
MIP-2 – macrophage inflammatory protein-2
MLD – minimum lethal dose
MNGC – multinucleated giant cells
MNL – mononuclear leucocytes
mRNA – messenger RNA
MW – molecular weight
NK – natural killer
NO – nitric oxide
OD – optical density
O-PS – O-antigenic polysaccharide
PBML – peripheral blood mononuclear leucocytes
PBP – penicillin-binding protein
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PEC – peritoneal exudate cells
PFGE – pulse field gel electrophoresis
PHA – phytohaemagglutinin
PMNL – polymorphonuclear leucocyte
*po* – per os, oral
PPD – tuberculin purified protein derivative
PPS – protein polysaccharide
RANTES – regulated upon activation, normal T-cell expressed and secreted chemokine
RAPD – randomly amplified polymorphic DNA
RNA – ribonucleic acid
RNI – reactive nitrogen intermediates
ROI – reactive oxygen intermediates
rRNA – ribosomal RNA
RT – room temperature
RT-PCR – reverse transcription PCR
SBA – sheep blood agar
*sc* – subcutaneous
SEM - standard error of the mean
TNF–α – tumor necrosis factor alpha
$T_H1$ – T Helper (type) 1
$T_H2$ – T Helper (type) 2
Ttox – tetanus toxoid