IMMUNE RESPONSES IN *BURKHOLDERIA PSEUDOMALLEI* INFECTION, RELAPSE, REACTIVATION AND PROTECTION

Thesis submitted by
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DECLARATION

I declare that this thesis is my own work and has not been submitted in any other form for another degree of diploma at any university or institution of tertiary education. Reagents obtained from other organisations and researchers have been acknowledged where appropriate. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Sharon Marie Lazzaroni
January 2009

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DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human (1999)*, the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice (1997)*, the *James Cook University Policy on Experimentation Ethics*. *Standard Practices and Guidelines (2001)*, and the *James Cook University Statement and Guidelines on Reseach Practice (2001)*. The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval numbers A709, A987 and H1659).

Sharon Marie Lazzaroni
January 2009

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So here we are.....4 years and 2 kids later, finally finishing one of the biggest challenges in my life....my thesis. There are many people who have contributed to this work that need to be mentioned. Firstly, DSTO (David Proll and Jane McAllister) for constructing the DNA vaccines that were used in this study and secondly a huge thanks to Helen Smith from QHPS for carrying out the PFGE when our machine decided to call it quits. And of course thanks to the blood donors who selflessly gave us blood to carry out our studies.

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PUBLICATIONS

Publications resulting from this Thesis:

1. McAllister J, Gauci P, Lazzaroni S, Barnes J, Ketheesan N and Proll D. 2007. Asssessment of a DNA vaccine encoding *Burkholderia pseudomallei* bacterioferritin (technical report). *Defence Science and Technology Organisation*.

2. McAllister J, Lazzaroni S, Barnes J, Ketheesan N and Proll D. 2007. Development of DNA vaccines against *Burkholderia pseudomallei* (technical report). *Defence Science and Technology Organisation*.

3. Lazzaroni S M, Barnes J L, Williams N L, Govan B L, Norton R E, LaBrooy J T and Ketheesan N. 2008. Seropositivity to *Burkholderia pseudomallei* does not reflect the development of cell-mediated immunity. *Transactions of the Royal Society of Tropical Medicine and Hygiene (in press)*.

Presentations:

1. Lazzaroni S, Inglis T, Currie B, Ketheesan N, Norton R. 2004. The characterisation of *Burkholderia pseudomallei* isolates from the Townsville region using pulsed-field gel electrophoresis. 4th World Melioidosis Congress (poster presentation).

2. Lazzaroni S, Ketheesan N and Norton R. 2005. Molecular characterisation of *Burkholderia pseudomallei* isolates from Queensland. Australian Society for Microbiology Queensland Branch Meeting (oral presentation).

3. Lazzaroni S, Barnes J, Govan B, Norton R, Ketheesan N. 2006. Immune responses in melioidosis. Australian Institute of Medical Scientists 21st Annual Conference (oral presentation).

4. McAllister J, Lazzaroni S, Barnes J, Shahin S, Gauci P, Govan B, Ketheesan N, Proll D. 2006. Assessment of DNA vaccines against *Burkholderia pseudomallei*. Australian Society of Microbiology (poster presentation).

ABSTRACT

Burkholderia pseudomallei is the bacterium that causes the potentially fatal disease melioidosis. Endemic regions include South-east Asia and northern Australia. The immunopathogenesis of melioidosis is not fully understood as the bacterium can evade the host immune responses surviving within cells becoming apparent when the host is immunocompromised. Since *B. pseudomallei* is an intracellular organism, cell-mediated immune (CMI) responses are important in clearance and protection. The processes involved in the induction of a protective immune response is necessary to understand how the disease would progress and for developing vaccine strategies against *B. pseudomallei*. Therefore, the focus of the research in this thesis is to determine the extent of exposure to *B. pseudomallei* in northern Queensland and to characterise the CMI responses of seropositive healthy individuals from this endemic region. The immune responses involved in relapse and latent *B. pseudomallei* infection will also be determined by analysing the clonality of isolates from patients with recurrent melioidosis and developing a murine model of latency. The protective effect of DNA constructs of putative virulence factors will be studied in an experimental model of melioidosis.

Using the indirect haemagglutination assay, 1500 blood donors from northern Queensland were assessed for antibodies to *B. pseudomallei*. The results demonstrated 2.47% (n=37) of the population had an antibody response to *B. pseudomallei* with a titre of ≥1:40. Using these seropositive donors, the Melioidosis IgG Rapid Cassette test was evaluated as a potential screening test for melioidosis. The results found only 32% were positive using this rapid test. This study demonstrated that individuals living in endemic regions may have been exposed to *B. pseudomallei* and consequently produced an antibody response. By characterising the CMI response of these seropositive individuals it will be possible to determine whether these individuals develop protective immunity against the bacterium.

Lymphocyte proliferation assays and flow cytometry were used to characterise the CMI response in seropositive individuals (n=8). Lymphocytes were isolated and stimulated

with *B. pseudomallei* antigen (BpLy1) the proliferative response was assessed, as well as activation of T cell subsets and cytokine production. Results demonstrated no significant differences in the proliferative response of lymphocytes in seropositive individuals when compared to seronegative individuals. There were no significant differences observed in CD4+ and CD8+ populations, interferon (IFN)-γ or interleukin (IL)-4 production. These results did not demonstrate an induction of a CMI response following stimulation with BpLy1. Although these seropositive individuals may have produced an antibody response to *B. pseudomallei*, the exposure was not sufficient to produce a CMI response.

Clonality of *B. pseudomallei* isolates from patients who had relapsed with melioidosis was determined using pulsed-field gel electrophoresis (PFGE). Fragment patterns produced following PFGE demonstrated clonal isolates were responsible for each relapse episode in four patients. One patient who had relapsed with melioidosis nine times over a period of eight years was due to the same *B. pseudomallei* isolate recrudescing rather than infection with a different isolate. These results suggest that the bacterium is able to evade the immune responses and cause continual episodes of melioidosis, therefore, treatment of the disease may need to be reassessed.

The development of a latent model of melioidosis involved C57BL/6 mice being infected with a low dose of a low virulence strain of *B. pseudomallei* (NCTC13179). The mice were allowed to clear the infection over a period of 30 days with bacterial load, delayed-type hypersensitivity (DTH) and lymphocyte proliferation responses assessed at days 10, 20 and 30. These results demonstrated that a CMI response had been produced with significant differences seen in the DTH response and proliferative response of lymphocytes following stimulation with BpLy1. There was also an apparent clearance of *B. pseudomallei*, with negative culture from spleen and liver of infected animals. Mice were then immunosuppressesed with dexamethasone and monitored for survival, DTH and lymphocyte proliferation responses and bacterial load. Following immunosuppression, there was a significantly reduced immune response demonstrated by the DTH response. The proliferative response of lymphocytes in infected, immunosuppressed mice was not reflective of observations seen *in vivo* with no

differences observed in proliferation of lymphocytes, following stimulation with BpLy1, in infected, immunosuppressed mice compared to infected, non-immunosuppressed mice. Interestingly, results from bacterial loads demonstrated that by bacterial culture, *B. pseudomallei* was not present, however using real-time PCR (qPCR) *B. pseudomallei* DNA was detected in the spleens of both groups of mice. There was apparent reactivation of *B. pseudomallei* infection with a significant decrease in survival of infected mice that had been immunosuppressed and infected mice that had not been immunosuppressed. This aspect of the investigation demonstrates the initial development of an animal model of latent melioidosis.

Potential DNA vaccine constructs were evaluated using a BALB/c mouse model of experimental *B. pseudomallei* infection to determine their protective potential. The DNA vaccine constructs were developed against selective putative virulence factors of *B. pseudomallei*. Mice were vaccinated at three time points using a gene gun and subsequently challenged with a low virulence strain of *B. pseudomallei* (NCTC13179). Mice were monitored for survival and bacterial load. There were no significant differences seen in survival of mice vaccinated with different DNA constructs. When a combination of DNA constructs or CpG, an immunostimulator, was added to the vaccination regime, no significant differences were seen in survival of mice in these groups when compared to control mice. Splenic bacterial loads were not reduced in vaccinated groups when compared to control mice.

The results of this present study have provided further knowledge into host-pathogen interactions of *B. pseudomallei*. There is evidence that although there is exposure to *B. pseudomallei* in northern Queensland, detected by assessing antibody production, this exposure does not produce a CMI response which is essential in protection against the bacterium. Initial studies in determining whether *B. pseudomallei* persists within the host has been modelled in a murine model of latency. To produce a successful vaccine to protect against *B. pseudomallei* infection, further understanding of the immune responses and interaction of the bacterium is required.

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LIST OF ABBREVIATIONS

APC – antigen presenting cell
BpLy1 – *B. pseudomallei* lysate (NCTC13179)
BSA – bovine serum albumin
CDC – Centre of Disease Control and Prevention
CFA – culture filtrate antigen
cfu – colony forming units
CMI – cell-mediated immunity
CO_2 – carbon dioxide
Con A – concavalin A
Da – dalton
DC – dendritic cell
DNA – deoxyribonucleic acid
EDTA – ethylene diamine tetraacetic acid
ELISA – enzyme linked immunosorbent assays
FACS – fluorescent-activated cell sorting
FBS – foetal bovine serum
ICT – immunochromatographic test
ID – intradermal
IFA – immunofluorescent antibody assays
IFN – interferon
Ig – immunoglobulin
IHA – indirect haemagglutination assay
IL - interleukin
ip – intraperitoneal
in – intranasal
iNOS – inducible nitric oxide synthase
iv – intravenous
kb – kilobases
kDa – kilodalton
LPS – lipopolysaccharide
MAb – monoclonal antibodies
MEE – multilocus enqyme electrophoresis
MLST – multilocus sequence testing
MNL – mononuclear leukocytes
mRNA – messenger RNA
NF – nuclear transcription factor
NK – natural killer
NO – nitric oxide
OD – optical density
ODN – oligodeoxynucleotides
PAMP – pathogen-associated molecular patterns
PBS – phosphate buffered saline
PC – physical containment
PCR – polymerase chain reaction
PFGE – pulsed field gel electrophoresis
PHA – phytohaemagglutinin
PMA – phorbol myristate acetate
PMED – particle-mediated epidermal delivery
PMNL – polymorphonuclear leukocytes
PPD – purified protein derivative
PS - polysaccharide
qPCR – real-time PCR
RAPD – random amplified polymorphic DNA
RNA – ribonucleic acid
rRNA – ribosomal RNA
sc – subcutaneous
SI – stimulation index
TE – Tris-EDTA
TBE – Tris-Boric-EDTA
TES – Tris-EDTA sarcosine
Th – T helper
TLRs – toll-like receptors
TNF – tumour necrosis factor
TTSS – type III secretion system