The Development and Characterisation of a Murine Model of Type 2 Diabetes and *Burkholderia pseudomallei* Infection

Thesis submitted by
Kelly Ann HODGSON BSc (Hons I),
James Cook University
in December 2013

for the degree of Doctor of Philosophy
in the School of Veterinary & Biomedical Sciences
James Cook University

Dedicated to my family and friends:
for always believing that graduation day would come.

DECLARATION OF ELECTRONIC COPY

I, the undersigned, author of this work, declare that the electronic copy of this thesis provided to the James Cook University Library is an accurate copy of the print thesis submitted, within the limits of the technology available.

Kelly Hodgson
December 2013

STATEMENT OF ACCESS

I, the undersigned, author of this work, understand that James Cook University will make this thesis available for use within the University Library and, via the Australian Digital Theses network, for use elsewhere.

I understand that, as an unpublished work, a thesis has significant protection under the Copyright Act and I do not wish to place any further restriction on access to this work.

Kelly Hodgson
December 2013

STATEMENT OF SOURCES

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 Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Kelly Hodgson
December 2013

DECLARATION OF ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics, Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review (#A1556).

Kelly Hodgson
December 2013

ACKNOWLEDGEMENTS

This thesis is the culmination of the efforts, support and guidance of numerous people for whom I will be forever indebted. I am especially grateful to have had the opportunity to conduct this research, which I owe to my wonderful supervisors: Professor Ketheesan, Dr Jodie Morris and Associate Professor Brenda Govan. This research simply would not have been possible if not for their tireless support, encouragement and mentorship.

Thank you Ketheesan for helping me to overcome my self-limiting beliefs. It has been a long journey (*for both of us*) since I started a summer research project in 2008, which led to Honours and then a PhD. Thank you for your advice and reassurance on so many occasions. It has been an arduous but enjoyable experience and I am extremely grateful to have made it this far, which I owe especially to you. Thank you for providing the encouragement I needed along the way and sharing your insights on science and research. Thanks for pushing me when necessary and always inspiring me to do better. Thanks also for the opportunity to be involved in additional research work and tutoring.

I also owe a tremendous thank you to Jodie for taking the time to teach me many of the research techniques necessary for this project. Your dedication to research is truly inspiring and I have learned so much from working with you over the past few years. Thanks for assisting me during the major experiments, which I could not have managed without your help and often required working at all hours of the night. Thanks for teaching me to think critically about experimental design and the interpretation of data. Thanks especially for your advice and giving up your time during the editing of this thesis. I would also like to thank Brenda for always being there to offer support and advice during my candidature. Thanks for providing some much needed perspective on experiments during the more difficult times and also for your contribution to data interpretation.

I am so grateful to have had the opportunity to work with (and learn from) such a talented and collaborative team of researchers within the Infectious Diseases and Immunopathogenesis Research Group. Thanks for making the past few years so

enjoyable. Thanks especially to Davina for always offering technical advice when needed and assistance with laboratory protocols. Thanks to Natasha for teaching me the basics of immunofluorescence microscopy and keeping me company in the office. Thanks also to Jacqui and Anne for taking care of my mice on the odd occasion. Thanks to Donna for her assistance with the measurement of lipoproteins and to Laurie for his assistance with histological processing and staining techniques.

I would also like to thank the Graduate Research School at James Cook University for a supplementary research grant and the Queensland Tropical Health Alliance for a travel award to attend the annual conference in Brisbane (2012). I am also grateful for the financial support afforded by an Australian Postgraduate Award, which made it possible for me to complete this degree.

Finally, I would like to thank my family and friends for their constant support, encouragement, advice and belief that this was possible. Thanks especially to my partner Kyle, who had to endure my wrath during the *never-ending* writing of this thesis. Thanks Kyle for your patience, support, providing a constant supply of caffeine and ultimately, never giving up hope that I would not be a professional student forever!

PUBLICATIONS

Publications resulting from this Thesis:

Hodgson, K. A., Govan, B. L., Ketheesan, N., and Morris, J. L. The role of glutathione deficiency in a murine model type 2 diabetes and melioidosis comorbidity. *In preparation*. For submission to *Infection and Immunity*.

Hodgson, K. A., Govan, B. L., Ketheesan, N., and Morris, J. L. The double burden of type 2 diabetes and infectious diseases. *In preparation*. For submission to *Immunology*.

Hodgson, K. A., Govan, B. L., Walduck, A. K., Ketheesan, N., and Morris, J. L. (2013). Impaired early cytokine responses at the site of infection in a murine model of type 2 diabetes and melioidosis comorbidity. *Infection and Immunity, 81*(2), 470-477.

Hodgson, K., Govan, B., Ketheesan, N., and Morris, J. (2013). Dietary composition of carbohydrates contributes to the development of experimental type 2 diabetes. *Endocrine, 43*(2), 447-451.

Hodgson, K. A., Morris, J. L., Feterl, M. L., Govan, B. L., and Ketheesan, N. (2011). Altered macrophage function is associated with severe Burkholderia pseudomallei infection in a murine model of type 2 diabetes. *Microbes and Infection / Institut Pasteur, 13*(14-15), 1177-1184.

Additional publications:

Morris, J. L., Hodgson, K. A., and Ketheesan N. (2012). Section VII.4 Development of protection. In N. Ketheesan (Ed.), *Melioidosis - A Century of Observation and Research*. The Netherlands: Elsevier B. V., pp 282-299.

ABSTRACT

Infections with *Burkholderia pseudomallei*, the causative agent of melioidosis, have been increasing steadily for the past two decades, earning its classification as an emerging tropical disease. Melioidosis is highly endemic in Northern Australia and Northeast Thailand, where annual incidence is approaching 50 cases per 100,000 individuals. In Northeast Thailand, melioidosis is the most common cause of death after infection with human immunodeficiency virus (HIV) and tuberculosis. Although rapid diagnosis, the availability of appropriate antibiotic therapy and intensive care facilities has recently improved outcomes from melioidosis in Australia, mortality rates remain high in many rural regions. Furthermore, therapeutic management of melioidosis involves prolonged antibiotic regimes, which do not always clear *B. pseudomallei* infection, frequently leading to relapse or reactivation of melioidosis following cessation of treatment. Therefore, the course of melioidosis is often protracted and can involve a range of clinical sequelae due to the protean manifestations of the infection.

A close association exists between melioidosis and type 2 diabetes (T2D). T2D has consistently been identified as the most significant risk factor predisposing to melioidosis. More than half of patients with melioidosis have pre-existing T2D and of those that do not, almost half have risk factors for pre-diabetes, including hypertension, dyslipidaemia and increased body mass index (BMI). Undoubtedly, the continued global prevalence of T2D will contribute to the increasing emergence of melioidosis as a tropical disease of significance. There is no vaccine available for melioidosis and no vaccine candidates have produced sterilising immunity, despite ongoing research. Furthermore, acquired antibiotic resistance by many bacteria, including *B. pseudomallei*, would have a significant impact on current therapeutic regimes, which rely on the availability of effective antibiotics and could subsequently result in increased mortality rates.

Future improvements in the therapeutic management of melioidosis will depend heavily on fundamental research to understand the host-pathogen interactions contributing to disease progression and how these are altered by T2D, leading to

increased susceptibility. *In vivo* experiments are essential for this research, due to the complexity of interactions between the metabolic and immune systems. While *in vitro* experiments can be useful for understanding individual cell functions and interactions with bacteria, it is difficult to interpret the overall effect of these processes in the dynamic physiological environment of the host without comparative *in vivo* studies. Therefore, an animal model of T2D and comorbid *B. pseudomallei* infection is essential for the investigation of fundamental early immune responses leading to the divergence in disease progression between individuals with and without T2D. To this end, several murine models of T2D were investigated in this study to develop a suitable animal model that can be used to identify the mechanisms contributing to comorbid *B. pseudomallei* infection.

At the time of this study, the leptin signalling deficient $Lept^{db}$ monogenic model of T2D was widely used in research to understand disease pathogenesis of T2D. $Lept^{db}$ mice are available on either a C57BL/6 or BKS genetic background, believed to result in distinct metabolic phenotypes. Therefore, metabolic parameters including body mass, blood glucose, lipid profiles and glucose tolerance were compared. Although the $Lept^{db}$ mutation resulted in comparable body mass and glucose intolerance between background strains, BKS homozygous (*db/db*) mice developed severe hyperglycaemia, while C57BL/6 *db/db* mice tended to maintain moderate blood glucose levels. Both strains of *db/db* mice were more susceptible to subcutaneous infection with *B. pseudomallei* than heterozygous (*db/+*) mice and the increased severity of infection was associated with decreased blood glucose, a phenomenon that frequently occurs in sepsis. Importantly, the BKS $Lept^{db}$ monogenic model of T2D is widely available and requires less time to develop overt hyperglycaemia relative to other polygenic murine models of T2D, enhancing its utility for this research.

Growth of *B. pseudomallei* and mortality from the infection was greater in *db/db* (diabetic) compared to *db/+* (non-diabetic) mice. However, these results need to be interpreted with caution due to the confounding complications of leptin signalling deficiency, which could be implicated in their increased susceptibility to infection. This is arguably the most significant limitation of this model. It is also a poor model in terms of the aetiology of T2D in humans, which is rarely caused by leptin

deficiency and other single gene mutations. Therefore, we sought to develop and characterise a murine model more reflective of the natural aetiopathology of T2D and subsequent infection with *B. pseudomallei*. A polygenic, diet-induced model of T2D is more reflective of the aetiopathology of T2D in humans and would arguably provide a more clinically relevant and comprehensive understanding of the immunological basis of melioidosis and comorbid T2D. Developing a murine model of diet-induced T2D was complicated by the vast inconsistencies in the literature, possibly owing to the variability and incomplete description of diets, genetic backgrounds, genders, age and duration of feeding regimes.

In this study, male mice were found to be more susceptible to metabolic complications induced by consuming a high fat diet (HFD) in comparison to female mice. This is similar to the gender susceptibility that has been documented in clinical T2D, possibly related to the involvement of sex hormones in regulating insulin sensitivity, though the mechanisms are incompletely understood. Despite the increased glucose intolerance in male B6D2F1 mice, blood glucose levels were only moderately elevated after 20 weeks of consuming a HFD. Hyperglycaemia is the most important clinical criteria for diagnosis of T2D in humans. Therefore, metabolic parameters were compared to male C57BL/6 mice on an identical HFD-feeding regime. Whilst body weight gain in B6D2F1 mice exceeded that in C57BL/6 mice fed a HFD, glucose intolerance was exacerbated in C57BL/6 mice. The genetic predisposition towards development of T2D in C57BL/6 mice at a lower degree of adiposity is clinically significant since most melioidosis patients, particularly in Asia, have T2D without extreme obesity. Optimisation of the HFD-feeding regime led to further increases in blood glucose levels in C57BL/6 mice, which was associated with abnormal albumin/creatinine ratio and increased baseline levels of inflammation, consistent with clinical T2D.

In the current study, diet-induced T2D in C57BL/6 mice was found to be the most reflective model of clinical T2D, in terms of aetiology and disease pathogenesis, and as such was selected for subsequent experiments that investigated differences in cytokine responses and disease progression of *B. pseudomallei* infection. Diabetic mice were significantly more susceptible to *B. pseudomallei* infection compared to non-diabetic littermates. Increased mortality in diabetic mice was associated with

greater dissemination and growth of *B. pseudomallei* in the spleen and liver within the first 24 hours post-infection and bacteraemia from day 3 post-infection, preceding death. Fatal outcome in diabetic mice was paralleled with delayed cytokine responses compared to non-diabetic littermates. At 12 hours post-infection, levels of TNF-α, IL-12 and IFN-γ were reduced in spleen of diabetic mice relative to non-diabetic mice. Impaired killing of *B. pseudomallei* by macrophages from diabetic mice was also observed following *in vitro* studies, compared to macrophages from non-diabetic mice. Given the essential role of macrophages in immune defence against *B. pseudomallei* infection, impaired macrophage function may contribute to increased *B. pseudomallei* growth and poor disease outcome observed in diabetic mice.

Macrophage function is influenced by the intracellular concentration of antioxidants such as glutathione (GSH), which regulates the cellular redox balance by cycling between reduced (GSH) and oxidised states (GSSG). In addition to the ability of GSH to regulate immune responses, there is also evidence that GSH has direct antimicrobial toxicity. The mechanisms for this and the sensitivity of *B. pseudomallei* to GSH are not known. Oxidative stress, defined by decreased GSH/GSSG levels, is central to the pathogenesis of T2D. However, the role of this in susceptibility to bacterial infections is unclear. Therefore, GSH concentrations were measured in blood and tissues of diabetic and non-diabetic mice prior to, and following, infection with *B. pseudomallei*. At baseline (uninfected), the GSH/GSSG ratio was significantly lower in diabetic mice compared to non-diabetic mice, primarily due to increased GSSG, indicative of oxidative stress.

The intracellular balance of GSH/GSSG levels in macrophages and other immune cells regulates many signalling pathways, including cytokine production. It was proposed that altered GSH/GSSG redox balance could be involved in the dysregulation of cytokine responses described in diabetic mice following infection with *B. pseudomallei*. GSH modulators were used to deplete or restore GSH in non-diabetic and diabetic mice, respectively, to determine the effect on disease progression of *B. pseudomallei* infection. The depletion of GSH in non-diabetic mice, prior to infection with *B. pseudomallei* and during the first 24 hours post-infection, did not increase susceptibility to infection. However, it is not known if

repletion of GSH occurred following the first 24 hours post-infection, which may have been sufficient to control the infection. Further research should investigate whether increased duration of treatment or use of other GSH modulators to deplete GSH influences susceptibility to *B. pseudomallei* infection. Furthermore, diabetic mice treated with a cysteine derivative to restore GSH did not improve overall disease outcome from melioidosis, nor did this reduce *B. pseudomallei* growth. However, it is again recognised that treatment schedules may require further optimisation, particularly since a variety of synthetic pathways involved in GSH metabolism appear to be altered in T2D. Further research is warranted to investigate the potential for GSH derivatives as more suitable treatment options.

In summary, this thesis describes and characterises the first polygenic murine model of T2D and comorbid *B. pseudomallei* infection. This novel model will be useful for further fundamental and translational research exploring the pathogenic mechanisms that link these two significant diseases. Identifying the mechanisms responsible for the increased susceptibility of people with T2D to melioidosis will enable the development of improved therapeutic strategies to reduce morbidity and mortality in a highly susceptible population. In addition to the value of this murine model for understanding the pathogenesis of the most important comorbidity of melioidosis, it may also be invaluable for research focusing on other infectious comorbidities of T2D, such as tuberculosis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACR	albumin creatinine ratio
ADA	American Diabetes Association
AGE	advanced glycation end-products
AIHW	Australian Institute of Health and Welfare
ANOVA	analysis of variance
API	analytical profile index
ATM	adipose tissue macrophage
ATP	adenosine triphosphate
AUC	area under glucose curve
BCG	bacillus Calmette-Guérin
BMI	body mass index
BSO	*L*-buthionine-sulfoximine
°C	degrees Celsius
CBA	cytometric bead array
CD	cluster of differentiation
CFU	colony forming units
CMI	cell-mediated immunity
COPD	chronic obstructive pulmonary disease
COX2	cyclooxygenase 2
CVD	cardiovascular disease
CXC5	CXC motif ligand 5
DC	dendritic cell
DEM	diethyl maleate
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
FFA	free fatty acids
γ-GCL	gamma-glutamylcysteine ligase
g	relative centrifugal force
G-CSF	granulocyte colony stimulating factor
GI	glycaemic index
GLUT4	glucose transporter 4
GSH	reduced glutathione

GSSG	oxidised glutathione
GTT	glucose tolerance test
HbA1c	glycosylated haemoglobin
HFD	high fat diet
HF/HGD	high fat, high glycaemic index diet
HIV	human immunodeficiency virus
H & E	haematoxylin and eosin
IκB	inhibitor of NFκB
ID_{50}	infectious dose (50%)
IDF	International Diabetes Federation
IFG	impaired fasting glucose
IFN	interferon
Ig	immunoglobulin
IGT	impaired glucose tolerance
IL	interleukin
IRS	insulin receptor substrates
LB4	leukotriene B4
LN	lymph node
LPS	lipopolysaccharide
M1	classically activated (proinflammatory) macrophages
M2	alternatively activated (anti-inflammatory) macrophages
MCP-1	monocyte chemoattractant protein-1
MNGC	multinucleated giant cells
mRNA	messenger ribonucleic acid
NAC	*N*-acetyl-*L*-cysteine
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NET	neutrophil extracellular traps
NF-κB	nuclear factor kappa B
NHMRC	National Health and Medical Research Council
NK	natural killer
NLRC4	NOD-like receptor caspase-activating domain 4
NLRP3	NOD-like receptor protein 3
NO	nitric oxide

OCT	optimal cutting temperature
OD	optical density
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PKC	protein kinase C
RAGE	receptor for advanced glycation end products
RBP4	retinol binding protein 4
ROS	reactive oxygen species
SAT	subcutaneous adipose tissue
SBA	sheep's blood agar
SEM	standard error of the mean
SSA	5-sulfosalicylic acid
T1D	type 1 diabetes
T2D	type 2 diabetes
T3SS	type III secretion system
TCA	tricarboxylic acid cycle
T_H0	naïve T helper cell
T_H1	T helper type 1
T_H2	T helper type 2
TLR	toll like receptor
TNF-α	tumour necrosis factor alpha
T_{reg}	regulatory T cell
TSB	tryptic soy broth
TTH	The Townsville Hospital
VAT	visceral adipose tissue
WHO	World Health Organisation

CHAPTER 1
GENERAL INTRODUCTION

Melioidosis is a tropical infection caused by the Gram negative soil bacterium, *Burkholderia pseudomallei.* The infection is acquired via inhalation or ingestion of *B. pseudomallei* or contamination of percutaneous abrasions with *B. pseudomallei* from soil or water (Currie *et al.* 2000b; Suputtamongkol *et al.* 1994). Since its first description in Burma a century ago, melioidosis has become a disease of public health significance in Southeast Asia and Northern Australia. Melioidosis is the most common cause of fatal community-acquired bacteraemic pneumonia and sepsis in the Northern Territory of Australia and the third most common infectious cause of death in Thailand (Currie *et al.* 2000a; Douglas *et al.* 2004; Limmathurotsakul *et al.* 2010b).

The incidence of melioidosis has been increasing incrementally since prospective clinical and epidemiological studies began in 1986 (Dance 2012; Limmathurotsakul *et al.* 2010b; Suputtamongkol *et al.* 1994). The most recent prospective cohort studies have shown that cases of melioidosis have trebled in Thailand and in the Northern Territory of Australia (Currie, Ward & Cheng 2010; Limmathurotsakul *et al.* 2010b). While there have been improvements in diagnostic techniques and subsequent detection of melioidosis during this period, there has also been dramatic increases in the prevalence of chronic, non-communicable diseases like type 2 diabetes (T2D). As the most significant risk factor of melioidosis, T2D substantially increases the susceptibility of individuals to *B. pseudomallei* infection (Currie, Dance & Cheng 2008).

Melioidosis is an extraordinarily clinically diverse disease. Infections can range from chronic or asymptomatic forms, which can remain latent for decades prior to activation, to acute fulminant disease with septic shock (Cheng & Currie 2005). Furthermore, the extensive organ tropism of *B. pseudomallei* accounts for the broad range of clinical presentations of melioidosis, which can include pneumonia, cutaneous or visceral abscesses, septic arthritis, osteomyelitis, genitourinary infections particularly prostatic abscesses, suppurative parotitis, bacteraemia and meningo-encephalitis (Currie, Chaowagul & Cheng 2012). This clinical diversity

impedes diagnosis, which is one of the strongest determinants of outcome due to the inherent resistance of *B. pseudomallei* to standard empiric antibiotic regimens (Currie, Chaowagul & Cheng 2012). However, even for patients who receive prolonged antimicrobial treatment and survive the initial infection, relapse of melioidosis is a significant complication (Limmathurotsakul *et al.* 2008; Limmathurotsakul *et al.* 2006).

Mortality rates of melioidosis are highly variable depending on disease severity, access to diagnostic and therapeutic facilities, and underlying risk factors. In Thailand, the average mortality rate of melioidosis is 49%, and although improved diagnosis and medical management of patients with melioidosis has reduced the case-fatality ratio in Australia, it remains between 19-25% (Cheng, Dasari & Currie 2004; Limmathurotsakul *et al.* 2006; Malczewski *et al.* 2005; White 2003). Furthermore, mortality rates in melioidosis patients with septic shock still range from 50% in Singapore, to over 90% in Thailand (Chan *et al.* 2005; Cheng *et al.* 2007; Cheng *et al.* 2008; Limmathurotsakul *et al.* 2006).

Although melioidosis can occur in otherwise healthy individuals, a number of predisposing host risk factors have been identified, including T2D, alcoholism, renal disease, chronic lung disease and immunosuppression (Cheng & Currie 2005; Leelarasamee & Bovornkitti 1989). While uncertainty remains over the mechanisms underlying increased susceptibility, fatalities occur predominantly in patients with the aforementioned predisposing risk factors, the most significant of which is T2D (Currie *et al.* 2004; Suputtamongkol *et al.* 1999). Approximately 37-42% of melioidosis patients in Australia, 60% in Thailand and 76% in India have concomitant T2D (Malczewski *et al.* 2005; Suputtamongkol *et al.* 1999; Vidyalakshmi *et al.* 2007). The risk of acquiring melioidosis for people with T2D is approximately 13 fold greater than people without this risk factor, which is a higher relative risk than other infectious comorbidities of T2D, such as tuberculosis (Baker *et al.* 2011b; Currie *et al.* 2004; Suputtamongkol *et al.* 1994). The significant association between T2D and melioidosis underscores the need for research to understand the pathogenesis of this comorbidity, to improve therapeutic management of melioidosis in populations most at risk.

One of the most pressing epidemics of the 21st Century is T2D (AIHW 2008; IDF 2011). As public health is challenged by the advent of non-communicable, chronic diseases like T2D, it becomes even more pertinent to address the double burden of associated communicable diseases such as melioidosis (IDF 2011). Australian Aboriginals have one of the highest rates of T2D in the world, while rates of T2D have increased 3 to 5-fold in Thailand and India over the preceding few decades (McDermott, Li & Campbell 2010; Yoon *et al.* 2006). As T2D continues to increase in melioidosis endemic areas, so too does the need for novel therapeutic strategies to improve outcomes from melioidosis in a highly susceptible population.

Murine models have already contributed significantly to our understanding of the immunopathogenesis of melioidosis and protective host immune responses during *B. pseudomallei* infection (Barnes & Ketheesan 2005; Barnes *et al.* 2001; Barnes, Williams & Ketheesan 2008; Leakey, Ulett & Hirst 1998; Ulett, Ketheesan & Hirst 2000a, 2000b). An animal model of T2D and comorbid *B. pseudomallei* infection is essential to unravel the complex immunopathogenic mechanisms governing this comorbidity and to facilitate the development and evaluation of the efficacy of novel therapeutic regimes. This provided the impetus for developing the first model of T2D and comorbid *B. pseudomallei* infection, enabling fundamental characterisation of the host-pathogen interactions *in vivo* during the very early stages of infection. Therefore, the aims of this thesis were:

Aim 1. To develop and characterise a monogenic murine model of T2D and *B. pseudomallei* infection using leptin receptor deficient ($Lept^{db}$) mice (Chapter 4).

Aim 2. To develop and characterise a polygenic murine model of T2D and *B. pseudomallei* infection. This included the:

a) comparison of metabolic profiles of B6D2F1 male and female mice after a high fat diet (HFD)-feeding regime (Chapter 5).
b) comparison of metabolic profiles of male B6D2F1 and C57BL/6 mice fed a HFD and their outcome following *B. pseudomallei* infection (Chapter 6).
c) comparison of metabolic profiles of male C57BL/6 mice fed a HFD with a low or high glycaemic index (GI) and their outcome following *B. pseudomallei* infection (Chapter 7).

Aim 3. To compare the early dissemination of *B. pseudomallei* and differences in inflammatory cytokine responses within the first 24 hours post-infection between diabetic and non-diabetic mice (Chapter 8).

Aim 4. To compare glutathione (GSH) levels in tissues of diabetic and non-diabetic mice and determine if GSH modulation influences survival following *B. pseudomallei* infection (Chapter 9).

CHAPTER 2
LITERATURE REVIEW

2.1 Melioidosis

2.1.1 Historical perspective

A century ago, Whitmore and Krishnaswami (1912) described a 'glanders-like' disease occurring in Rangoon, Burma. The novel disease was later termed melioidosis by Stanton and Fletcher, a name derived from the Greek etymology of 'melis' (a diseased condition resembling glanders), in lieu of its similarity to the equine infectious disease, glanders (1921). The similarities between melioidosis and glanders have since been confirmed with the advent of molecular techniques, which have indicated that the two aetiological agents are indeed closely related (Godoy *et al.* 2003; Holden *et al.* 2004; Nierman *et al.* 2004).

Since its first description, the Gram negative bacteria that causes melioidosis has been known by a variety of names. Some of these include *Bacillus pseudomallei*, *Bacillus whitmorii*, *Malleomyces pseudomallei* and *Pseudomonas pseudomallei* (Cheng & Currie 2005). *Burkholderia pseudomallei*, the current name as it stands today, originated in 1992 (Yabuuchi *et al.* 1992). After its initial description in Burma, reports of melioidosis in humans and animals began to emerge from Malaysia, Singapore, Vietnam and Indonesia between 1913 and 1929 (Cheng & Currie 2005; Dance 1991; White 2003). The first documented case of melioidosis in Australia was in 1949, in sheep at Winton in Western Queensland (Cottew, Sutherland & Meehan 1952). A year later, the first human case of melioidosis was described in Townsville and in 1960, the first human case in the Northern Territory was reported (Crotty *et al.* 1963; Rimington 1962). Since then, melioidosis has become an important infectious disease in Northern Australia.

2.1.2 Geographical distribution

B. pseudomallei is a Gram negative environmental bacteria, located in soil and surface water in tropical regions throughout the world (Cheng & Currie 2005). The

geographical location of *B. pseudomallei* endemnicity is extraordinarily diverse, consisting of highly developed regions, along with some of the world's least developed countries, as illustrated in Figure 2.1. Melioidosis is frequently observed in Malaysia and Singapore and has been described in China, Taiwan, Brunei, Vietnam, Laos, Cambodia, Philippines, Papua New Guinea, Fiji, New Caledonia, Sri Lanka, Bangladesh and Pakistan (Cheng & Currie 2005; Le Hello *et al.* 2005; Leelarasamee 2000; Liu *et al.* 2006; Puthucheary, Parasakthi & Lee 1992; Wuthiekanun *et al.* 2008; Yap *et al.* 1991). There is also increasing recognition of melioidosis in India (Barman, Kaur & Kumar 2013; Saravu *et al.* 2010; Vidyalakshmi *et al.* 2012). The true geographical range and incidence may be under-represented, due to asymptomatic or misdiagnosed infections (Cheng & Currie 2005).

2.1.3 Incidence

Melioidosis has been classified as an emerging disease (Dance 2012). Epidemiological studies suggest that the incidence of melioidosis has been steadily increasing since 1981 (Currie *et al.* 2000a; Dance 2012; Rode & Webling 1981). Between 1989 and 1999, the average annual incidence of melioidosis in the Northern Territory of Australia was 16.5 per 100,000 individuals (Currie *et al.* 2000b). However, the annual incidence was more than double this during the 1997 monsoon, which was marked by high rainfall and two cyclonic events (Currie *et al.* 2000b). Meanwhile, an incidence of 40 cases per 100,000 individuals has been described in the Torres Strait in Northern Queensland (Faa & Holt 2002). The annual incidence of melioidosis in Ubon Ratchathani province, Northeast Thailand, was reported to be 4.4 cases per 100,000 individuals (Currie *et al.* 2000b; Dance 1991; Suputtamongkol *et al.* 1994). However, this is likely to be an underestimate of the true burden of melioidosis due to inadequate access to hospitals in rural areas and misdiagnosis based on similarities to clinical features of other infectious diseases (Currie *et al.* 2000b; Dance 1991; Suputtamongkol *et al.* 1994).

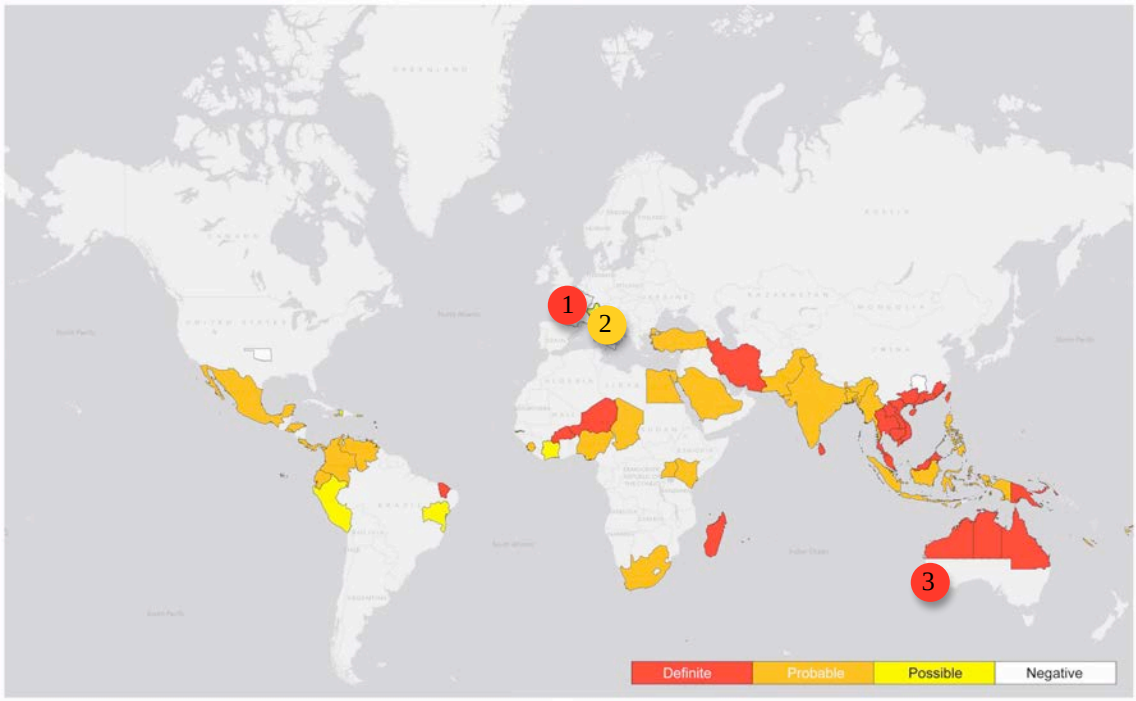

Figure 2.1 Global distribution of *B. pseudomallei*
Proposed global distribution of *B. pseudomallei*, as adapted from Limmathurotsakul *et al.* (2013). Definite regions are defined as where *B. pseudomallei* has been isolated from environmental samples, probable regions indicate areas where there is clinical evidence to support acquisition of melioidosis and possible regions are where *B. pseudomallei* has thought to have been isolated but identification was not confirmed and there is no evidence for acquisition of melioidosis. Numbers 1, 2 and 3 designate outbreaks of melioidosis in Paris, Italy and Southwest Western Australia, respectively.

Currently, melioidosis is a significant public health concern in Northern Australia and Southeast Asia (Currie, Chaowagul & Cheng 2012; Dance 2012). The greatest number of cases of melioidosis, between 2,000 to 3,000 annually, are documented in Thailand, due to the higher population density compared to Australia (Chaowagul *et al.* 1989; Currie *et al.* 2000a; Leelarasamee 2000; Vuddhakul *et al.* 1999). Melioidosis is the third most common infectious cause of death in Thailand and the most common cause of fatal community-acquired bacteraemic pneumonia and sepsis in the Northern Territory of Australia (Currie *et al.* 2000a; Douglas *et al.* 2004; Limmathurotsakul *et al.* 2010b).

There is a widely recognised link between the incidence of melioidosis and high annual rainfall (Suputtamongkol *et al.* 1994). In Australia and Thailand, 75-85% of melioidosis cases occur during the monsoonal wet season (Currie *et al.* 2000b; Suputtamongkol *et al.* 1994). This is attributed to the increased probability of human exposure to *B. pseudomallei* in soil and groundwater after heavy rainfall events (Baker *et al.* 2011a). Additionally, *B. pseudomallei* is able to persist in the environment despite harsh conditions such as low nutrient levels, low pH and high temperatures (Dejsirilert *et al.* 1991; Wuthiekanun, Smith & White 1995). It is surmised that global warming and subsequent increases in severity of climatic events will favour expansion of the ecological niche of *B. pseudomallei*, potentially extending the geographic range of endemnicity (Dance 2012). Increased urbanisation of, and population growth, in tropical regions will also contribute to the continued emergence of melioidosis as an important infectious disease (Inglis & Sousa 2009).

2.1.4 Clinical pathology

Melioidosis is a clinically diverse disease with a wide spectrum of manifestations. Several infectious routes have been proposed for the acquisition of melioidosis and may contribute to the variety of clinical presentations, as illustrated in Figure 2.2. Percutaneous inoculation of skin abrasions and wounds exposed to contaminated soil and surface water is presumed to be the predominant route of infection with *B. pseudomallei* (Currie *et al.* 2000b). Inhalation and ingestion have also been implicated as potential routes of infection with *B. pseudomallei*, however the importance of these is still under investigation (Cheng *et al.* 2003; Currie 2003; Currie & Jacups 2003; Mayo *et al.* 2011). Continued research on the routes of infection of *B. pseudomallei* is important to enable the implementation of public health measures to minimise acquisition of *B. pseudomallei* infection in endemic regions (Limmathurotsakul & Peacock 2011). However, due to the widespread environmental persistence of *B. pseudomallei*, it is unlikely that complete prevention of melioidosis will ever be achieved.

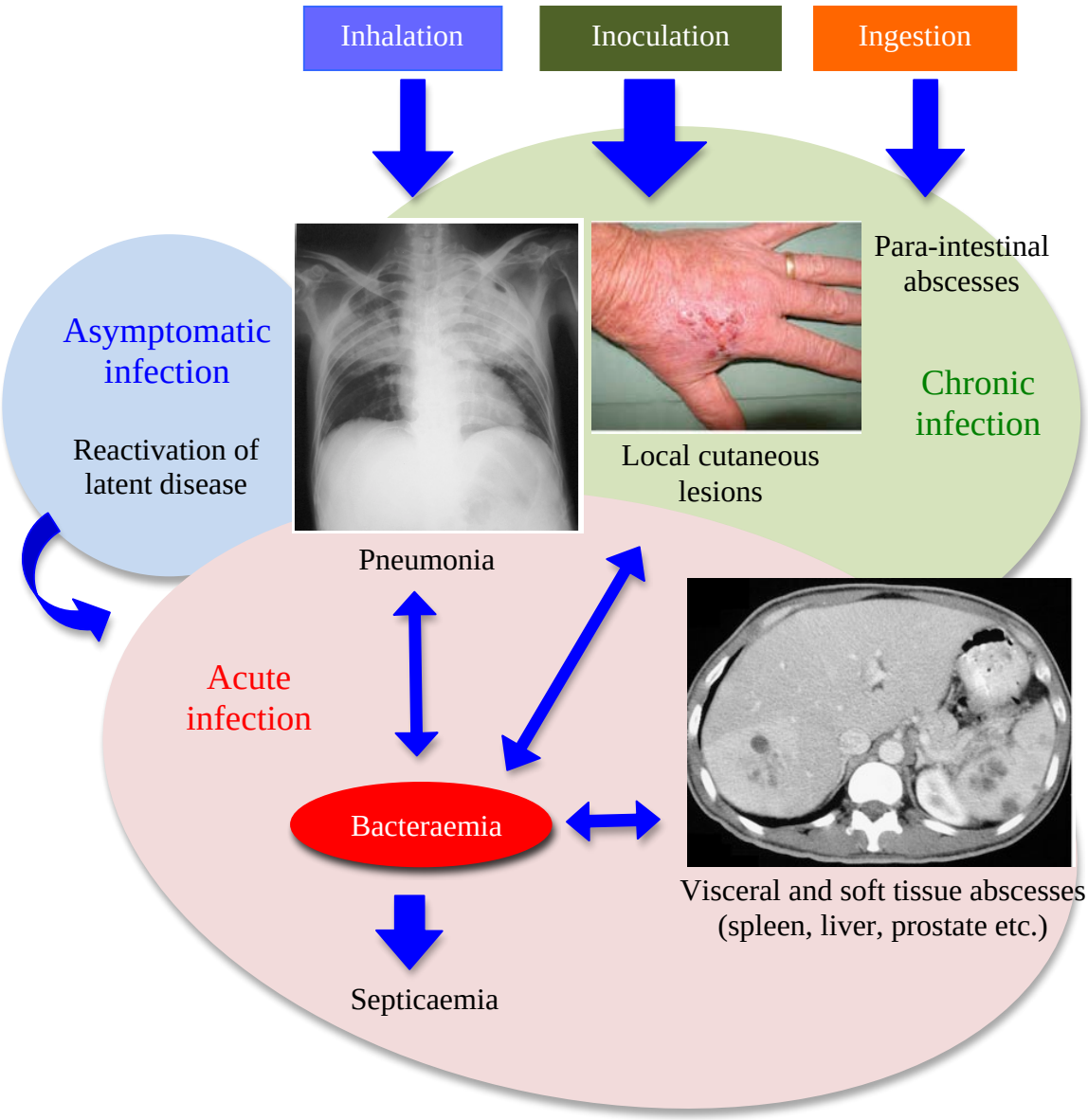

Figure 2.2 Acquisition and proposed relationship with clinical features of melioidosis
Melioidosis is a clinically diverse disease that can be acquired through a variety of routes and has a complex pathogenesis that can involve multiple organs and manifest as acute, chronic or asymptomatic (latent) infections. Images sourced from Burivong *et al.* (2012), Muttarak *et al.* (2009) and Gibney, Cheng and Currie (2008).

While pulmonary complications are a common feature of melioidosis, perhaps providing support for inhalation as a predominant route of infection, pneumonia may also arise from secondary haematogenous spread of *B. pseudomallei* (Figure 2.2) (Cheng *et al.* 2013; Currie, Ward & Cheng 2010; Tan *et al.* 2008). Additional clinical manifestations of melioidosis frequently observed in Australia and Thailand

are listed in Table 2.1. Variations in clinical presentation may be due to differences in strain virulence and tropism, route of acquisition, size of the inoculum and/or host immune status (Currie, Ward & Cheng 2010).

Table 2.1 Clinical spectrum of melioidosis in Australia and Thailand

Clinical Presentation	Australia (%)[a]	Thailand (%)[b]
Pneumonia	51	45
Bacteraemia	55	58
Septic shock	21	NR
Genitourinary infection	14	7
Skin or soft tissue infection	13	13
Neurological	3	3
Splenic abscess	4	2
Liver abscess	2	7
Prostatic abscess	18*	0.3
Parotid abscess	0	2
Septic arthritis and/or osteomyelitis	4	5
Mortality	**14**	**38-61**

Updated from Cheng and Currie (2005) with reference to Currie, Ward and Cheng (2010). [a] Royal Darwin Hospital Series (*n* = 540); [b] Infectious Diseases Association of Thailand Series (*n* = 686); * of males; NR – not recorded.

In addition to the wide variety of clinical symptoms, melioidosis can vary in degree of severity, which can be broadly subdivided into acute, chronic, subclinical and latent or asymptomatic infections, described in Table 2.2 (Cheng & Currie 2005). Acute infection is the most severe manifestation of melioidosis, which can rapidly progress to fulminating septicaemia, within 24 hours of hospital admission, and has a mortality rate of up to 50% (Cheng *et al.* 2007; Currie, Ward & Cheng 2010; Gan 2005). In contrast, chronic melioidosis can persist for decades as localised abscesses in almost any organ, causing a variety of symptoms depending on the organ/s affected (Cheng & Currie 2005). In reality, these classifications of melioidosis severity are not so clear-cut, and chronic, subclinical and asymptomatic infections

have the potential to develop into acute disease after prolonged periods of quiescence. Reactivation usually follows an immunosuppressive event and has been documented as long as 62 years after initial exposure (Ngauy *et al.* 2005). Intensive research has been focused on identifying the site and mechanisms of latency, which remain to be defined (Gan 2005). Importantly, the lack of cardinal symptoms and potential involvement of virtually any organ substantially complicates diagnosis of melioidosis.

Table 2.2 Range in severity of melioidosis

Clinical Features	Acute	Chronic	Asymptomatic
Progression	Rapid	Slow	Nil
Presentation	Acute septicaemia and/or pneumonia	Chronic pneumonia or extrapulmonary involvement*	Identified by serological studies
Septic shock	Frequent	Rare	Nil
Mortality	6-25% (sepsis 49-84%)	4%	Nil

Adapted from Burivong *et al.* (2012). * eg. visceral abscesses, skin lesions, osteomyelitis.

2.1.5 Diagnosis and treatment

Rapid diagnosis and effective antibiotic treatment are the key determinants for recovery from *B. pseudomallei* infection. Colloquially termed 'the great imitator,' melioidosis is non-discriminative from other pyogenic infections based on signs and symptoms, making preliminary diagnoses difficult (Vishnu Prasad *et al.* 2012). Since *B. pseudomallei* exhibits resistance to a range of first- and second-generation antibiotics, definitive diagnosis is critical for commencement of effective treatment.

The 'gold standard' for identification of *B. pseudomallei* relies on colony morphology on Ashdown agar, a selective and differential medium, combined with standard biochemical tests (Limmathurotsakul *et al.* 2010a). Commercial kit-based systems, such as the Analytical Profile Index (API)-20E and API-20NE and Vitek® 1 have also been demonstrated to have a reliably high success rate for identification of

B. pseudomallei: 98%, 99% and 99%, respectively (Lowe, Engler & Norton 2002). The major limitation of these methods of identification is the time required for culture and definitive diagnosis (at least 48 hours), by which time 50% of septicaemic melioidosis patients may have succumbed to infection due to the rapid course of acute disease (Anuntagool *et al.* 2000; Currie, Ward & Cheng 2010). Serological techniques are also problematic due to the high background seropositivity in the general population of endemic regions (Limmathurotsakul & Peacock 2011). Relative cost and required expertise is an important factor to consider in the development of new diagnostic tests, since this may severely limit their practicality in many resource poor regions where *B. pseudomallei* is endemic. The development of rapid immunoassays with high specificity and sensitivity for *B. pseudomallei* antigens is an active area of research and shows great promise in expediting diagnosis of melioidosis to improve patient outcome from acute disease (Cooper *et al.* 2013; Harris, Engler & Norton 2011; Harris *et al.* 2009; Harris *et al.* 2011).

As previously mentioned, *B. pseudomallei* is intrinsically resistant to empiric antibiotic therapy. This is attributed to the expression of multiple multidrug efflux pumps (*AmrAB-OprA*, *BpeAB-OprB* and *BpeEF-OprC*) and includes resistance to some third-generation cephalosporins, penicillins, rifamycins, aminoglycosides, quinolones and macrolides (Chan, Ong & Chua 2007; Cheng & Currie 2005). Prior to 1989, the standard treatment regimen for melioidosis consisted of high combined doses of chloramphenicol, doxycycline, trimethoprim-sulphamethoxazole and occasionally kanamycin, for periods of up to 6 months (White *et al.* 1989). Despite this, mortality rates for melioidosis remained high, often exceeding 70%, until the implementation of ceftazidime following successful clinical trials in Thailand (White *et al.* 1989).

Improvements in diagnosis and access to appropriate antibiotic therapies have significantly reduced mortality rates from melioidosis. Currently, the treatment of choice for melioidosis consists of an intensive phase of intravenous antibiotics (ceftazidime, meropenem or imipenem), followed by an eradication phase of oral antibiotics (co-amoxiclav or co-trimoxazole and doxycycline) for at least 3 months to maximise clearance of the infection and minimise the potential for relapse (Brett

& Woods 2000; Currie *et al.* 2000a; Simpson & White 1999). Mortality rates have been considerably reduced with the implementation of this treatment regime. However, mortality rates still remain at 14% and 50% in Australia and Thailand, respectively, and recurrent disease due to relapse occurs in 6-13% of cases, despite prolonged antibiotic therapy (Currie, Ward & Cheng 2010; Maharjan *et al.* 2005; Suputtamongkol *et al.* 1999; Suputtamongkol *et al.* 1994). Furthermore, the mortality rate for melioidosis-induced septic shock is still unacceptably high, at 50% in Australia and even higher in regions without access to advanced intensive care facilities (Chou *et al.* 2007; Currie, Ward & Cheng 2010; Limmathurotsakul *et al.* 2010b). In contrast, other causes of septic shock in Australia have a mortality rate of 18.4% (Kaukonen *et al.* 2014; Khwannimit & Bhurayanontachai 2009; Peake *et al.* 2009; Tanriover *et al.* 2006).

The potential severity of disease, ease of transmission and treatment limitations, have earned *B. pseudomallei* classification as a Category B bioterrorism agent given the substantial risk it poses to global health (Rotz *et al.* 2002). The advent of widespread antibiotic resistance has given rise to doubt on the future reliance on antibiotic therapy as the sole treatment modality (Behera *et al.* 2012; Sarovich *et al.* 2012; Schweizer 2012). There is increasing recognition of the need to consider novel therapies such as immunomodulating agents to facilitate advancements in treatment for melioidosis. Such developments rely heavily on ascertaining a better understanding of the immunopathogenesis of this disease.

2.1.6 Host-pathogen interactions

2.1.6.1 Bacterial pathogenicity

A number of virulence factors have been identified that enable *B. pseudomallei* to overcome host defences, establish infection and evade immune detection, as listed in Table 2.3. The polysaccharide capsule of *B. pseudomallei* is resistant to lysosomal defensins and complement deposition (Gan 2005). This interferes with complement-mediated phagocytosis and lysis, thereby enabling prolonged survival of *B. pseudomallei* in the blood. Sun *et al.* (2005) also described a caspase-1 dependent killing mechanism used by *B. pseudomallei* to induce apoptosis of monocytes,

macrophages and dendritic cells (DC). Bacterial-induced death of phagocytes limits both their microbicidal and antigen presenting capacity, thereby facilitating bacterial persistence and dissemination. Furthermore, several morphological variations of *B. pseudomallei* have been described, which are reflective of changes to the expression of surface determinants (Chantratita *et al.* 2007). This may have important clinical implications for vaccine development given that specific adaptive immune responses rely on the unique recognition of surface antigens.

Due to the presence of a type III secretion system (T3SS; *Bsa*), *B. pseudomallei* can avoid immune detection by invading both phagocytic and non-phagocytic cells, where it can multiply and spread intracellularly (Allwood *et al.* 2011; Stevens *et al.* 2002). This is dependent on the escape of *B. pseudomallei* from the phagosome to the cytosol and the formation of cell protrusions by actin polymerisation (Stevens *et al.* 2002). *B. pseudomallei* also induces host cell fusion, leading to the formation of multinucleated giant cells (MNGC), further facilitating cell to cell spread (Horton *et al.* 2013; Kespichayawattana *et al.* 2000; Muangsombut *et al.* 2008; Suparak *et al.* 2005; Utaisincharoen *et al.* 2006). The lipopolysaccharide (LPS) structure of *B. pseudomallei* is unique in that it has been shown to be a less potent activator of innate defence mechanisms compared to other Gram negative bacteria, potentially through incomplete stimulation of toll like receptor (TLR)-4 (West *et al.* 2008). Matsuura *et al.* (1996) found that *B. pseudomallei* LPS was 100 times less pyrogenic than LPS of *Salmonella* species, resulting in a 10-fold reduction in the release of proinflammatory cytokines and nitric oxide (NO) from macrophages. Ultimately, the ability of *B. pseudomallei* to remain inconspicuous to the various components of the host immune response facilitates its persistence and potentially explains the propensity for establishment of latent and chronic forms of this disease and the high rates of recrudescence and relapse.

Table 2.3 Significant virulence factors of *B. pseudomallei*

Virulence Factor	Putative Role	References
Capsule	Epithelial attachment and resistance to complement	Ahmed *et al.* (1999) Reckseidler-Zenteno, DeVinney and Woods (2005)
LPS	Resistance to complement and defensins	DeShazer, Brett and Woods (1998) Burtnick and Woods (1999)
Flagella	Motility	DeShazer *et al.* (1997)
Pili	Epithelial attachment and microcolony formation	Essex-Lopresti *et al.* (2005) Brown *et al.* (2002) Boddey *et al.* (2006)
Quorum sensing	Stationary phase gene regulation	Valade *et al.* (2004) Lumjiaktase *et al.* (2006)
T3SS (*Bsa*)	Cell invasion and vacuolar escape	Stevens *et al.* (2003) Stevens *et al.* (2002) Sun *et al.* (2010) Muangsombut *et al.* (2008)
Morphotype switching	Alteration of surface determinants	Chantratita *et al.* (2007)
Caspase-1 dependent cell death	Lysis of monocytes, macrophages and dendritic cells	Sun *et al.* (2005)

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

2.1.6.2 Immune responses

a) Innate immunity

Murine models of melioidosis have been instrumental in discerning protective immune responses to *B. pseudomallei* infection (Titball *et al.* 2008). Evidence from murine studies using BALB/c and C57BL/6 mice, which resemble the acute and chronic forms of melioidosis, respectively, suggests that the progression and clinical manifestation of melioidosis is largely determined by the immunological status of the host (Table 2.4). Resistance to melioidosis in C57BL/6 mice is associated with earlier immune activation and greater control of *B. pseudomallei* growth compared to BALB/c mice, which succumb to overwhelming sepsis within days of infection (Koo & Gan 2006; Leakey, Ulett & Hirst 1998; Liu *et al.* 2002; Tan *et al.* 2008; Ulett, Ketheesan & Hirst 1998, 2000a).

Table 2.4 Immune responses in acute and chronic murine models of melioidosis

BALB/c (acute model)	C57BL/6 (chronic model)	References
Hyperproduction of proinflammatory cytokines	Moderate proinflammatory cytokine production	Hoppe *et al.* (1999) Ulett, Ketheesan and Hirst (2000a) Ulett, Ketheesan and Hirst (2000b) Barnes *et al.* (2001)
Cytokines peak early (24-48h)	Cytokines peak later (48-72h)	Ulett, Ketheesan and Hirst (2000a) Ulett, Ketheesan and Hirst (2000b)
Reduced macrophage and lymphocyte recruitment	Initial influx of neutrophils, followed by macrophages and lymphocytes	Santanirand *et al.* (1999)
Poor *B. pseudomallei* clearance by macrophages	Efficient *B. pseudomallei* clearance by macrophages	Breitbach *et al.* (2006) Barnes and Ketheesan (2007)
Significant bacterial loads and tissue necrosis	Lower bacterial loads with evidence of focal containment	Hoppe *et al.* (1999) Leakey, Ulett and Hirst (1998)

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

Neutrophils and macrophages are the first cells to encounter invading pathogens and their combined involvement is required for effective control of *B. pseudomallei* infection. Pathogen associated molecular patterns (PAMP) are recognised by TLR on the surface of these cells, leading to rapid activation of inflammatory genes (Takeuchi & Akira 2010). There is evidence that TLR-2, TLR-4 and CD14 are involved in recognition of *B. pseudomallei* (Wiersinga *et al.* 2007b). Intracellular activation of the inflammasome, involving NOD-like receptor caspase-activating domain 4 (NLRC4) and NOD-like receptor protein 3 (NLRP3) complexes, in macrophages is also initiated in response to *B. pseudomallei* (Wiersinga, Currie & Peacock 2012). This leads to activation of caspase-1 and secretion of active interleukin (IL)-1β and IL-18 (Wiersinga, Currie & Peacock 2012). Caspase-1 initiates pyroptosis, a form of cell death that may help restrict intracellular bacterial growth due to the ability of *B. pseudomallei* to persist and replicate in the cytoplasm (Ceballos-Olvera *et al.* 2011; Sun *et al.* 2005).

The early cytokine response initiated by macrophages and neutrophils is critical in driving the development of protective cell-mediated immune (CMI) responses (Figure 2.3). Production of IL-12 is essential to induce interferon (IFN)-γ production by natural killer (NK) cells and bystander $CD8^+$ T cells early after infection with *B. pseudomallei* (Haque *et al.* 2006b). There is significant evidence to support the absolute requirement of IFN-γ in protective immune responses against *B. pseudomallei* infection (Goodyear *et al.* 2009; Koo & Gan 2006; Santanirand *et al.* 1999). IFN-γ is a potent activator of macrophages, which is required for the intracellular killing of *B. pseudomallei* (Santanirand *et al.* 1999). Increased bacterial dissemination associated with acute infection has been attributed to impaired IFN-γ production and subsequent reduction in macrophage microbicidal activity, as has been demonstrated in BALB/c mice, which ultimately succumb to infection within 72-96 hours post-infection (Barnes, Williams & Ketheesan 2008; Breitbach *et al.* 2006; Leakey, Ulett & Hirst 1998). IFN-γ-activation of macrophages markedly improves killing of *B. pseudomallei* and is believed to contribute to the increased bacterial clearance observed in C57BL/6 mice following *B. pseudomallei* infection (Barnes & Ketheesan 2007; Breitbach *et al.* 2006). Consistent with this, the depletion of macrophages in C57BL/6 mice significantly reduces their resistance to *B. pseudomallei* infection (Barnes, Williams & Ketheesan 2008; Breitbach *et al.* 2006).

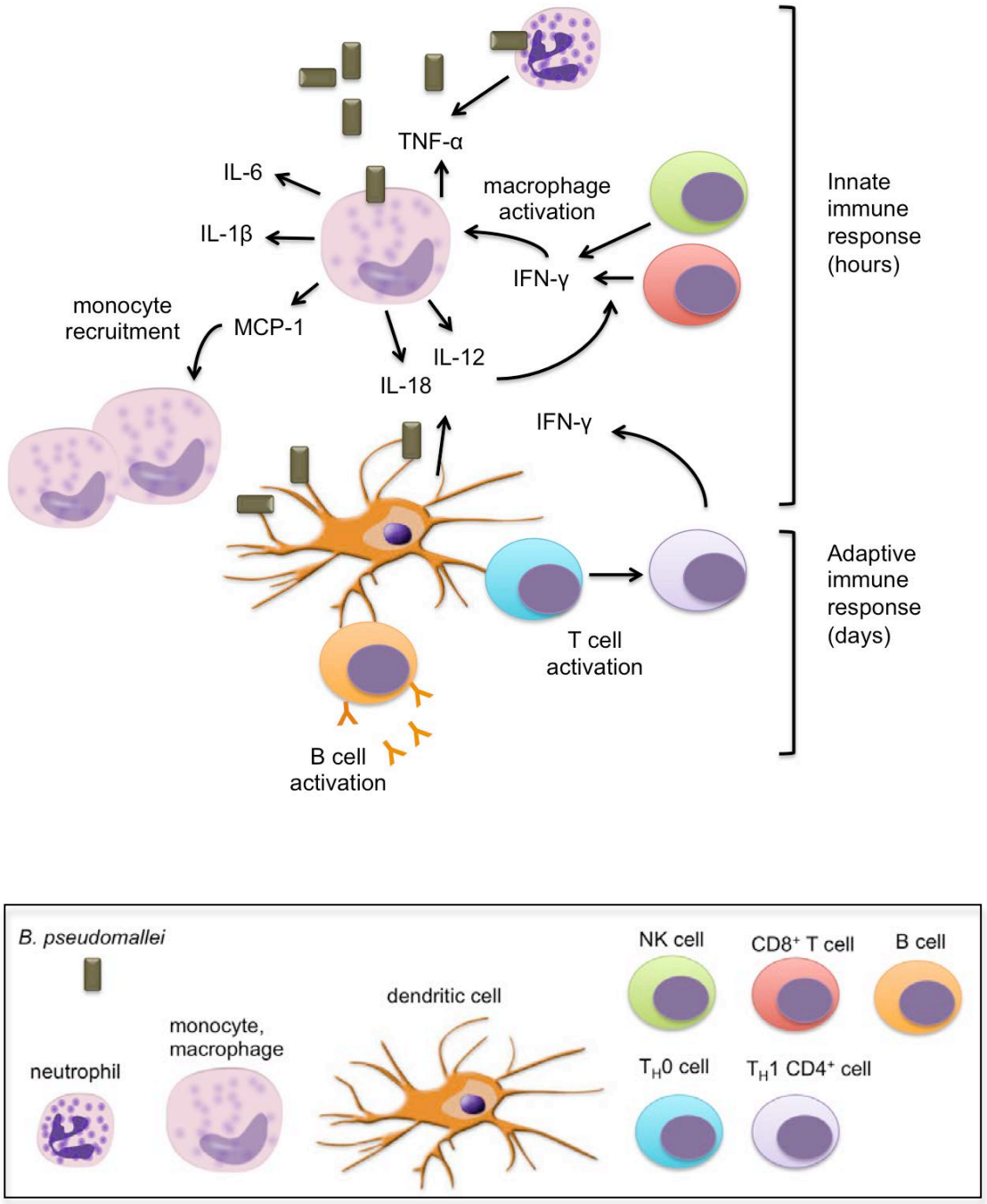

Figure 2.3 Immune responses to *B. pseudomallei*
Following recognition of *B. pseudomallei* by neutrophils, macrophages and dendritic cells (DC), transcription of inflammatory genes is initiated through nuclear factor kappa B (NF-κB) signalling. Cytokines such as IL-12 and IL-18 are essential for inducing IFN-γ production by natural killer (NK) and T cells early post-infection. IFN-γ activates macrophages, enhancing microbicidal mechanisms and thereby limiting intracellular persistence of *B. pseudomallei.* Antigen presentation by DC, in the presence of IL-12, promotes development of protective cell-mediated immune (CMI) responses, involving $CD4^+$ T helper type 1 (T_H1) cells and further production of IFN-γ.

There is still controversy over the precise role of neutrophils in the immune response to *B. pseudomallei* infection. It has been shown that they are rapidly activated, recruited to sites of infection and may contribute to early containment of *B. pseudomallei* (Barnes *et al.* 2001; Easton *et al.* 2007; Riyapa *et al.* 2012). However, excessive neutrophil infiltration may be involved in host pathology through hyperinflammation and increased tissue damage (Barnes *et al.* 2001). The kinetics of cytokine production must be tightly regulated during infections to achieve a balance that maximises bacterial clearance, whilst minimising damage to host tissues.

While appropriate immune responses are necessary to combat infections, widespread immune activation, if left unchecked, can do more harm than good. Sepsis involves a systemic inflammatory response to infection, which is associated with a number of immunological, metabolic and haemostatic derangements and contributes significantly to mortality in melioidosis (Wiersinga *et al.* 2007a). There is evidence that IL-1β may be detrimental in melioidosis, through its contribution to excessive neutrophil recruitment, tissue damage and inhibition of IFN-γ (Ceballos-Olvera *et al.* 2011). Overproduction of IFN-γ, TNF-α, IL-1β and IL-6 at 24 hours after infection, and peaking prior to host death, has been demonstrated in the susceptible BALB/c model of acute melioidosis (Koo & Gan 2006; Ulett, Ketheesan & Hirst 2000a, 2000b). This is consistent with the observed correlation between mortality and high serum concentrations of proinflammatory cytokines in patients with septic melioidosis (Suputtamongkol *et al.* 1992; Wiersinga *et al.* 2007a; Wiersinga *et al.* 2008c). Whilst inflammation is necessary for the early containment of bacteria and initiation of adaptive immunity, uncontrolled amplification of the inflammatory process can result in widespread tissue damage and multi-organ failure, underscoring the importance of immune regulation to limit self-induced tissue damage, while still mounting an effective immune response to pathogens (Rittirsch, Flierl & Ward 2008).

b) Adaptive immunity

Host protection from intracellular pathogens such as *B. pseudomallei* relies on development of CMI responses. DC are especially critical for the initiation of T cell

responses following infection. There is growing evidence that antigen-specific $CD4^+$ T helper type 1 (T_H1) cells are an important source of cytokines during the latter phase of *B. pseudomallei* infection and play an important role in host protection (Haque *et al.* 2006a; Ketheesan *et al.* 2002). In particular, *B. pseudomallei*-specific $CD4^+$ T_H1 cells mediate protection in mice through secretion of IFN-γ, which is critical for macrophage activation (Haque *et al.* 2006a). Development of an adaptive CMI response characterised by activation of *B. pseudomallei*-specific $CD4^+$ and $CD8^+$ T cells has also been demonstrated clinically (Barnes *et al.* 2004). Barnes *et al.* (2004) showed that lymphocyte proliferation and IFN-γ production from asymptomatic, seropositive individuals was significantly higher following *in vitro* stimulation with *B. pseudomallei* antigens than lymphocytes from individuals with a history of clinical melioidosis.

While immunisation strategies for *B. pseudomallei* in C57BL/6 mice produce strong CMI responses, complete clearance of *B. pseudomallei* is rarely achieved (Peacock *et al.* 2012; Sarkar-Tyson & Titball 2010; Ulett *et al.* 2005). Adoptive transfer of lymphocytes to naïve mice fails to confer protection to subsequent infection (Barnes & Ketheesan 2007). The lack of evidence to support an association between HIV and melioidosis further brings in to question the relative importance of T cell responses in protection from *B. pseudomallei* infection (Morris, Hodgson & Ketheesan 2012). Undoubtedly, a comprehensive cellular response with combined involvement of early innate immune mechanisms is pivotal to the containment and control of *B. pseudomallei* infection. The potential role of other T cell subsets, including T_H17 cells and regulatory T cells (T_{reg}), in the immunopathogenesis of *B. pseudomallei* infection is yet to be described (Morris, Hodgson & Ketheesan 2012; Patel *et al.* 2011).

As an intracellular pathogen, humoral responses alone cannot provide complete protection from *B. pseudomallei* infection. However, development of humoral responses following *B. pseudomallei* infection, in combination with CMI responses, may additionally contribute to host protection to melioidosis (Patel *et al.* 2011). There is evidence to suggest that high titres of *B. pseudomallei*-specific immunoglobulins (Ig) correlate with improved clinical outcome and may contribute to increased survival, in combination with CMI responses, following

B. pseudomallei infection in mice (Healey *et al.* 2005; Sarkar-Tyson & Titball 2010). This may be attributed to antibody-mediated phagocyte killing of *B. pseudomallei*, which has been demonstrated *in vitro* (Ho *et al.* 1997). However, the absolute importance of humoral responses in host defence to *B. pseudomallei* infection has been questioned given that depletion of B cells *in vivo* has no impact on susceptibility to *B. pseudomallei* infection in mice (Haque *et al.* 2006b). Furthermore, recurrent *B. pseudomallei* infections are common in melioidosis patients regardless of high specific antibody levels (Vasu, Vadivelu & Puthucheary 2003). Serological studies have shown that the seroprevalence of antibodies for *B. pseudomallei* is high in areas endemic for melioidosis (Gilmore *et al.* 2007). While it is possible that antibodies to related but avirulent *Burkholderiaceae* species are cross-reactive, it is thought that healthy hosts are frequently able to clear the infection (Cheng & Currie 2005; Gilmore *et al.* 2007). In contrast, those patients with severe disease and poor outcomes tend to have underlying comorbidities (Currie, Ward & Cheng 2010).

2.1.7 Comorbidities

The strong association between melioidosis and diabetes has been recognised since the 1980's (Guard *et al.* 1984). The most recent evidence originating from the largest prospective study in the Northern Territory of Australia indicated that 39% of patients with melioidosis had pre-existing diabetes (Table 2.5) (Currie, Ward & Cheng 2010). Similarly, high rates of comorbid diabetes have been reported in other endemic regions, including 42% in North Queensland, 57% in Thailand, 48% in Singapore, 60% in Taiwan and 38% in Malaysia (Limmathurotsakul *et al.* 2006; Lo *et al.* 2009; Malczewski *et al.* 2005; Puthucheary, Parasakthi & Lee 1992; Shih *et al.* 2009). The relative risk of melioidosis in people with diabetes is similar in Australia and Thailand, estimated to be 13 fold higher than people without this risk factor (Currie *et al.* 2004; Suputtamongkol *et al.* 1994). In particular, it is recognised that almost all patients with melioidosis and comorbid diabetes have T2D (Currie *et al.* 2000a; Currie, Ward & Cheng 2010; Simpson *et al.* 2003). Additional risk factors for melioidosis are also documented in Table 2.5. While excessive alcohol intake has been reported as a significant risk factor for melioidosis in Australia, this association is not observed in Thailand, Malaysia and Singapore, possibly a reflection of

reduced alcohol consumption rates in these countries (Chaowagul *et al.* 1989; Currie *et al.* 2000a; Puthucheary, Parasakthi & Lee 1992; Rode & Webling 1981; Suputtamongkol *et al.* 1999; Suputtamongkol *et al.* 1994). It is possible that similar mechanisms are responsible for the predisposition of individuals to melioidosis, given many of these risk factors may be associated with immune abnormalities (Currie *et al.* 2000a).

Table 2.5 Risk factors and mortality rate associated with melioidosis in Australia from 1989 to 2009

Risk Factor	Prevalence		Mortality	
	number of patients	% of total	number of deaths	% who died
Diabetes	**213**	**39**	**33**	**15**
Excess alcohol intake	211	39	33	16
Chronic lung disease	140	26	27	19
Chronic renal disease	65	12	13	20
Rheumatic heart disease[a]	39	7	9	23
Immunosuppression	31	6	6	20
Other[b]	6	1	2	33
Nil	106	20	2	2

Data derived from the Royal Darwin Hospital Series (n = 540) as described by Currie, Ward and Cheng (2010). [a] includes other heart conditions; [b] includes chronic liver disease.

While it is widely recognised that individuals with T2D are predisposed to acquiring *B. pseudomallei* infection, there is still controversy regarding the implications of pre-existing T2D on disease outcome. It was documented in a recent study in Malaysia that the mortality rate in patients with comorbid T2D was twice that of patients without this risk factor (Hassan *et al.* 2010). However, no correlation between increased rates of mortality and co-existing T2D was found in the most recent prospective study in Australia (Currie, Ward & Cheng 2010). It has since been recognised that this may be confounded by the survival advantage afforded by glyburide, a common medication for the treatment of T2D (Koh *et al.* 2011).

Nevertheless, it is clear that the presence of at least one of the risk factors for melioidosis is an independent predictor of mortality (Table 2.5).

Regardless of overall mortality rates, melioidosis disease severity does appear to be exacerbated in patients with comorbid T2D. This is supported by clinical data from The Townsville Hospital (TTH) in North Queensland, which suggests a correlation between patients with melioidosis and comorbid T2D and the subsequent development of bacteraemia (*personal communication,* Dr R. Norton, TTH Melioidosis Database), as has been described in the Northern Territory and in Thailand (Currie, Ward & Cheng 2010; Suputtamongkol *et al.* 1999). Furthermore, relapse of melioidosis following appropriate antibiotic therapy is a significant feature of patients with comorbid T2D compared to patients without this risk factor (64% vs 38%) (Currie, Ward & Cheng 2010).

Despite 30 years of epidemiological studies consistently drawing links between T2D and melioidosis, critical questions regarding this comorbidity remain to be answered. Why are individuals with T2D predisposed to melioidosis? What mechanisms contribute to the increased disease severity of melioidosis in those with coexisting T2D? Answering these questions is of prime importance for the development of novel adjunctive therapeutics for melioidosis in the population at most risk of this infectious disease. Additionally, diagnostics and drug development are typically founded utilising non-diabetic animal models. Considering the distinct course of melioidosis and immunopathogenesis associated with T2D, there is a need for preventative and therapeutic strategies that are targeted and proven to be efficacious in a model of T2D. The continued emergence of this comorbidity as the T2D epidemic continues is a driving force for this research, particularly with the rising incidence of T2D in countries where melioidosis is already a significant burden.

2.2 Type 2 Diabetes

2.2.1 Historical and current perspective

Diabetes is a heterogeneous metabolic disease characterised by hyperglycaemia and glucose intolerance. It was one of the first diseases to be described (Eknoyan &

Nagy 2005). The term ‘diabetes’ was first used in 230 BC, derived from the Greek etymology ‘to pass through’, indicative of one of its most prominent symptoms of frequent micturition (Poretsky 2009). Due to the seminal research by Himsworth (1936) more than 70 years ago, diabetes was subdivided and classified into Type 1 (T1D, previously known as insulin-dependent) and Type 2 (T2D, previously known as insulin-independent) diabetes, based on their distinctive aetiologies. However, the major pathological determinant of T2D proposed by Himsworth, insulin resistance, was not widely accepted for another 40 years, until insulin-mediated glucose disposal could be definitively measured and subsequent prospective studies confirmed that insulin resistance antedated the development of hyperglycaemia and was the best predictor of developing T2D (Ginsberg, Olefsky & Reaven 1974; Lillioja *et al.* 1993; Shen, Reaven & Farquhar 1970; Sicree *et al.* 1987).

Although once a rare disease, diabetes is now considered a global epidemic (IDF 2011). In 2011, 366 million people were estimated to have diabetes and the most recent statistics from the International Diabetes Federation (IDF) indicate this will escalate to 552 million people by 2030 (IDF 2011). In Australia, diabetes is the sixth leading cause of death according to the Australian Institute of Health and Welfare (AIHW 2011). In 2008, diabetes affected approximately 4% of the Australian population, more than double the prevalence in 1990 (Figure 2.4) (AIHW 2011). Furthermore, this is likely to be an underestimate, since many cases may be undiagnosed due to the chronic nature of the disease, which can remain asymptomatic for years (AIHW 2011). The trend of increasing prevalence of diabetes in Australia is reflected in many developed countries throughout the world (IDF 2011). Importantly, T2D is the most common form of diabetes, constituting at least 85-95%, and is responsible for the burgeoning epidemic (IDF 2011).

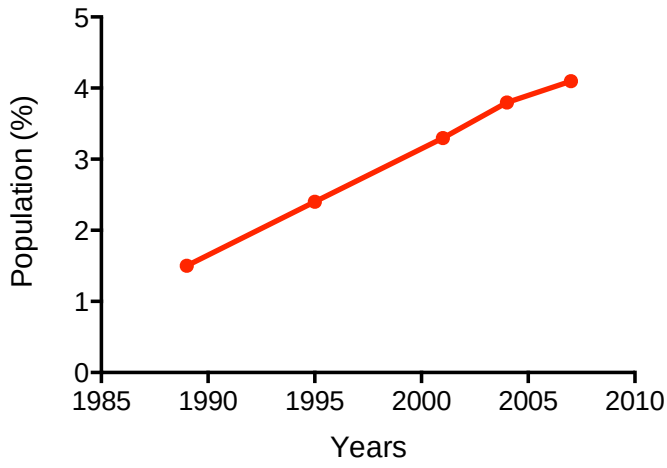

Figure 2.4 The increasing prevalence of diabetes in Australia
Age-standardised data sourced from the Australian Institute of Health and Welfare (2011), which illustrates the growing trend of diabetes in Australia, attributed predominantly to type 2 diabetes (T2D).

Hyperglycaemia is a key diagnostic criterion for T2D. There are slight discrepancies in the criteria for diagnosis of T2D established by the World Health Organisation (WHO) and IDF, compared to the criteria used by the American Diabetes Association (ADA), shown in Table 2.6 (ADA 2013; WHO/IDF 2006). The difficulty in defining hyperglycaemia is that blood glucose is a continuous variable, leading to disagreement over the arbitrary cut-off point for designating hyperglycaemia (WHO/IDF 2006). The current hyperglycaemia cut-off for T2D is generally based on the increased risk of adverse complications, such as microvascular and cardiovascular disease (CVD) (WHO/IDF 2006). In particular, there is insufficient data with which to distinguish normal from intermediate hyperglycaemic levels, which can vary greatly between individuals and populations depending on age, gender and ethnicity (WHO/IDF 2006). Glycosylated haemoglobin (HbA1c), formed by non-enzymatic glycation of haemoglobin, which is accelerated when blood glucose is high, is a useful measurement for assessing prolonged hyperglycaemia over a period of 2-3 months. Measuring HbA1c is advantageous for monitoring glycaemic control in people with T2D, since it overcomes the dynamic fluctuations that may occur when measuring blood glucose. However, HbA1c is not considered suitable as a diagnostic test due to its limited

availability, inconsistencies based on measurement techniques, lack of standardisation and the possibility of inaccuracies caused by haemoglobinopathies (WHO/IDF 2006).

Table 2.6 Diagnostic criteria for type 2 diabetes

Diagnostic Criteria	WHO/IDF (2006)	ADA (2003)
Diabetes Fasting glucose 2hr glucose*	 ≥ 7.0 mmol/L *or* ≥ 11.1 mmol/L	 ≥ 7.0 mmol/L *or* ≥ 11.1 mmol/L
IGT Fasting glucose 2hr glucose*	< 7.0 mmol/L *and* ≥ 7.8 and < 11.1 mmol/L	*Not required* ≥ 7.8 and < 11.1 mmol/L
IFG Fasting glucose 2hr glucose*	6.1 to 6.9 mmol/L *and (if measured)* < 7.8 mmol/L	5.6 to 6.9 mmol/L *Not required*

* 2 hours after ingestion of 75g oral glucose load; WHO – World Health Organisation; IDF – International Diabetes Federation; ADA – American Diabetes Association; IGT – impaired glucose tolerance; IFG – impaired fasting glucose.

Whilst rising prevalence of T2D is widely recognised as a public health concern in high-income countries, such as Australia, approximately 80% of people with T2D currently live in low- and middle-income countries, with the largest increases in incidence predicted to occur in these regions (IDF 2011). This has important economic and public health implications, considering the limited health care available to appropriately manage both T2D and the double burden of communicable diseases within the diabetic population. Infectious diseases still contribute significantly to morbidity and mortality in these areas (IDF 2011). The two regions with the highest burden of T2D are the Western Pacific and Southeast Asia, with 131.9 and 71.4 million people affected, respectively, as illustrated in Figure 2.5 (IDF 2011). Furthermore, this number is anticipated to increase up to 69% by 2030 (IDF 2011). Meanwhile, the Indigenous population in Australia has been recognised as having one of the highest incidence rates of T2D globally (McDermott, Li &

Campbell 2010). As previously described, the majority of melioidosis cases also occur in the Asia Pacific region. Increases in the number of people with T2D in areas of *B. pseudomallei* endemnicity will facilitate the continued emergence of this infectious disease and will further complicate disease management.

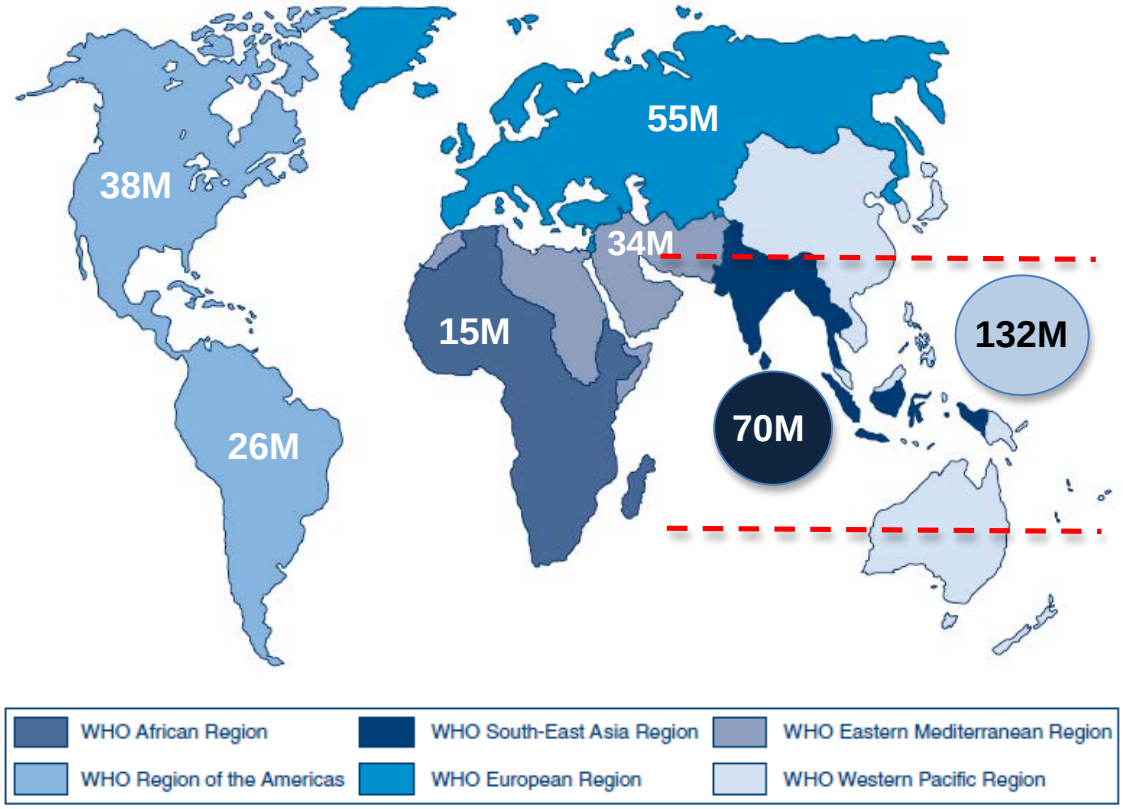

Figure 2.5 The global prevalence of type 2 diabetes
The highest burden of type 2 diabetes (T2D) is in Southeast Asia (132 million) and the Western Pacific (70 million), with many of the countries in these regions also highly endemic for *B. pseudomallei* (between broken red lines). Map sourced from Shaw (2013) and adapted using data from the International Diabetes Federation (IDF 2011).

2.2.2 Aetiopathogenesis

In healthy individuals, blood glucose levels normally fluctuate depending on food consumption, the gluconeogenic activity of hepatocytes and the uptake of glucose by peripheral tissues such as muscle and adipose tissue (Saltiel & Kahn 2001). This

intricate balance is achieved by the anabolic actions of insulin, a metabolic hormone released in to the circulation from beta cells in the pancreatic islets in response to increased blood glucose. The insulin receptor, particularly abundant on myocytes, hepatocytes and adipocytes, is a tyrosine kinase that undergoes autophosphorylation upon binding of insulin (Saltiel & Kahn 2001). This initiates a phosphorylation cascade, involving the activation of insulin receptor substrates (IRS) and the translocation of glucose transporter 4 (GLUT4) to the membrane to enable glucose uptake (Figure 2.6) (Saltiel & Kahn 2001). Through this mechanism, insulin maintains blood glucose concentrations within a narrow range of 4.0-7.8mmol/L. In the absence of insulin binding, GLUT4 is sequestered in the lipid bilayer of intracellular vesicles and glucose cannot be absorbed from the blood leading to hyperglycaemia (Bryant, Govers & James 2002). Multiple other phosphorylation and dephosphorylation events initiated by insulin binding regulate the expression of genes and activity of enzymes, which inhibit gluconeogenesis, increase protein and lipid synthesis and promote cell growth. T1D usually commences in childhood and is attributable to autoimmune or otherwise idiopathic processes (IDF 2011). It is characterised by the destruction of the pancreatic beta cells, thereby resulting in absolute insulin deficiency. In contrast, T2D has a distinct pathogenesis, which involves a combination of insulin resistance and abnormal beta cell function, eventually culminating in hyperglycaemia (Figure 2.7).

2.2.2.1 Visceral adiposity, inflammation and insulin resistance

The global epidemic of T2D has been attributed to obesogenic lifestyle changes, such as increased energy intake and decreased physical activity; a correlation particularly evident in urbanised regions (IDF 2011). It is anticipated that by 2030 the prevalence of overweight and obesity will have doubled (Gnacinska *et al.* 2009). Extensive cohort and cross-sectional studies worldwide have revealed a strong positive correlation between increased weight gain, body mass index (BMI) and the incidence of T2D (Chan *et al.* 1994; Colditz *et al.* 1995; Colditz *et al.* 1990; Drivsholm *et al.* 2001; Field *et al.* 2001). In fact, it has been reported that the risk of T2D increases 4.5-9% per kg of weight gain (Ford, Williamson & Liu 1997). In contrast, weight loss confers a substantial reduction in risk of T2D (Heymsfield *et al.* 2000; Sjostrom 2013; Sjostrom *et al.* 2004). Approximately 90% of people with T2D

are overweight or obese, emphasising the importance of adiposity as a risk factor for T2D and supporting the close relationship between the concurrent epidemics of T2D and obesity (IDF 2011).

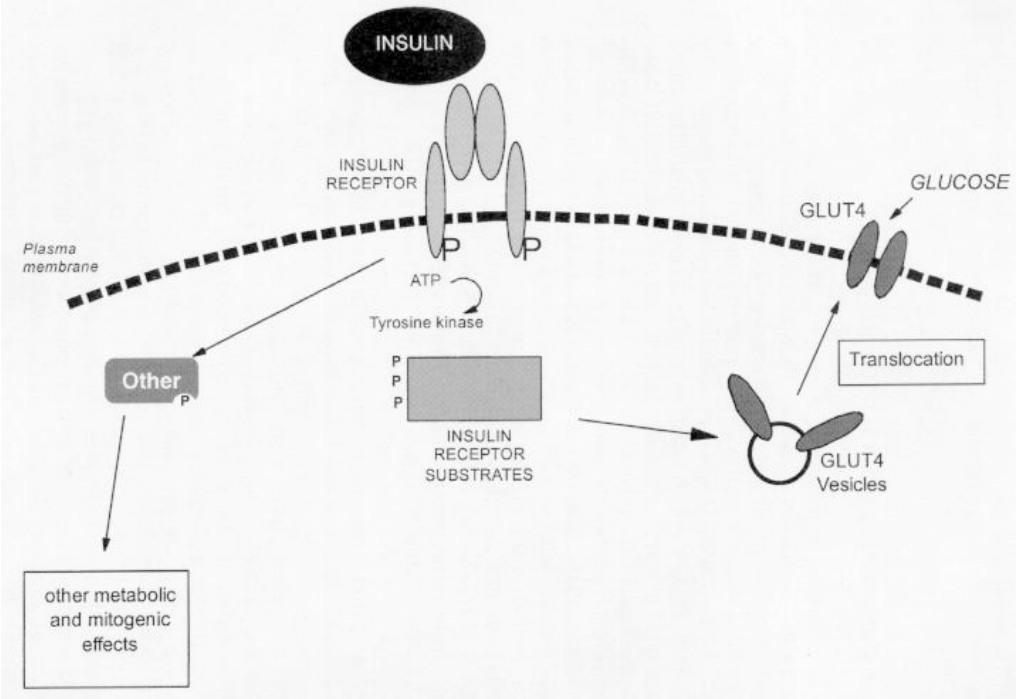

Figure 2.6 Insulin signalling pathway
The insulin receptor undergoes autophosphorylation upon binding of insulin, initiating a phosphorylation cascade involving insulin receptor substrates (IRS), which culminates in the translocation of glucose transporter 4 (GLUT4) to the membrane to enable glucose uptake. Additional phosphorylation cascades initiated by insulin binding regulate gluconeogenesis, protein and lipid synthesis and cell growth. Adapted from McCowen, Malhotra and Bistrian (2001). P – phosphate group; GLUT4 – glucose transporter 4.

Despite an undeniably strong link, the causative role of obesity in development of T2D has been challenged by the fact that metabolic complications can arise, albeit with less frequency, in non-obese individuals (Lee *et al.* 2007). For instance, a similar or higher prevalence of T2D has been documented in many regions of Southeast Asia, despite relatively low BMI in this population (He *et al.* 2002; Huxley *et al.* 2008; Yoon *et al.* 2006). It has since been documented that certain ethnicities, such as Asian and Indigenous populations, are more susceptible to visceral adiposity and have greater insulin resistance and impairments in beta cell

function (Aekplakorn *et al.* 2006; Boyko *et al.* 2000; Chan *et al.* 2004; Fukushima, Suzuki & Seino 2004; Kuroe *et al.* 2003; Nakagami *et al.* 2003; Nishi *et al.* 2005; Ramachandran *et al.* 2005; Reynolds *et al.* 2007; Stolk *et al.* 2005). This has been described as a 'metabolically obese' phenotype, which results in impaired glucose tolerance with only minimal weight gain (Chan *et al.* 2009; Ruderman *et al.* 1998; Ruderman, Schneider & Berchtold 1981). The original thresholds for BMI demarcating overweight and obesity were derived largely from data pertaining to Caucasians. However, it has become increasingly evident that it is not valid to generalise these thresholds across all ethnicities. Certainly there is a need for more reliable definitions of obesity, specific to individual populations, particularly since visceral adiposity is more predictive of metabolic complications and adipose tissue dysfunction, as opposed to total adipose tissue mass (Chandalia & Abate 2007).

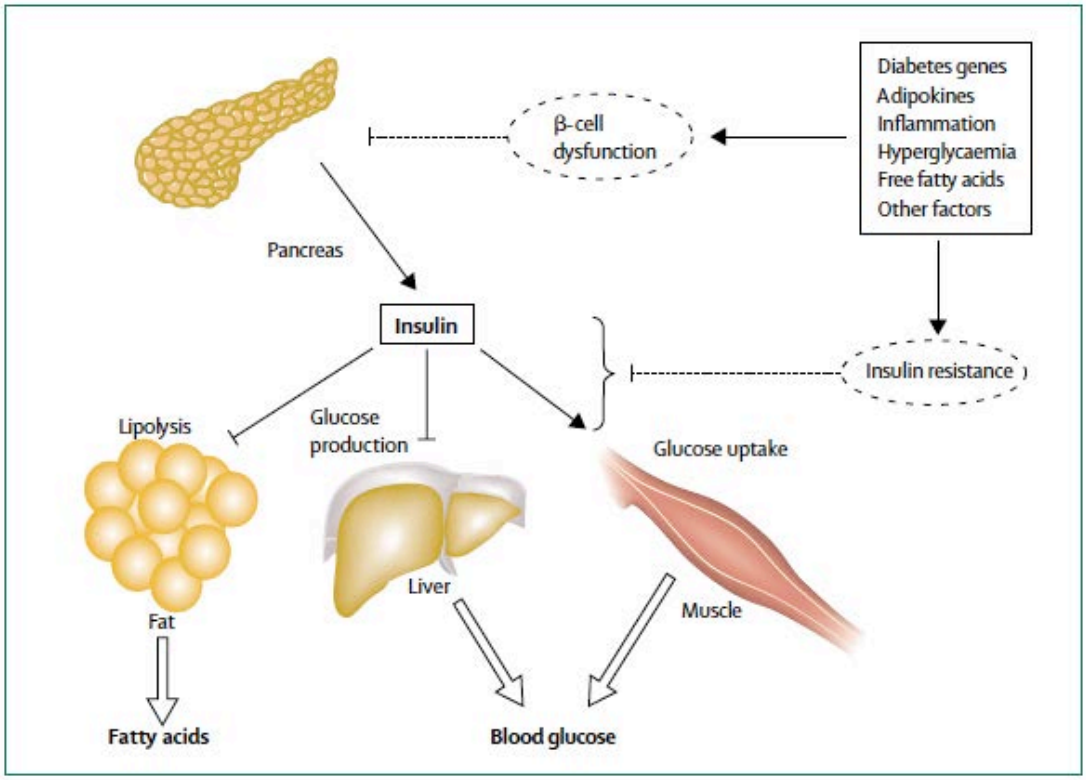

Figure 2.7 The aetiopathogenesis of type 2 diabetes
Distinct from type 1 diabetes (T1D), type 2 diabetes (T2D) develops primarily from insulin resistance, whereby the activity of insulin is impaired, followed by progressive damage to the pancreatic beta cells. Adapted from Stumvoll, Goldstein and van Haeften (2005).

The inflammatory nature of obesity has been widely described and is testimony to the highly integrated pathways connecting the metabolic and immune systems (Wellen & Hotamisligil 2003). In addition to the storage of triglycerides, adipose tissue functions as an endocrine organ, secreting many hormones, cytokines and bioactive lipids (Table 2.7), which have pleiotropic effects on both the metabolic and immune systems (Fukuhara *et al.* 2005; Lehrke *et al.* 2004; Ouchi *et al.* 2003; Steppan & Lazar 2004). It is widely accepted that obesity, particularly excess visceral adipose tissue (VAT), is characterised by a chronic state of low-grade inflammation due to the secretion of proinflammatory cytokines by stressed adipocytes and adipose tissue macrophages (Furuhashi *et al.* 2008; Lumeng *et al.* 2007; Nieto-Vazquez *et al.* 2008; Shah 2007).

Table 2.7 Major sources and functions of adipokines

Adipokine	Primary Source	Function
Leptin	Adipocytes	Appetite control, inflammation
Resistin	Adipocytes (mouse), PBMC (humans)	Promotes insulin resistance and inflammation through TNF and IL-6
RBP4	Adipocytes, macrophages hepatocytes	Implicated in systemic insulin resistance
Lipocalin 2	Adipocytes, macrophages	Promotes insulin resistance and inflammation through TNF
TNF-α	Adipocytes, macrophages	Inflammation, antagonism of insulin signalling
IL-6	Adipocytes, macrophages, hepatocytes	Inflammation
IL-18	Macrophages	Inflammation
MCP-1	Adipocytes, macrophages	Monocyte recruitment
CXCL5	Macrophages	Antagonism of insulin signalling
Adiponectin	Adipocytes	Anti-inflammatory, insulin sensitising

Adapted from Ouchi *et al.* (2011). RBP4 – retinol binding protein 4; CXCL5 – CXC motif ligand 5.

During excessive weight gain, extreme hypertrophy of adipocytes can lead to significant changes in adipocyte metabolism. It is hypothesised that once a threshold of adiposity is reached, a level that varies genetically between individuals, changes in adipocyte metabolism occur. This includes increased lipolysis and secretion of inflammatory factors, such as monocyte chemotactic protein (MCP)-1 and TNF-α, which triggers recruitment and activation of adipose tissue macrophages (ATM), illustrated in Figure 2.8. Increased adipocyte lipolysis leads to higher circulating levels of free fatty acids (FFA), which can directly contribute to insulin resistance (Despres & Lemieux 2006; Mittelman *et al.* 2002). Skeletal muscle insulin resistance reduces the uptake of circulating glucose; hepatic insulin resistance results in increased gluconeogenesis; and adipose tissue insulin resistance promotes the release of FFA that forms a positive feedback loop further exacerbating systemic insulin resistance and increasing circulating glucose levels (Eckardt, Sell & Eckel 2008; Ruan & Lodish 2003).

Evidence suggests that infiltration of macrophages in obese adipose tissue is positively correlated with BMI and adipocyte size in obese humans and animal models (Weisberg *et al.* 2003). The percentage of ATM increases from less than 10% of the total cell population in adipose tissue of lean hosts, to 40-50% in obese humans and mice (Weisberg *et al.* 2003). It is thought that endoplasmic reticulum (ER) stress and hypoxia due to adipocyte hypertrophy in obese states may contribute to increased necrosis and subsequent ATM accumulation (Hosogai *et al.* 2007). Recent research indicates that T cells may also be involved in ATM recruitment and activation (Zeyda *et al.* 2010). The accumulation of ATM further exacerbates lipolysis and inflammation within adipose tissue (Olefsky & Glass 2010). This is attributed to the phenotypic switch of ATM from a primarily anti-inflammatory (M2) to a proinflammatory (M1) state (Olefsky & Glass 2010). Activated M1 macrophages typically form ‘crown like’ structures in adipose tissue (Figure 2.9) and secrete high levels of inflammatory mediators (TNF-α, IL-1β, IL-6 and MCP-1) (Grimble 2002). It is proposed that systemic spillover of these cytokines, indicated by elevated circulating levels, is central to the development of systemic insulin resistance (Cai *et al.* 2005; Shoelson, Lee & Yuan 2003; Wellen & Hotamisligil 2003).

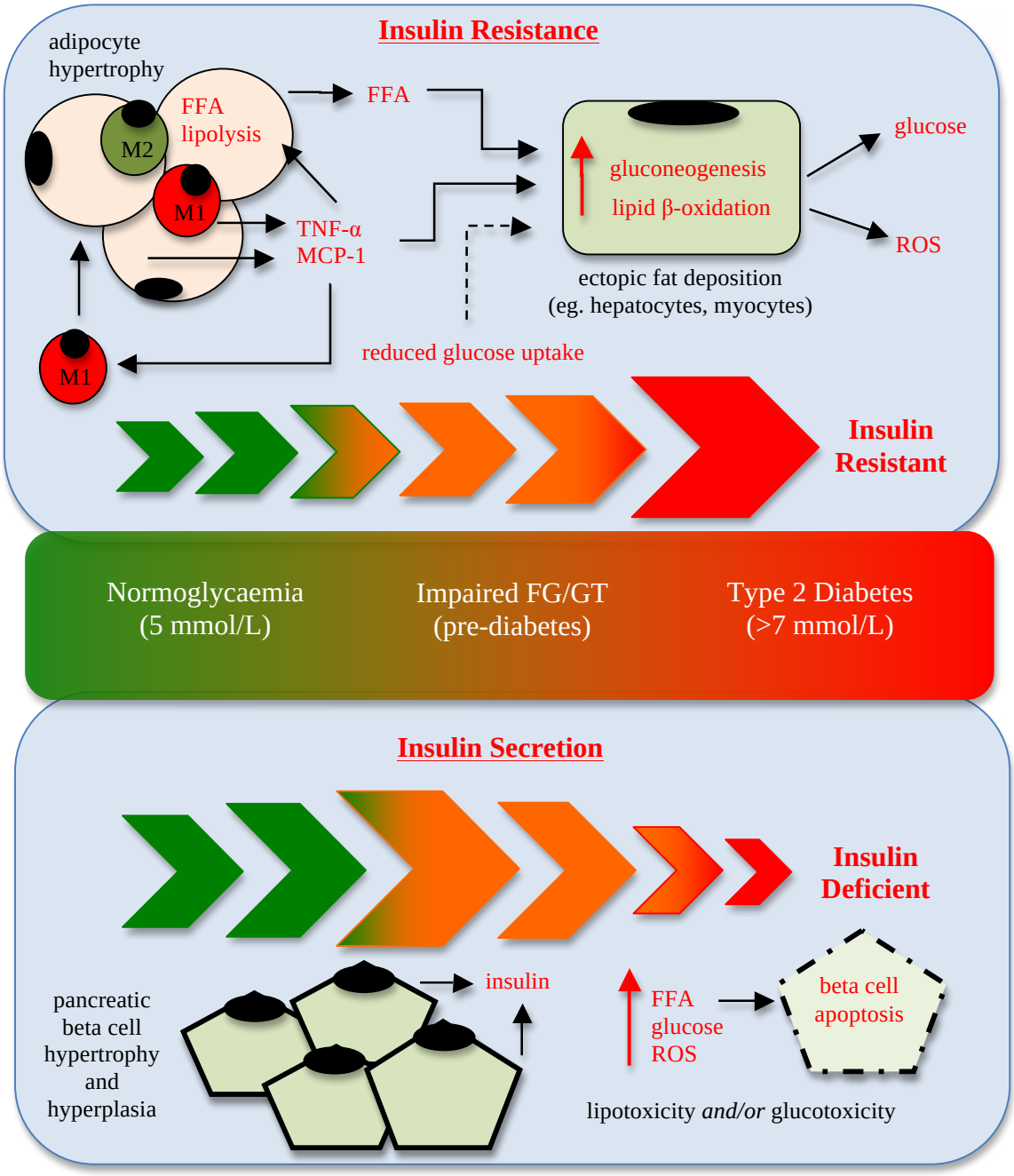

Figure 2.8 The multi-stage model of progressive development of type 2 diabetes
Excessive weight gain induces adipocyte hypertrophy, free fatty acid (FFA) lipolysis and recruitment of classically activated (M1) macrophages, increasing the ratio of M1 relative to alternatively activated (M2) macrophages. Inflammatory cytokines, such as TNF-α and MCP-1, are produced by stressed adipocytes and M1 macrophages, which exacerbates insulin resistance caused by increased FFA levels. Development of overt type 2 diabetes (T2D) occurs when insulin secretion decreases, or is not sufficient to compensate for increasing insulin resistance, due to oxidative stress and glucolipotoxicity. FFA – free fatty acids; FG – fasting glucose; GT –glucose tolerance; ROS – reactive oxygen species.

a) b)

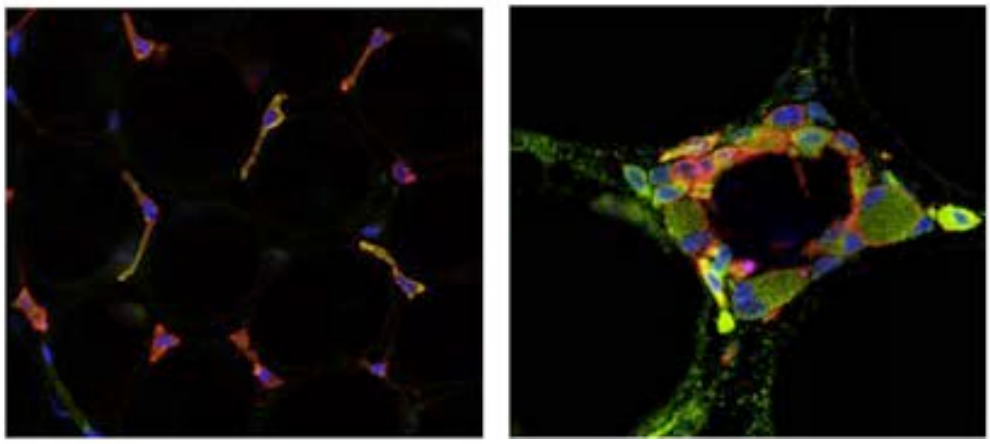

Figure 2.9 Macrophage accumulation in adipose tissue
Compared to adipose tissue from a) lean mice, accumulation of activated macrophages (red; F4/80; green; RelA/p65) was observed in adipose tissue from b) obese mice, forming characteristic 'crown like' structures around adipocytes (blue; nuclei). Adapted from Chiang *et al.* (2009).

The hypothesis that chronic activation of the innate immune system is involved in the pathogenesis of T2D was first suggested in the late 1990's (Pickup 2004). Over-expression of the proinflammatory cytokine, TNF-α, in obese adipose tissue was the first documented link to inflammation and was later determined to be a key feature mediating insulin resistance (Grimble 2002; Hotamisligil, Shargill & Spiegelman 1993; Moller 2000; Pickup & Crook 1998). TNF-α directly interferes with insulin signalling by suppressing insulin-induced receptor and IRS-1 tyrosine phosphorylation, while promoting proteasome-mediated degradation of IRS-1 (Feinstein *et al.* 1993; Kanety *et al.* 1995; Pirola, Johnston & Van Obberghen 2004). In mouse models of obesity, inflammation is evident following increased adiposity but before overt insulin resistance (Xu *et al.* 2003). Furthermore, genetic depletion of M1 macrophages or inhibition of inflammatory pathways in adipose tissue is able to normalise glucose tolerance and insulin sensitivity (Patsouris *et al.* 2008; Schenk, Saberi & Olefsky 2008; Xu *et al.* 2003). The improved insulin sensitivity associated with weight loss is also accompanied by a concomitant decrease in M1 macrophages, providing further support that dysfunctional adipose tissue and inflammatory responses contribute to the development of insulin resistance and ultimately T2D (Cancello *et al.* 2005).

Insulin resistance is a central pathogenic determinant of T2D. However, evidence from longitudinal studies indicates that relative insulin secretion is the prevailing factor dictating disease progression (Bonora *et al.* 2007). Although approximately 25% of adults are estimated to be insulin resistant, compensatory hyperinsulinaemia in most of these individuals is able to maintain relatively normal glycaemic levels (Reaven 2005). Insulin sensitivity is as heterogeneous as glycaemia, with a range varying more than 6-fold in the general population (Yeni-Komshian *et al.* 2000). The dynamic interaction between insulin sensitivity and secretion was emphasised in an otherwise healthy cohort of women whose insulin resistance was equivalent to individuals with T2D yet, due to an augmented insulin secretory response, exhibited no signs of glucose intolerance (Diamond *et al.* 1995). Therefore, insulin resistance does not necessarily result in overt T2D unless the insulin secreting capacity of beta cells is also impaired, as illustrated in Figure 2.8. It has been demonstrated that in individuals with T2D, beta cell function and volume is 50% lower than in non-diabetic individuals and is primarily responsible for the loss of glycaemic control (Butler *et al.* 2003; Marchetti *et al.* 2006; United Kingdom Prospective Diabetes Study Group 1995). The genetic factors determining the functional capacity of beta cells are thought to account for some of the genetic variability in the development of T2D and may explain why not all overweight or insulin resistant individuals develop T2D. A combination of genetic susceptibility to beta cell failure, in addition to an obesogenic environment, is likely to be important (Abbasi *et al.* 2002; Astrup & Finer 2000).

2.2.2.2 Lipotoxicity, glucotoxicity and oxidative stress

The mechanisms by which insulin resistance can alter beta cell functional capacity are not completely understood. Two concepts for the cause of beta cell failure have been described: lipotoxicity and glucotoxicity. The relative importance of each process or combined involvement remains unclear. Lipotoxicity induces apoptosis of beta cells through multiple mechanisms including increased lipid oxidation, accumulation of fatty-acyl-coenzyme A, increased ceramide synthesis and activation of ER stress through production of reactive oxygen species (ROS) (Del Prato 2009). It has also been shown that FFA incorporation into phospholipid or mitochondrial membranes can influence membrane fluidity and directly contribute to cell death,

although the implications of this need to be further investigated (Gehrmann, Elsner & Lenzen 2010; Newsholme *et al.* 2007). Similarly, glucotoxicity, which impairs insulin secretion and action, is also mediated through ER stress and subsequent production of ROS (Del Prato 2009; Newsholme *et al.* 2007). Hyperglycaemia, both *in vivo* and *in vitro,* has been shown to have a proapoptotic effect on beta cells (Del Prato 2009). Beta cells are particularly sensitive to oxidative stress, due to low endogenous antioxidant levels (Lenzen 2008; Lenzen, Drinkgern & Tiedge 1996; Tiedge *et al.* 1997). Therefore, regardless of which process predominates, oxidative stress is a common link in the pathogenesis of pancreatic beta cell destruction.

T2D is associated with a range of severe complications as a result of micro- and macro-vascular abnormalities, including retinopathy, nephropathy, neuropathy, cardio- and cerebro-vascular disease. Many of these abnormalities can persist for years before clinical signs of T2D become apparent (Vendrame & Gottlieb 2004). Vascular complications of T2D are thought to evolve from the combination of alterations originating from hyperglycaemia and insulin resistance (Ginsberg & Huang 2000; Wei *et al.* 1998). The mechanisms are complex and not completely understood although oxidative stress and chronic inflammation are centrally involved, possibly through increased polyol pathway flux and the formation of advanced glycation end products (AGE), illustrated in Figure 2.10 (Brownlee 2001). Hyperglycaemia, a key feature of T2D, is associated with increased rates of non-enzymatic protein glycation, which gives rise to AGE. AGE have been implicated in multiple T2D complications due to their ability to functionally alter proteins and stimulate production of inflammatory proteins and ROS (Brownlee 2001, 2005; Turk *et al.* 2001; Vlassara *et al.* 2002). In T2D, the expression of the receptor for advanced glycation end products (RAGE) is upregulated on the surface of immune cells, leading to dysfunction through increased NF-κB activation in response to rising levels of AGE (Ahmed *et al.* 2005; Giacco & Brownlee 2010).

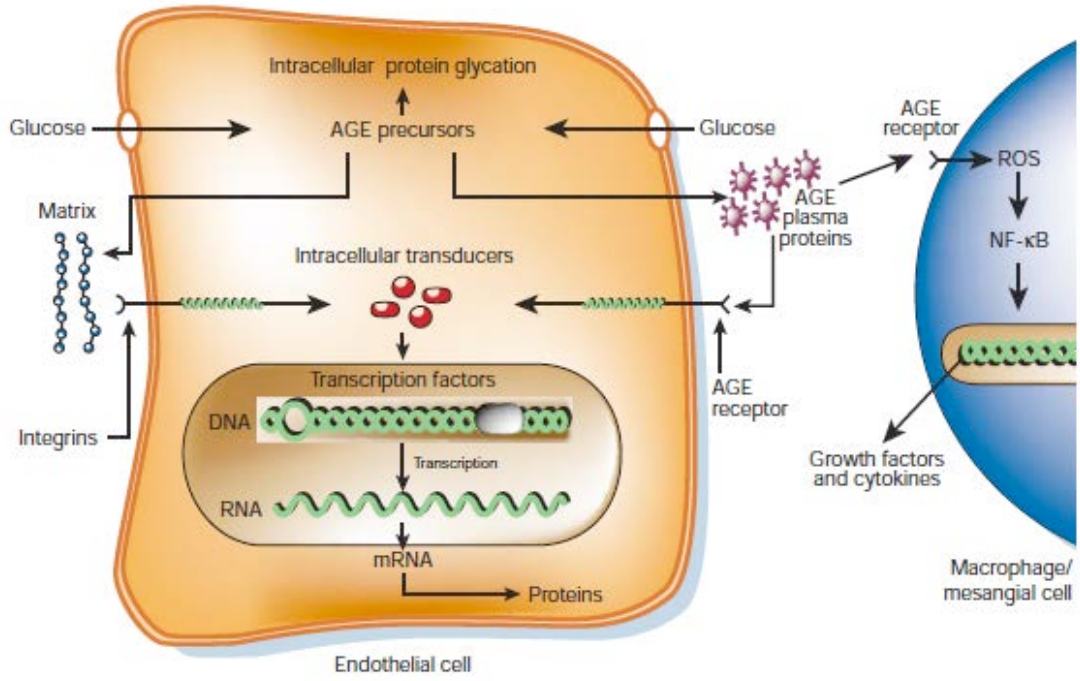

Figure 2.10 Molecular mechanisms of advanced glycation end products
Advanced glycation end products (AGE) generated by hyperglycaemia cause modifications to intracellular proteins as well as extracellular matrix proteins. This may result in abnormal interaction with integrins and binding of AGE receptors, which modifies gene expression and initiates inflammatory responses in endothelial cells and macrophages. Adapted from Brownlee (2001). AGE – advanced glycation end products; ROS – reactive oxygen species.

One of the most abundant and important endogenous cellular antioxidants is the tripeptide GSH (Griffith 1999; Hayes & McLellan 1999; Tran *et al.* 2004). Regulation of GSH is exquisitely controlled (Hentze *et al.* 1989; Hwang, Sinskey & Lodish 1992). The primary and rate-limiting enzyme involved in *de novo* GSH synthesis is γ-glutamylcysteine ligase (γ-GCL). GSH acts as an antioxidant through its ability to reduce ROS and in the process, cycles between reduced GSH and oxidised glutathione (GSSG) states in a highly dynamic process. The intracellular GSH/GSSG ratio defines the GSH redox state of the host cell and is crucial for normal cellular function. Metabolism of GSH involves a complex network of enzymes and pathways, illustrated in Figure 2.11. Net loss of intracellular GSH occurs during the reduction of ROS by GSH peroxidase, leading to the generation of GSSG. GSH is also depleted through the activity of GSH transferase, an enzyme involved in conjugation of GSH to toxic compounds and xenobiotics facilitating

their extracellular transport from the cell (Malmezat *et al.* 2000). GSH reductase regenerates GSH by reducing GSSG through a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent pathway. During oxidative stress, GSH is consumed and GSSG is produced, shifting the intracellular redox state to a lower GSH/GSSG ratio. Ultimately, GSH concentration and overall cellular redox status are dependent on the combined activity of these processes (Malmezat *et al.* 2000).

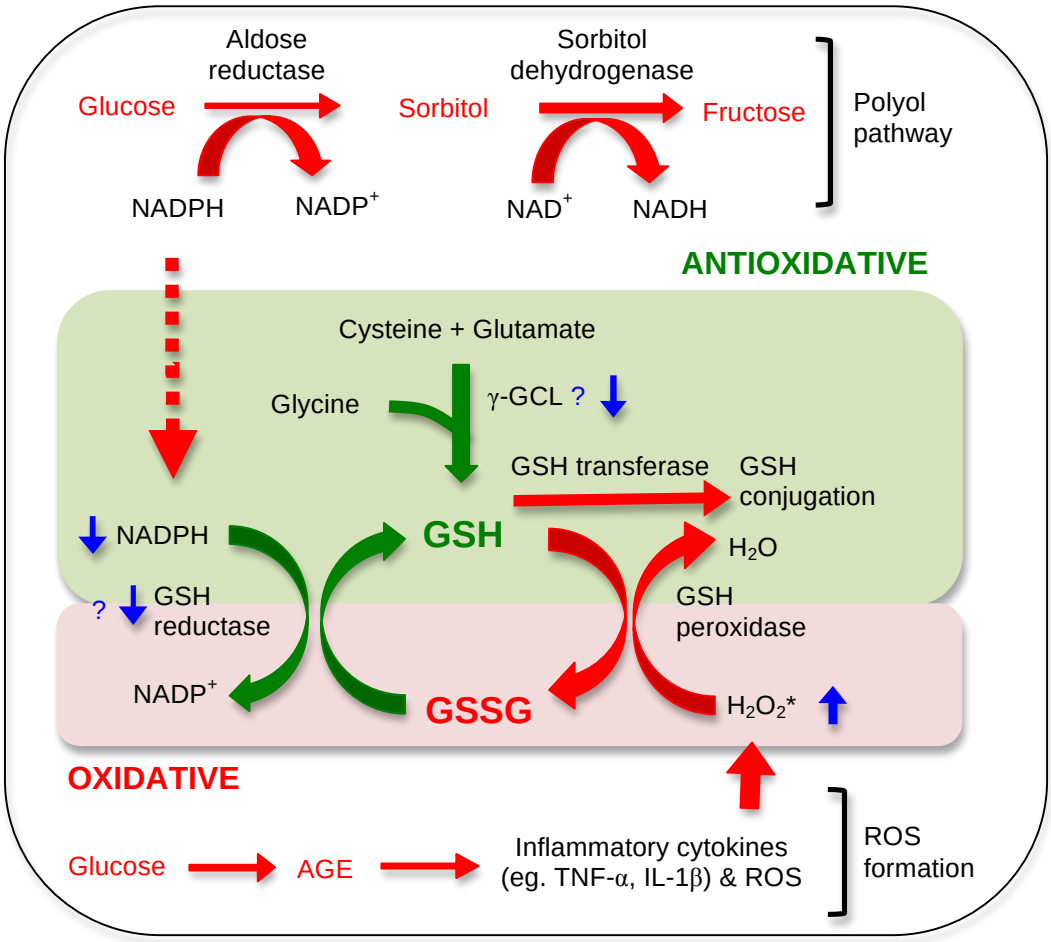

Figure 2.11 Dysregulation of the major intracellular biosynthetic pathways of glutathione in type 2 diabetes
Type 2 diabetes (T2D) is associated with dysregulation of glutathione pathways leading to depletion of reduced glutathione (GSH) and accumulation of oxidised glutathione (GSSG). This may occur through a number of mechanisms including increased oxidative stress and GSH peroxidase activity, which depletes GSH. Dysregulation may also arise through reduced resynthesis and synthesis of GSH due to potentially impaired activity of γ-glutamylcysteine ligase (γ-GCL) and GSH reductase. The polyol pathway is activated in T2D primarily due to hyperglycaemia. Increased flux of glucose through the polyol pathway, which consumes NADPH as a cofactor, may also deplete reconversion of GSSG to GSH leading to an oxidative state.

GSH deficiency has been described in many human diseases, including T2D (Atkuri *et al.* 2007; Townsend, Tew & Tapiero 2003). Specifically, clinical studies have documented decreased GSH levels and increased GSSG levels in erythrocytes and plasma of patients with T2D (Bravi *et al.* 2006; De Mattia *et al.* 1998; De Mattia *et al.* 1994; Jain & McVie 1994; Murakami 1991; Murakami *et al.* 1989; Paolisso *et al.* 1993a; Paolisso *et al.* 1993b; Paolisso *et al.* 1992; Samiec *et al.* 1998). Altered GSH/GSSG ratio has also been correlated to worsening hyperglycaemia (Jain & McVie 1994; Murakami *et al.* 1989; Sharma *et al.* 2000; Yoshida *et al.* 1995). The mechanisms contributing to the depletion of intracellular GSH and the consequences of this in development of T2D is currently an active area of research.

The role of oxidative stress in the pathogenesis of T2D through increased inflammation and production of ROS is widely accepted (Collier *et al.* 1990; Ghiselli *et al.* 1992; Jennings *et al.* 1987a; Jennings *et al.* 1987b; Tran *et al.* 2004). Increasing circulating GSH levels can improve insulin sensitivity in people with T2D, emphasising the importance of inflammation and oxidative stress in the pathogenesis of glucose intolerance in T2D (Osawa & Kato 2005; Paolisso *et al.* 1993a; Paolisso *et al.* 1993b; Paolisso *et al.* 1992). The current understanding of the regulation of GSH in T2D is incomplete. However, it is hypothesised that the decreased circulating GSH/GSSG ratio observed in individuals with T2D may be a reflection of the increased consumption of GSH due to oxidation to GSSG, induced by chronic exposure to ROS and increased levels of inflammatory cytokines, such as TNF-α (Bravi *et al.* 2006; Malmezat *et al.* 2000).

Alteration in the GSH/GSSG ratio may also occur following interference with GSH biosynthetic pathways and mechanisms involved in the reduction of GSSG to regenerate GSH. Decreased activity of γ-GCL has been documented in erythrocytes from individuals with T2D, perhaps contributing to the lower GSH levels that have been described in this population (Murakami 1991; Murakami *et al.* 1989; Yoshida *et al.* 1995). It is believed that decreased activity of γ-GCL could be caused by glycation directly related to the generation of AGE (Murakami 1991). There is also evidence that the activity of GSH reductase is decreased in T2D, in addition to the availability of the critical substrate NADPH, which is consumed by over-stimulation of the polyol pathway (Bravi *et al.* 1997; Brownlee 2005; Goldin *et al.* 2006;

Murakami 1991; Murakami *et al.* 1989). While glucose is predominantly metabolised via glycolysis and the tricarboxylic acid cycle (TCA), during hyperglycaemia, excess glucose is shunted through the polyol pathway (Brownlee 2001; Du *et al.* 2000). Increased activation of this pathway is well documented in T2D and may contribute to the dysregulation of GSH/GSSG pathways through consumption of NADPH, required for the resynthesis of GSH from GSSG (Figure 2.11) (Greene, Stevens & Feldman 1999; Lee & Chung 1999). Under normal cellular conditions, GSH reductase maintains a high GSH/GSSG ratio by reconversion of GSSG to GSH, but in cases of extreme oxidative stress, GSSG can also rapidly be exported from the cell (Barycki 2008). Therefore, the reduction in adenosine triphosphate (ATP)-dependent extracellular transport of GSSG that has been described in T2D may further contribute to increased GSSG levels (Murakami 1991; Murakami *et al.* 1989).

The function of GSH extends beyond protection from oxidative stress, to synthesis of DNA precursors, regulating sulfhydryl-dependent enzymes, gene expression, facilitating protein folding and maintaining normal protein function (Barycki 2008; Cappel & Gilbert 1988; Gerard-Monnier & Chaudiere 1996; Kosower & Kosower 1969; Malmezat *et al.* 2000; Morris *et al.* 2012a; Staal *et al.* 1990; Zhang, Forman & Choi 2005). A role for GSH in regulating immune responses has also been described. The immunomodulatory activities of GSH are believed to occur primarily through regulation of the activation of NF-κB (Staal *et al.* 1990). One of the earliest responses to infection or inflammation is activation of the transcription factor, NF-κB, which regulates the expression of many genes related to cytokine production and inflammatory processes (Libermann & Baltimore 1990). In unstimulated cells, NF-κB is present in the cytoplasm in association with its inhibitor (IκB). IκB prevents NF-κB from migrating to the nucleus where it activates transcription of target genes involved in inflammation (Baeuerle & Baltimore 1988a, 1988b; Ghosh & Baltimore 1990). Activation of protein kinase C (PKC) phosphorylates the IκB/NF-κB complex to liberate NF-κB. GSH levels can potentially influence multiple regions along the signal transduction pathway for NF-κB. GSH may i) block activation of PKC preventing liberation of NF-κB; ii) interfere directly with IκB phosphorylation or transport of activated NF-κB to the nucleus; iii) be involved indirectly by scavenging ROS that would otherwise activate NF-κB (Staal *et al.* 1990). Whilst the exact

mechanisms have not been clearly defined, it is apparent that depletion of GSH facilitates cytokine-stimulated NF-κB activation (Staal *et al.* 1990). The influence of altered GSH/GSSG ratio on cytokine production both in aetiology of T2D and effect on ability to respond to subsequent immune insults, such as *B. pseudomallei* infection, is yet to be described.

2.3 Type 2 Diabetes and Infectious Diseases

It is widely acknowledged that people with T2D are at increased risk of infections (Shah & Hux 2003). In one of the largest prospective studies conducted, subjects with T2D were more susceptible to lower respiratory tract infections and skin and soft tissue infections; incidentally the two major clinical presentations associated with melioidosis (Muller *et al.* 2005). T2D is a multifaceted disease with a complex pathogenesis, connected to a vast array of metabolic alterations. Due to the progressive and multifactorial nature of T2D and its close association with other metabolic alterations, it is extremely difficult to isolate the underlying mechanisms responsible for the comorbidity between metabolic and infectious diseases such as that observed between T2D and melioidosis.

While evidence is mounting that innate immunity is compromised in T2D, there is still disagreement on the exact mechanisms and cell subsets involved (Geerlings & Hoepelman 1999; Gupta *et al.* 2007; Joshi *et al.* 1999). While some studies have reported decreased proinflammatory cytokines, ROS and NOS production, other studies have found the opposite trends (Delamaire *et al.* 1997; Desfaits, Serri & Renier 1998; Naguib *et al.* 2004; Noritake *et al.* 1992; Park *et al.* 2009; Ptak *et al.* 1998; Saiki *et al.* 1980; Scatena *et al.* 1998; Stalenhoef *et al.* 2008; Vallerskog, Martens & Kornfeld 2010; Yamashiro *et al.* 2005; Zykova *et al.* 2000; Zykova *et al.* 2004). These discrepancies may be a result of differences between the infecting pathogen and kinetics of disease progression, aetiological differences of diabetes and level of glycaemic control in the host. Often studies that investigate comorbid diabetes do not distinguish between T2D and T1D, despite their distinct aetiologies, nor the potential confounding effect of the use of hypoglycaemic agents and other diabetic treatments. This is important to consider especially since certain hypoglycaemic treatments have been shown to improve outcome from

B. pseudomallei infection (Koh *et al.* 2011). One of the most commonly used drugs to treat T2D is the sulfonylurea, glyburide, which has been shown to provide a survival advantage in patients with sepsis (Esper, Moss & Martin 2009; Moss *et al.* 2000). This has been attributed to the known anti-inflammatory properties of glyburide, specifically involving inhibition of the inflammasome (Lamkanfi *et al.* 2009). The inflammasome is particularly important in driving macrophage inflammatory responses, in which it activates caspase-1 leading to secretion of IL-1β and IL-18. Inflammasome activation also indirectly promotes recruitment of neutrophils, which is believed to contribute to excessive tissue damage (Martinon, Burns & Tschopp 2002; Meduri *et al.* 1995). The survival benefit of glyburide in patients with comorbid T2D and melioidosis could also be secondary to the improved control of T2D. This highlights the difficulty in deriving conclusions from patient studies, representing a range of different stages of T2D and level of control of the disease and the added pharmacological influences of different treatments. Some of these potentially confounding factors can be overcome through the use of animal models of T2D to understand the pathogenesis of comorbid infections.

2.3.1 Type 2 diabetes and tuberculosis comorbidity

Mycobacterium tuberculosis is an intracellular bacterium that shares many similarities with *B. pseudomallei*. In contrast to the paucity of research on melioidosis, there is substantial evidence supporting defects in the immune response to *M. tuberculosis* infection in patients with T2D (Dooley & Chaisson 2009). People with T2D have a 3-fold greater risk of acquiring tuberculosis and similarly to melioidosis, a greater risk of relapse post-treatment (Baker *et al.* 2011b). The risk of mortality from tuberculosis is 5-fold greater in individuals with T2D, compared to those without this risk factor (Baker *et al.* 2011b).

Clearance of *M. tuberculosis* infection relies heavily on development of CMI responses. This involves production of IL-12 by antigen presenting cells, which favours development of T_H1 cells that are involved in the secretion of IFN-γ necessary for effective macrophage activation (Figure 2.12). Impaired T_H1-type responses in T2D are believed to contribute to increased susceptibility to *M. tuberculosis* infection, however the precise mechanisms responsible for defective

T_H1 responses are not known (Millman *et al.* 2008; Stalenhoef *et al.* 2008). It has been postulated that a decreased GSH/GSSG ratio within immune cells of patients with T2D may be responsible for reduced IL-12 production (Alam *et al.* 2010). The intracellular GSH/GSSG redox state of macrophages in particular, has been shown to influence the cytokine profile generated in response to an immune challenge, thereby affecting the development of either T_H1 or T helper type 2 (T_H2) responses (Kato, Mikami & Natsuno 2008; Murata *et al.* 2002a; Murata, Shimamura & Hamuro 2002; Peterson *et al.* 1998). Following stimulation with LPS, reductive macrophages, with a high concentration of GSH, produce IL-12, which drives T_H1 responses (Murata, Shimamura & Hamuro 2002). In contrast, IL-12 production is lower following stimulation of oxidative macrophages, with comparatively low intracellular GSH levels, which instead produce cytokines such as IL-4 and IL-10 that stimulate T_H2 responses (Murata *et al.* 2002a; Parmely, Wang & Wright 2001). This potentially explains why macrophages with high levels of GSH exhibit enhanced microbicidal mechanisms against intracellular pathogens such as *M. tuberculosis* (Venketaraman *et al.* 2003) and *Leishmania* (Buchmuller-Rouiller *et al.* 1995). Similarly, high intracellular GSH concentrations within macrophages may be beneficial for the control of *B. pseudomallei* infection, though this is yet to be investigated. Conversely, it is tempting to speculate that lower GSH levels in macrophages of diabetic hosts may interfere with the generation of protective T_H1 cell responses, thereby facilitating persistence of the bacterium.

In addition to its role in cytokine regulation, there is some evidence to suggest GSH may exert direct microbicidal activity against *Mycobacteria* (Green, Seth & Connell 2000; Venketaraman *et al.* 2003). The basis of this toxicity and the extent to which it affects other microbes is unknown (Green, Seth & Connell 2000). Since GSH is present in high concentrations in eukaryotic cells, it is possible that such antibacterial properties may contribute to increased killing of other intracellular bacteria such as *B. pseudomallei*, though to our knowledge this has not been studied.

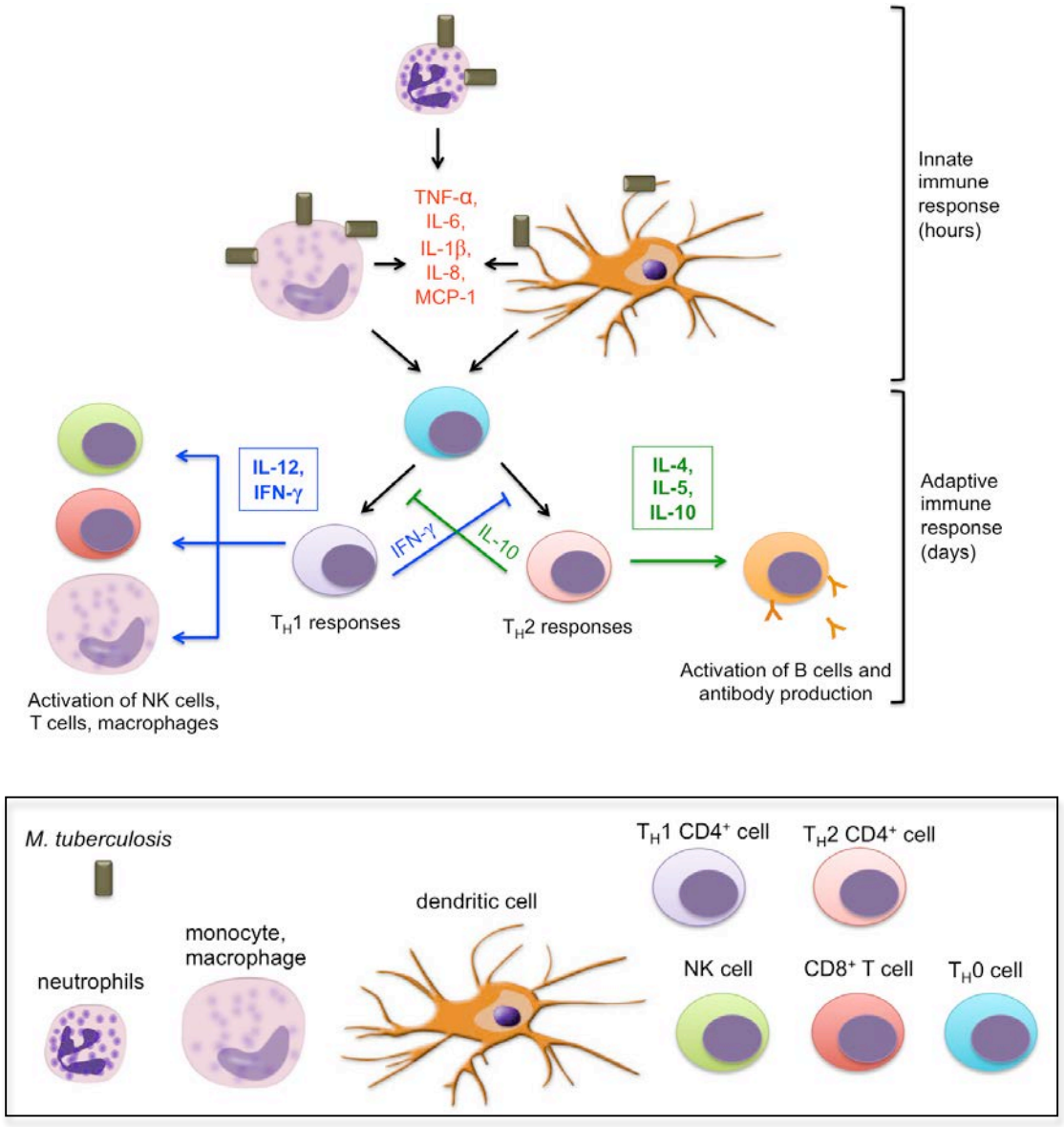

Figure 2.12 Immune responses to *M. tuberculosis*
Cell-mediated immunity (CMI) is critical for host protection against *M. tuberculosis*. Development of T_H1 responses is critical for activation of CMI and relies on production of the cytokines, IL-12 and IFN-**γ.** Protective immune responses to other intracellular bacteria, such as *B. pseudomallei,* are thought to be similar. Adapted from Askeland *et al.* (2012).

2.3.2 Type 2 diabetes and melioidosis comorbidity

Despite being the most common underlying risk factor for melioidosis, the implications of metabolic and immunological alterations associated with T2D on the immune response generated following infection with *B. pseudomallei* is poorly

understood. To date, most research has focused on characterising the immune responses to *B. pseudomallei* infection in hosts with T2D through *in vitro* or *ex vivo* experiments. One of these initial *ex vivo* studies found that migration in response to IL-8 and stimulation with *B. pseudomallei* in culture was impaired in neutrophils derived from individuals with T2D compared to neutrophils obtained from non-diabetic individuals (Chanchamroen *et al.* 2009). The authors postulated that this might result in delayed accumulation of neutrophils at the foci of infection and impaired early control of *B. pseudomallei* in individuals with T2D. However, it is difficult to confirm this without the availability of an animal model of T2D to confirm the importance of these findings *in vivo*. Phagocytosis of *B. pseudomallei* was significantly impaired in neutrophils from individuals with T2D and poor glycaemic control (HbA1c levels greater than 8.5%), with a trend for defective oxidative burst activity, compared to neutrophils from non-diabetic individuals (Chanchamroen *et al.* 2009). Neutrophils from individuals with T2D also had a reduced ability to delay apoptosis following *B. pseudomallei* exposure, which may contribute to increased persistence of *B. pseudomallei* in the diabetic host (Chanchamroen *et al.* 2009). Adding to these findings is the recent description of impaired production of neutrophil extracellular traps (NET) in individuals with T2D in response to *B. pseudomallei* (Riyapa *et al.* 2012). Given neutrophils are necessary for initial containment of *B. pseudomallei* infection, reduced bactericidal activity and functional longevity of neutrophils in individuals with T2D may have important implications in early *B. pseudomallei* dissemination and establishment of infection.

Altered early activation of neutrophils and monocytes with *B. pseudomallei* may underpin the increased susceptibility of individuals with T2D to melioidosis, particularly those with poorly controlled glycaemia. Using an *ex vivo* human whole-blood model, contrasting inflammatory profiles were observed in blood from individuals with poorly controlled T2D, compared to individuals with well controlled T2D and non-diabetic individuals following stimulation with *B. pseudomallei* (Morris *et al.* 2012b). Reduced expression of pathogen recognition receptors on monocytes and neutrophils from poorly controlled individuals with T2D was observed in the first 4 hours following exposure to *B. pseudomallei* (Morris *et al.* 2012b). This reduced leukocyte activation potentially contributes to the impaired migratory capacity previously described in patients with T2D (Chanchamroen *et al.*

2009). Impaired activation of neutrophils and monocytes from poorly controlled diabetics following *ex vivo* stimulation with *B. pseudomallei* also coincided with impaired functional and cytokine responses (Morris *et al.* 2012b).

In another recent *ex vivo* study, impairments in IL-12 and IFN-γ production and poor *B. pseudomallei* killing was observed in peripheral blood mononuclear cells (PBMC) from patients with T2D, compared to PBMC from non-diabetic individuals (Tan *et al.* 2012). Similar impairments in IL-12 and IFN-γ production in PBMC from patients with T2D have been described in response to *M. tuberculosis* infection (Al-Attiyah & Mustafa 2009). The impaired production of IL-12 in PBMC from patients with T2D in response to stimulation with *B. pseudomallei* correlated directly with reduced intracellular GSH content (Tan *et al.* 2012). Restoration of GSH levels using *N*-acetyl *L*-cysteine (NAC) and other GSH derivatives was shown to improve IL-12 and IFN-γ production in PBMC and increased bacterial killing of *B. pseudomallei* and *M. tuberculosis* (Tan *et al.* 2012). This is an exciting avenue of research to pursue. Modulation of GSH levels to improve macrophage responses to intracellular bacterial infections potentially provides a novel therapeutic approach to improve control of *B. pseudomallei* infection in individuals with T2D. Availability of a suitable animal model of comorbid T2D and melioidosis would facilitate further investigations of the influence of GSH on the pathogenesis of *B. pseudomallei* infection and evaluation of the efficacy of GSH modulatory agents as adjunctive therapies.

2.4 Murine Models of Type 2 Diabetes

A complete understanding of the pathogenesis of complex diseases like T2D cannot be obtained from *in vitro* or *ex vivo* studies alone. Animal models are essential in the development and application of therapeutics, which will facilitate advancements in treatment and management of T2D and comorbid melioidosis. Prior to this thesis, the only murine study of the comorbidity between T2D and *B. pseudomallei* infection involved a streptozotocin-induced diabetes model (Chin, Monack & Nathan 2012; Williams *et al.* 2011). This is a chemically-induced model which causes diabetes by destroying the pancreatic beta cells, akin to the pathology of T1D. The caveat of this model is that the majority of patients with comorbid melioidosis have T2D (Simpson

et al. 2003), which has an aetiology and pathogenesis distinct from that of T1D. While streptozotocin-induced T1D does lead to hyperglycaemia, the metabolic complications of T2D are closely associated with dyslipidaemia and insulin resistance, features that are not represented in this model. This may have important implications when investigating downstream mechanisms that contribute to increased susceptibility to melioidosis. The variability in streptozotocin dosing quantities and schedules between studies and inadvertent toxicity of streptozotocin on the renal and hepatic systems further complicates findings using this model (Imaeda *et al.* 2002; Kazumi *et al.* 1978). It is critical that a murine model of T2D and melioidosis comorbidity is characterised that incorporates the clinical metabolic complications of T2D in humans, including dyslipidaemia and insulin resistance. Murine models are complicated by the intrinsic resistance of many strains to the development of T2D and associated metabolic complications, and the complex, multifactorial aetiopathogenesis of T2D involving interplay between genetic susceptibility and environmental risk factors (Inadera 2013).

2.4.1 Monogenic murine models of type 2 diabetes

Since its discovery in 1965 (Hummel, Dickie & Coleman 1966), the leptin receptor deficient *Lept*^{db} mouse has been one of the most widely used monogenic murine models of T2D (Panchal & Brown 2011). A single recessive genetic mutation in the leptin receptor gene located on chromosome 4 causes homozygous (*db/db*) mice to develop morbid obesity, as shown in Figure 2.13, and progressive glucose intolerance (Coleman 2010). Heterozygous (*db/+*) mice maintain normal body weight and glucose tolerance and are used as littermate controls (Panchal & Brown 2011). The *Lept*^{db} mouse model has been used previously to examine the effects of T2D on the immunopathogenesis of infectious comorbidities, including Gram negative and Gram positive bacterial infections (Mastropaolo *et al.* 2005; Park *et al.* 2009). Until now, this model has not been used in the field of melioidosis research.

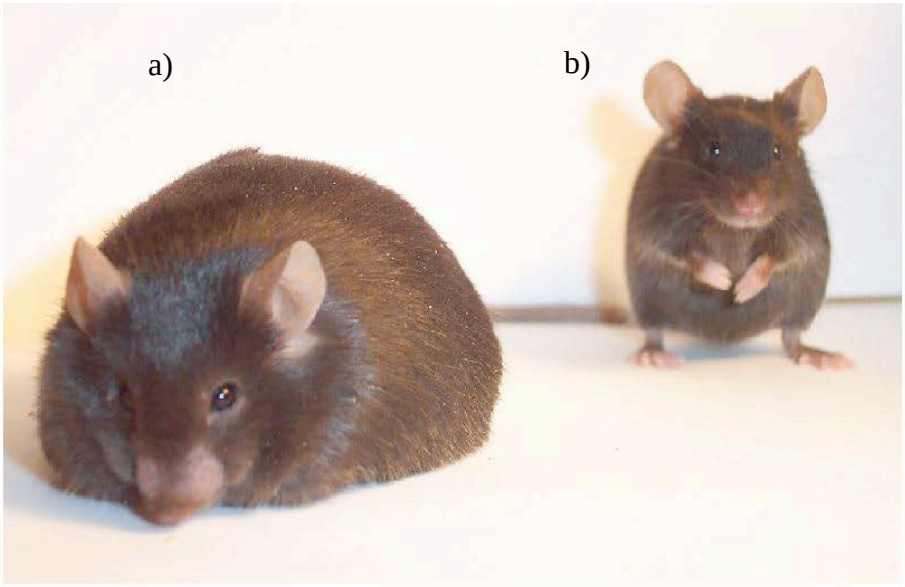

Figure 2.13 Gross difference in body weight between *db/db* and *db/+* mice
a) Homozygous (*db/db*) mice have defective leptin signalling and develop severe obesity compared to b) metabolically normal heterozygous (*db/+*) littermates.

2.4.2 Polygenic murine models of type 2 diabetes

Genetically altered mouse models, such as the *Lept*^{db} mice, fail to incorporate the significant nutritional and polygenic determinants involved in the aetiology of T2D in humans, as previously described. Diet-induced animal models of T2D have been used in the past few decades since they are considered to be more reflective of the clinical aetiopathology of the disease in humans (Kanasaki & Koya 2011). However, there is considerable discordance between studies using diet-induced models and metabolic phenotypes associated with these models (Kanasaki & Koya 2011; Karasawa *et al.* 2009). Inconsistencies between studies are likely to be complicated by differences in dietary composition and fat content, age of mice and duration of feeding, along with the rodent strain and gender (Scroyen, Hemmeryckx & Lijnen 2013).

Surwit *et al.* (1988) were the first to describe a diet-induced model of T2D in C57BL/6 mice fed a HFD (60% energy from fat). However, the progression to overt hyperglycaemia using this diet is moderate, arguably complicated by the genetic resistance of C57BL/6 mice to diet-induced metabolic alterations (Karasawa *et al.*

2009; Kooptiwut *et al.* 2002). To overcome this limitation, Karasawa *et al.* (2009) described a diet-induced model of T2D using B6D2F1 mice (the first generation [F1] cross of C57BL/6 and DBA/2 mice). B6D2F1 mice fed a HFD (52.1% energy from fat) developed hyperglycaemia after 8 weeks of feeding. Importantly, the fat content in the diet used by Karasawa *et al.*, (2009) markedly exceeds global dietary intakes, which range from 23-34% of energy, and consists predominantly of saturated fats (WHO 2003). There are limited studies that have reported the development of T2D in mice fed a moderately HFD (40% of energy from fat) that is more reflective of typical human dietary intakes, particularly given that fat intake may be even lower in developing nations.

The usefulness of animal models of disease is determined by the degree to which they closely reflect clinical disease. Importantly, T2D is so much more than a state of hyperglycaemia. A diet-induced model of T2D provides the opportunity to incorporate the complex metabolic and immunological mechanisms that occur concurrently in clinical T2D associated with dietary modification, including dyslipidaemia, hypertension, adipose tissue abnormalities and chronic inflammatory changes. Encompassing the vast range of complications in T2D that potentially sum together to alter the course of *B. pseudomallei* infection is not possible without *in vivo* studies and the development of a suitable animal model.

2.5 Conclusion

Although the connection between melioidosis and T2D has been recognised for decades, the mechanisms behind it are poorly understood. Melioidosis is currently responsible for significant morbidity and mortality in the tropics, particularly in the population with T2D. As T2D is anticipated to become the biggest health concern of this century, the paucity of research investigating the immunological mechanisms contributing to T2D and comorbid bacterial infections, such as melioidosis, needs to be addressed. The rising incidence of T2D in melioidosis endemic regions provides even greater impetus for this research. Undoubtedly, the immune response to *B. pseudomallei* infection in individuals with T2D is altered. Nevertheless, it is not clear what specific mechanisms are responsible for impaired immune responses and how this affects the development of protection to *B. pseudomallei* infection.

Currently, diagnostic and therapeutic research utilises non-diabetic models and the translatability of this to hosts with T2D is questionable given the likely differences in immune responses and disease mechanisms. Ideally, an animal model reflective of T2D is urgently needed to facilitate advancements in disease management in the population most at risk of infectious diseases, such as melioidosis. Significant advancements in the treatment and management of melioidosis will come from understanding the critical mechanisms involved in the underlying comorbidities, like T2D, that dramatically influence morbidity and mortality. A murine model reflective of T2D and comorbid *B. pseudomallei* infection will be particularly important for the development and evaluation of novel therapeutic strategies for melioidosis and potentially other infectious comorbidities of T2D.

CHAPTER 3
GENERAL MATERIALS AND METHODS

3.1 Bacterial Isolates

3.1.1 *B. pseudomallei* strain

B. pseudomallei strain NCTC13178, from the Infectious Diseases and Immunopathogenesis Research Group collection at James Cook University, was used for the studies described in this thesis. NCTC13178 is a highly virulent clinical isolate that originated from a fatal case of melioidosis at TTH (Ulett *et al.* 2001). The subcutaneous infectious dose (ID_{50}) for this *B. pseudomallei* isolate has previously been determined to be 1×10^3 colony forming units (CFU) and 9×10^5 CFU in BALB/c and C57BL/6 mice, respectively (Barnes & Ketheesan 2005).

3.1.2 Preparation of bacterial inoculum

All experimental work with *B. pseudomallei* was conducted within a Class II biosafety cabinet in the Physical Containment 3 Laboratory at the School of Veterinary and Biomedical Sciences, James Cook University. *B. pseudomallei* was cultured on sheep's blood agar (SBA; Appendix 1.1.5) for 48 hours at 37°C. Pure colonies were selected and cultured aerobically for 24 hours in 20ml of tryptic soy broth (TSB; Oxoid, Australia) at 37°C. The culture broth was diluted 10-fold in fresh TSB and incubated at 37°C for a further 3 hours to bring bacteria to logarithmic phase. The bacteria were then washed twice with phosphate buffered saline (PBS; pH 7.2; Appendix 1.1.1), centrifuged at 4000*g* for 10 minutes and resuspended in PBS. The concentration of bacteria was determined by absorbance at an optical density (OD) of 650nm (Biochrom Libra S22 UV/Vis Spectrophotometer) and confirmed retrospectively by plating 10µl triplicates of 10-fold serial dilutions on Ashdown agar (Appendix 1.1.4) and enumerating colonies after incubation at 37°C for 24-48 hours.

3.2 Experimental Animals

3.2.1 Ethics approval

Animal experiments were conducted according to the National Health and Medical Research Council (NHMRC) guidelines, under Ethics Approval #A1556 granted by the James Cook University Animal Ethics Review Committee. All mice were obtained from the Small Animal Breeding Facility at the School of Veterinary and Biomedical Sciences at James Cook University, Townsville. Animals were housed in a 12-hour light controlled room and supplied with water *ad libitium*.

3.2.2 Feeding regimes

B6D2F1 and C57BL/6 mice (6 weeks of age) were randomly separated into dietary groups. The control group received standard rodent chow (4.8% fat, 20% protein; Specialty Feeds, Australia), while the experimental groups received a HFD based on a typical 'Western diet' (23% fat, 19% protein; #SF00-219 and #SF03-030, Specialty Feeds, Australia) for 10, 15 and 20 weeks as described. Food consumption was measured bi-weekly for the duration of the diet intervention. Food consumption was determined by subtracting the amount of food remaining or spilled in the cage after 24 hours from the initial amount of food supplied. To account for differences in consumption rates due to palatability of the diets, food intake was isometrically controlled. Control mice received an isometric quantity of standard rodent chow, based on the daily consumption of paired mice fed a HFD that was calculated during the previous week.

3.2.3 Assessment of metabolic parameters

Blood glucose, total cholesterol and triglycerides (n = 5 per group) were measured in blood from the lateral tail vein (Accutrend® Plus Cobas®, Roche Diagnostics, Germany). The glucose tolerance test (GTT) is widely used in the clinical diagnosis of T2D. Although this typically involves overnight fasting, it is a comparatively long time for mice. Furthermore, unlike humans, mice do most of their feeding during the night.

Initial findings from this study indicated that overnight fasted mice had severely depressed glucose levels. It has previously been determined that overnight fasting of mice increases insulin sensitivity, depletes glycogen stores and results in the release of FFA (Andrikopoulos *et al.* 2008). Therefore, a 6-hour morning fasting regimen was used prior to performing GTT in all subsequent experiments (n = 5 per group), with blood glucose measured at 0, 15, 30, 60 and 120 minutes following intraperitoneal glucose challenge (2g/kg body weight). Area under the curve (AUC), as calculated using the trapezoidal rule, was used to compare glucose tolerance between experimental groups. Blood glucose clearance rate was also calculated for each animal by determining the slope of the line ($m = (y1-y2) \times (x1-x2) - 1$) for blood glucose measurements between 15 and 60 minutes.

3.2.4 Infection with *B. pseudomallei*

Age-matched mice were inoculated subcutaneously above the right hindquarter with *B. pseudomallei*. The challenge dose was suspended in a volume of 100µl of PBS and was injected using a 1ml syringe and a 27 gauge needle. An additional group of uninfected, control mice received injections of an equivalent volume of PBS. Animals were monitored twice daily for 10 days post-infection and moribund mice were euthanased by carbon dioxide (CO_2) asphyxiation. Persistent infection with *B. pseudomallei* at the end of the experimental period was confirmed on necropsy of mice by the presence of gross visceral abscesses or determination of bacterial loads in tissue homogenates when abscesses were not present.

3.2.5 Organ bacterial loads

To compare dissemination and growth of *B. pseudomallei* within organs after subcutaneous infection, mice were euthanased and blood was collected in microtubes with heparin (Appendix 1.2.2) by cardiac puncture. Organs were removed aseptically, weighed and macerated in stomacher bags (Disposable Products, South Australia) with 500µl (lymph node; LN), 1ml (spleen, subcutaneous adipose tissue, lung), or 3ml (liver) of PBS (pH7.2). Serial 10-fold dilutions of whole blood and organ homogenates were plated on Ashdown agar in triplicate. In addition, 100µl of undiluted homogenate was plated where bacterial numbers were anticipated to be

low. Colonies were enumerated after 24-48 hours of incubation at 37°C and the mean concentration of bacteria were calculated in CFU/ml. The minimum and maximum limit of detection was 10 CFU/ml and 5×10^5 CFU/ml, respectively, for the spleen, liver, lung, blood and LN, and 5×10^3 CFU/ml and 5×10^8 CFU/ml, respectively for the subcutaneous adipose tissue (SAT) at the site of infection.

3.2.6 Histological analysis

Tissues collected for histological analysis were fixed in 10% neutral-buffered formalin for a minimum of 24 hours before processing on a 6-hour cycle using a Shandon Citadel 2000 Tissue Processor (Shandon Southern Products Ltd, United Kingdom). After embedding in paraffin wax, 5µm sections were cut and stained with haematoxylin and eosin (H & E; Appendix 1.1.2). Staining of liver with Herxheimer's solution (Appendix 1.1.3) was performed on formalin fixed tissues that were cryosectioned in optimal cutting temperature (OCT) medium (Tissue Tek, The Netherlands) and counterstained with haematoxylin.

3.3 Statistical Analysis

Statistical analysis was performed using GraphPad Software Version 6.0b. Kaplan-Meier survival curves were used to compare susceptibility to subcutaneous infection with *B. pseudomallei* between groups of mice. Additional statistical tests included unpaired Student's T-tests with Welch's correction for unequal variances where appropriate or Mann-Whitney tests for nonparametric data. These tests were used when comparing parameters between diabetic and non-diabetic mice such as cytokine levels. One- or two-way analysis of variance (ANOVA) with Bonferonni post hoc tests were used to compare normally distributed data from more than two groups or was otherwise analysed by Kruskal-Wallis nonparametric tests. For example, one-way ANOVA was used for comparing blood glucose between 3 dietary groups at the end of diet intervention, while two-way ANOVA was used for comparing differences in bacterial dissemination between diabetic and non-diabetic mice at multiple time-points. Significance was determined by $P \leq 0.05$ and data are presented as mean ± standard error of the mean (SEM).

CHAPTER 4
CHARACTERISATION OF A MONOGENIC MURINE MODEL OF TYPE 2 DIABETES AND *B. pseudomallei* INFECTION

4.1 Introduction

Animal models have been invaluable for understanding the immunopathogenesis and treatment of melioidosis. Since 1925, various animal models of melioidosis have been described, ranging from non-human primates to non-mammalian hosts (Gan *et al.* 2002; Miller, Pannell & Ingalls 1948; O'Quinn, Wiegand & Jeddeloh 2001; Stanton & Fletcher 1925). Large mammalian models of melioidosis have been less frequently described, with the last 50 years of melioidosis research predominantly involving small animal models; rats, mice and hamsters in particular (Dannenberg & Scott 1958; Gauthier *et al.* 2001; Leakey, Ulett & Hirst 1998; Titball *et al.* 2008; Woods 2002). Murine models of melioidosis have been most widely utilised for understanding host-pathogen interactions since inbred and outbred mouse strains display varying degrees of resistance to *B. pseudomallei* infection and develop a spectrum of clinical presentations similar to humans (Sivalingam, Ulett & Nelson 2012).

First described in 1998, the models of chronic and acute melioidosis using C57BL/6 and BALB/c mice, respectively, have been extensively characterised (Barnes & Ketheesan 2005; Barnes *et al.* 2001; Gauthier *et al.* 2001; Hoppe *et al.* 1999; Jeddeloh *et al.* 2003; Leakey, Ulett & Hirst 1998; Liu *et al.* 2002; Tan *et al.* 2008; Ulett, Ketheesan & Hirst 2000b). The C57BL/6-BALB/c animal model has been crucial for understanding the immunological determinants behind susceptibility to melioidosis and subsequent disease progression. Resistance to melioidosis in C57BL/6 mice is associated with earlier immune activation and greater control of *B. pseudomallei* growth compared to BALB/c mice, with the latter succumbing to overwhelming sepsis within days of infection (Koo & Gan 2006; Leakey, Ulett & Hirst 1998; Liu *et al.* 2002; Tan *et al.* 2008; Ulett, Ketheesan & Hirst 1998, 2000a).

Melioidosis is associated with a number of significant risk factors, which are strong determinants for disease progression and poor outcome (Currie, Chaowagul &

Cheng 2012; Currie, Ward & Cheng 2010). The most significant risk factor that has been consistently correlated with melioidosis is T2D (Currie *et al.* 2000b; Currie *et al.* 2004; Currie, Ward & Cheng 2010; Suputtamongkol *et al.* 1999; Suputtamongkol *et al.* 1994). The global incidence of T2D has reached unprecedented levels and is projected to continue to increase (IDF 2011). The molecular mechanisms responsible for the comorbidity between T2D and melioidosis are not known. T2D is a complex disease, with wide-ranging complications affecting multiple organ systems, including vascular pathologies, hepatic alterations, nephropathy, neuropathy and potential immunological dysfunction (Forbes & Cooper 2013). *In vitro* studies are limited in that they fail to incorporate this complex pathophysiology. Consequently, an animal model of T2D is critical to enable *in vivo* studies that can unravel the mechanisms contributing to the increased susceptibility to melioidosis.

Prior to the current study, the only *in vivo* investigation of the comorbidity between melioidosis and diabetes involved a streptozotocin-induced T1D model, whereby pancreatic beta cells are chemically destroyed (Williams *et al.* 2011). The caveat of this model is that the majority of patients with comorbid melioidosis have T2D (Simpson *et al.* 2003), which has an aetiology and pathogenesis distinct from that of T1D. This could have important implications when investigating the underlying molecular and immunological mechanisms that contribute to the increased susceptibility of T2D to melioidosis. Importantly, T2D is a chronic disease requiring sufficient time to become established and progressing in association with other metabolic alterations, including dyslipidaemia, adipose tissue abnormalities, chronic inflammation and insulin resistance, which are not represented in the streptozotocin-induced model. The variability in streptozotocin dosing quantities and schedules between studies and inadvertent toxicity of streptozotocin on the renal and hepatic systems further complicates findings using this model (Deeds *et al.* 2011).

It is crucial that a murine model of T2D and comorbid *B. pseudomallei* infection is characterised that incorporates the range of metabolic complications associated with T2D, including dyslipidaemia, insulin resistance and hyperglycaemia. The intrinsic resistance of many mouse strains to the development of T2D and associated metabolic complications, and the complex, multifactorial aetiopathogenesis of T2D involving interplay between genetic susceptibility and environmental risk factors,

complicates the development of a suitable animal model (Inadera 2013). At the time this project commenced, one of the most widely used murine models of T2D was the leptin receptor deficient *Lept*^{db} mouse (Panchal & Brown 2011). Since its initial description in 1966, the *Lept*^{db} mouse has been used for over 40 years as a model of T2D (Hummel, Dickie & Coleman 1966). A single recessive genetic mutation in the leptin receptor gene located on chromosome 4 causes homozygous (*db/db*) mice to develop morbid obesity and insulin resistance (Coleman 2010). Heterozygous (*db/+*) mice maintain normal body weight and glucose tolerance and are routinely included as littermate controls (Panchal & Brown 2011). Variations in the development and severity of diabetic complications have been reported depending on the genetic background on which the *db/db* mutation is expressed; BKS.Cg-*Dock7*^{m} *Lepr*^{db} /+ +/J (herein referred to as BKS) or C57BL/6J-*Dock7*^{m} *Lepr*^{db} /+ + (herein referred to as C57BL/6) strains (Coleman & Hummel 1973). Therefore, the specific aims of Chapter 4 were to:

i) compare the metabolic profile of *db/db* and *db/+* mice on C57BL/6 and BKS genetic backgrounds
ii) compare survival and organ bacterial loads after *B. pseudomallei* infection in *db/db* and *db/+* mice on C57BL/6 and BKS genetic backgrounds

4.2 Materials and Methods

4.2.1 Comparison of metabolic profiles of mice

All experiments described in this Chapter involved BKS.Cg-*Dock7*^{m} *Lepr*^{db} /+ +/J and C57BL/6J-*Dock7*^{m} *Lepr*^{db} /+ + mice. Eight week-old homozygous *db/db* mice, herein referred to as diabetic mice, were compared to gender and age-matched heterozygous *db/+* control littermates, herein referred to as non-diabetic mice. Body and organ weights, GTT, blood glucose, total cholesterol and triglycerides levels were compared as described in Chapter 3 (n = 5 mice per group). Intracellular lipid accumulation in hepatocytes was also determined by staining with Herxheimer's solution (Thermo Fischer Scientific, Australia; Appendix 1.1.3).

4.2.2 Comparison of susceptibility of mice to *B. pseudomallei* infection

Mice (8-12 weeks of age) were subcutaneously infected with 4.5×10^5 CFU (equivalent to $0.5\times ID_{50}$) of *B. pseudomallei* as described in Chapter 3 (Barnes & Ketheesan 2005). Survival was monitored for 10 days (n = 5 mice per group) and bacterial loads were determined at 24 and 72 hours post-infection (n = 5 mice per group) according to methods described in Chapter 3. Data for bacterial loads were logarithmically transformed and expressed as the mean $\log_{10}$ CFU ± SEM.

4.2.3 Statistical analysis

Statistical analysis was performed using GraphPad Prism Software Version 6.0b. Kaplan–Meier survival curves were used to compare susceptibility to infection with *B. pseudomallei* between diabetic and non-diabetic mice. All other metabolic parameters were compared by two-way ANOVA with Bonferonni post hoc tests. The trapezoidal rule was used to determine AUC for the GTT. Statistical significance was determined by $P \leq 0.05$ and data was expressed as mean ± SEM.

4.3 Results

4.3.1 Metabolic profiles of C57BL/6 and BKS diabetic and non-diabetic mice

C57BL/6 diabetic mice had greater total body mass compared to non-diabetic littermates, consisting of an increase in VAT, SAT and liver mass ($P < 0.05$; Table 4.1). The increased weight gain in diabetic mice was associated with increased daily energy intake, which was more than 60% higher compared to non-diabetic littermates ($P < 0.05$; Table 4.1) and accompanied by extensive intracellular lipid accumulation in hepatocytes compared to non-diabetic mice (Figure 4.1a). Weight gain in C57BL/6 diabetic mice was also accompanied by impaired glucose tolerance with a higher peak in blood glucose at 30 and 60 minutes following challenge compared to non-diabetic mice ($P < 0.05$; Figure 4.2a) and delayed glucose clearance (AUC, $P < 0.05$; Table 4.1) indicative of insulin resistance. Fasting blood glucose tended to be higher in C57BL/6 diabetic mice ($P = 0.051$), although

cholesterol ($P = 0.156$) and triglyceride ($P = 0.178$) levels were comparable with non-diabetic littermates (Table 4.1).

Table 4.1 Metabolic profiles of C57BL/6 and BKS diabetic and non-diabetic mice

Metabolic Parameter	C57BL/6		Significance	BKS		Significance
	Diabetic ($n = 5$)	Non-diabetic ($n = 5$)		Diabetic ($n = 5$)	Non-diabetic ($n = 5$)	
Body mass (g)	32.1 ± 0.6	23.2 ± 0.9	* $P < 0.001$	34.4 ± 1.1	25.1 ± 0.4	* $P < 0.001$
VAT (g)	2.1 ± 0.1	0.2 ± 0.0	* $P < 0.001$	1.9 ± 0.1	0.2 ± 0.1	* $P < 0.001$
SAT (g)	0.4 ± 0.0	0.1 ± 0.0	* $P = 0.003$	0.5 ± 0.1	0.1 ± 0.0	* $P < 0.001$
Liver (g)	2.5 ± 0.2	1.0 ± 0.0	* $P < 0.001$	2.2 ± 0.1	1.1 ± 0.1	* $P < 0.001$
Daily energy consumption (kJ/mouse)	99.3 ± 2.8	60.9 ± 1.2	* $P < 0.001$	108.1 ± 3.8	57.9 ± 2.6	* $P < 0.001$
Cholesterol (mmol/L)	3.0 ± 0.5	2.2 ± 0.2	$P = 0.156$	2.7 ± 0.3	2.44 ± 0.1	$P = 0.988$
Triglycerides (mmol/L)	1.2 ± 0.2	0.9 ± 0.1	$P = 0.178$	2.0 ± 0.2	1.2 ± 0.1	* $P = 0.002$
Blood glucose (mmol/L)	11.5 ± 1.4	5.7 ± 0.4	$P = 0.051$	15.8 ± 2.6	6.7 ± 0.3	* $P = 0.006$
Glucose intolerance (AUC)	2,642 ± 492	1,065 ± 177	* $P = 0.010$	2,533 ± 260	1,195 ± 32	* $P = 0.024$

AUC – area under the glucose curve; SAT – subcutaneous adipose tissue; VAT – visceral adipose tissue; mean ± SEM.

Similar to C57BL/6 mice, BKS diabetic mice were significantly heavier compared to their non-diabetic littermates, accompanied by increases in VAT, SAT and liver mass ($P < 0.05$; Table 4.1). Intracellular lipid accumulation was observed in hepatocytes from BKS diabetic mice (Figure 4.1b), which is thought to be a primary mechanism contributing hepatic insulin resistance. Consistent with this, BKS diabetic mice were hyperglycaemic compared to non-diabetic littermates ($P < 0.05$;

Table 4.1) and exhibited severe impairments to glucose tolerance, with higher blood glucose levels within 15, 30 and 60 ($P < 0.05$) minutes following glucose challenge (Figure 4.2b) and greater AUC ($P < 0.05$; Table 4.1), indicative of insulin resistance. Whilst total cholesterol was comparable ($P = 0.988$), serum triglyceride levels were higher in BKS diabetic mice compared to non-diabetic littermates ($P < 0.05$; Table 4.1). Triglycerides were also significantly higher in diabetic mice on a BKS compared to a C57BL/6 background ($P < 0.05$; Table 4.1).

a) C57BL/6 b) BKS

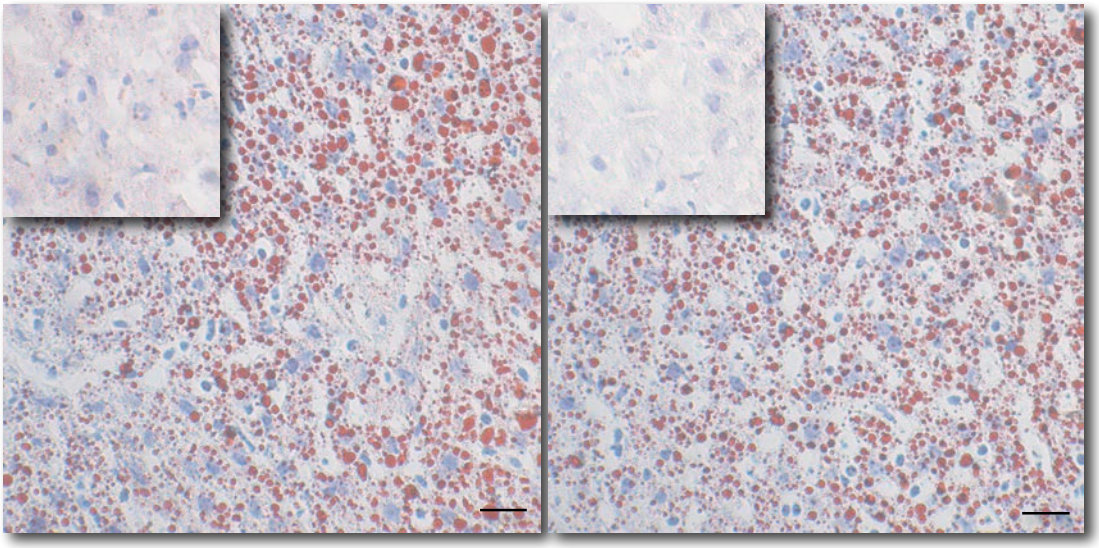

Figure 4.1 Hepatic lipid accumulation in C57BL/6 and BKS diabetic mice
Compared to hepatocytes of non-diabetic littermates (inset), accumulation of ectopic lipids (red) was observed in hepatocytes of a) C57BL/6 and b) BKS diabetic mice. Magnification: 400×; scale: 20μm.

4.3.2 Susceptibility to *B. pseudomallei* infection and bacterial dissemination

C57BL/6 and BKS diabetic mice were significantly more susceptible to subcutaneous infection with *B. pseudomallei* than their non-diabetic littermates. Within 96 hours post-infection, 60% of C57BL/6 diabetic mice ($P < 0.05$; Figure 4.3a) and 100% of BKS diabetic mice ($P < 0.05$; Figure 4.3b) succumbed to *B. pseudomallei* infection. In contrast, no deaths occurred in non-diabetic mice during the 10 day experimental period. Establishment of *B. pseudomallei* infection was confirmed in surviving non-diabetic mice by the presence of abscesses in the

spleen and liver. It was also noted that diabetic mice became severely hypoglycaemic preceding death, while homeostatic glycaemic levels were maintained in non-diabetic mice during the experimental period. After 72 hours post-infection, blood glucose was almost 3-fold lower in C57BL/6 diabetic mice ($P < 0.05$; Figure 4.2c) and almost 5-fold lower in BKS diabetic mice compared to baseline levels ($P < 0.05$; Figure 4.2d).

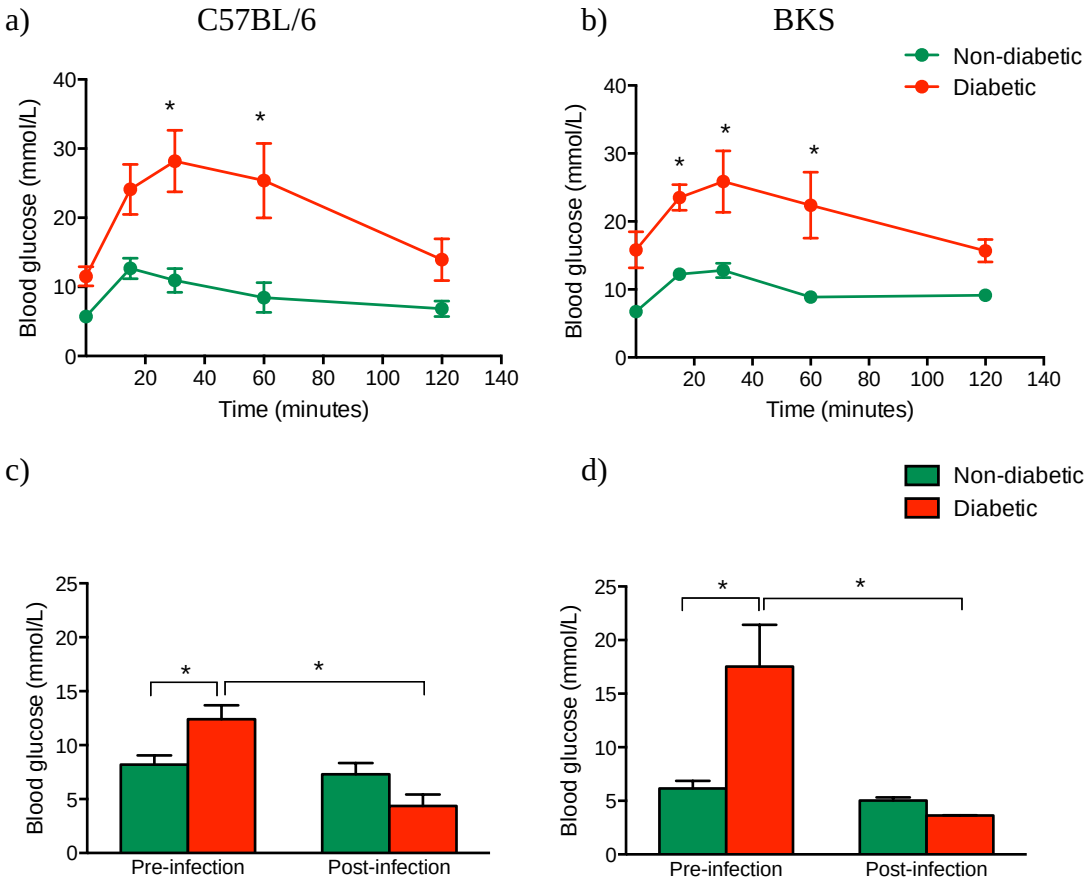

Figure 4.2 Glucose tolerance in C57BL/6 and BKS diabetic and non-diabetic mice and blood glucose following infection
Glucose clearance following an intraperitoneal glucose challenge (2g/kg body mass) was severely impaired in diabetic mice compared to non-diabetic mice on both the a) C57BL/6 and b) BKS background. Mice were infected subcutaneously with 4.5×10^5 CFU of *B. pseudomallei.* Decreased blood glucose at 72 hours post-infection in c) C57BL/6 and d) BKS diabetic mice compared to uninfected diabetic mice coincided with increased mortality compared to non-diabetic littermates. $n = 5$ per group; * $P < 0.05$; mean ± SEM.

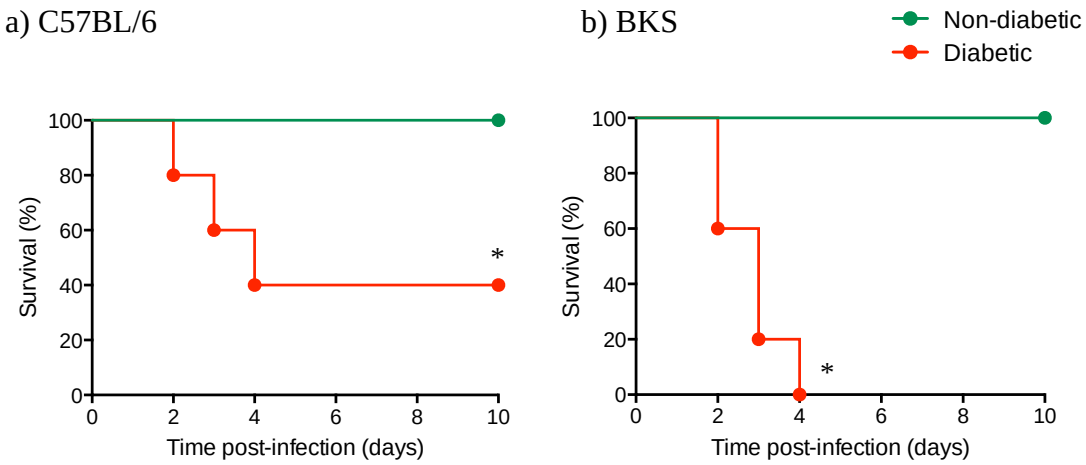

Figure 4.3 Survival of C57BL/6 and BKS diabetic and non-diabetic mice following infection with *B. pseudomallei*
Following subcutaneous infection with 4.5×10^5 CFU of *B. pseudomallei*, diabetic mice on both the a) C57BL/6 and b) BKS backgrounds were significantly more susceptible than their non-diabetic littermates. $n = 5$ per group; * $P < 0.05$.

At 24 hours post-infection, *B. pseudomallei* levels were comparable between C57BL/6 diabetic and non-diabetic mice in the spleen ($P = 0.457$), liver ($P > 0.999$) and SAT at the site of infection ($P > 0.999$; Appendix 2.1). By 72 hours post-infection, *B. pseudomallei* growth was significantly higher in the SAT of C57BL/6 diabetic mice compared to non-diabetic littermates ($P < 0.05$; Figure 4.4a). Similar to C57BL/6 mice at 24 hours post-infection, *B. pseudomallei* levels were comparable in the spleen ($P = 0.254$), liver ($P = 0.276$) and SAT ($P = 0.110$) of BKS diabetic and non-diabetic mice (Figure 4.4b). Due to rapid mortality of BKS diabetic mice following infection with *B. pseudomallei*, bacterial loads could not be determined at 72 hours post-infection.

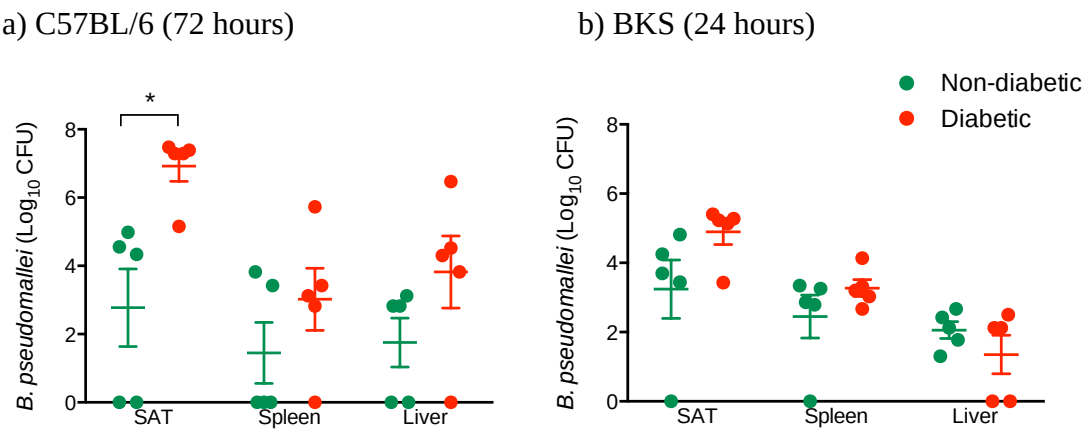

Figure 4.4 Dissemination of *B. pseudomallei* in C57BL/6 and BKS diabetic and non-diabetic mice
Mice (n = 5 per group) were infected subcutaneously with 4.5×10^5 CFU of *B. pseudomallei*. Levels of *B. pseudomallei* were greater in subcutaneous adipose tissue (SAT) at the site of infection in a) C57BL/6 diabetic mice by 72 hours post-infection compared to non-diabetic littermates ($P < 0.05$) and tended to be higher in the spleen and liver. b) At 24 hours post-infection, growth of *B. pseudomallei* also tended to be higher in the SAT of BKS diabetic mice at the site of infection ($P = 0.110$) compared to non-diabetic littermates, with all BKS diabetic mice succumbing to infection within 72 hours. * $P < 0.05$; mean Log_{10} CFU ± SEM.

4.4 Discussion

At the time of this project, leptin receptor deficient *Lept*^{db} mice were used widely as a model for obesity and T2D (Lindstrom 2007). Benefits of the *Lept*^{db} monogenic model included its widespread availability and the relatively short time required to develop complications characteristic of diabetes compared to other models. Obesity in this model is caused by hyperphagia, a direct consequence of leptin signalling-deficiency. It has also been shown that they have increased food efficiency due to reduced energy expenditure (Lindstrom 2007). Leptin is a pleiotropic hormone that has a major role in regulating energy intake through anorectic signalling (Fernandez-Fernandez *et al.* 2006; Prodi & Obici 2006). Leptin and insulin signalling pathways are intricately connected and impaired leptin signalling can exacerbate insulin resistance independently of increased energy intake by initiating the accumulation of ectopic lipids as was observed in the liver of diabetic mice in the current study (Brabant *et al.* 2005; Dyck, Heigenhauser & Bruce 2006; Kellerer *et al.* 2001; Rattarasarn 2006). A major cause of insulin resistance is believed to be the ectopic accumulation of intracellular lipids in myocytes and hepatocytes, which inhibits

glucose uptake and mitochondrial function leading to cell death (Choo *et al.* 2006; Petersen & Shulman 2006; Rattarasarn 2006). This process, known as lipotoxicity, is central to the development of insulin resistance and subsequent T2D (Lindstrom 2007).

Although the *db/db* mutation resulted in comparable body mass and glucose intolerance between background strains, BKS diabetic mice developed more severe hyperglycaemia compared to C57BL/6 diabetic mice. This is consistent with previous studies (Coleman 1978; Ranheim *et al.* 1997). It has been suggested that C57BL/6 mice are less susceptible to lipotoxicity and can maintain a high insulin secretory capacity in the face of increasing insulin resistance (Clee, Nadler & Attie 2005). Studies in diabetic mice on the C57BL/6 background have shown that islet hyperplasia and hyperinsulinaemia compensates for the increased demand for insulin to maintain glucose homeostasis (Garris and Garris, 2004). In contrast, the severity of hyperglycaemia in diabetic mice on the BKS background has been attributed to an inherent susceptibility to lipotoxic islet damage leading to the early destruction of pancreatic islet integrity and impaired beta cell function (Garris & Garris 2004). The moderate hyperglycaemia observed in C57BL/6 diabetic mice, despite severe glucose intolerance, suggests that susceptibility to defects in beta cell function is the decisive factor determining development of T2D. Similarly, there are strong genetic determinants for the development of T2D in humans (Despres & Lemieux 2006). Since the maintenance of an adequate insulin-producing capacity in C57BL/6 mice prolongs moderate blood glucose levels despite insulin resistance, this is arguably a better model of pre-diabetes.

Whilst the *db/db* mutation in BKS mice clinically resembles T2D with respect to insulin resistance, glucose intolerance and hyperglycaemia, the wider impact of leptin signalling deficiency and how this applies clinically imparts doubt in the suitability of a monogenic model of T2D. The aetiology of obesity and T2D in humans involves a multifactorial combination of genetic and environmental factors and less than 5% of human obesity is caused by single genetic mutations represented in the $Lept^{db}$ model (Scroyen, Hemmeryckx & Lijnen 2013). Therefore, polygenic models of T2D may be more clinically relevant when investigating downstream aetiopathogenic mechanisms contributing to comorbid infections. While leptin

resistance can be a consequence of obesity in humans, this is in complete contrast to the total absence of leptin signalling conferred by the *db/db* mutation in these mice. The widespread complications of leptin deficiency are many, including alterations in hematopoiesis, osteogenesis and angiogenesis, infertility and impaired immune responses such as decreased macrophage activation, proinflammatory responses and proliferative capacity of T cells (Coleman 2010; Dixit *et al.* 2003; La Cava & Matarese 2004; Lindstrom 2007).

Disease severity was exacerbated in diabetic mice following subcutaneous infection with *B. pseudomallei* compared to non-diabetic mice, regardless of the genetic background. BKS diabetic mice tended to be more susceptible to *B. pseudomallei* infection with 100% succumbing to infection within 4 days, compared to the 60% mortality rate of C57BL/6 diabetic mice. Dissemination of *B. pseudomallei* was evident by 24 hours post-infection in both diabetic and non-diabetic mice. At 72 hours post-infection, growth of *B. pseudomallei* was higher in C57BL/6 diabetic mice at the site of infection and tended to be higher in the spleen and liver compared to non-diabetic mice. Although growth of *B. pseudomallei* and mortality from the infection was greater in diabetic compared to non-diabetic mice, these results need to be interpreted with caution due to the confounding complications of leptin signalling deficiency. Absence of leptin signalling may be implicated in the heightened susceptibility of diabetic *db/db* mice to infection.

The importance of leptin in the immune system is indicated by the presence of leptin receptors on immune cells (Matarese, Moschos & Mantzoros 2005). Leptin is increased in response to bacterial infections and is involved in activation and phagocytic activity of monocytes and macrophages and their production of leukotriene B_4 (LB_4), cyclooxygenase 2 (COX2), NO and proinflammatory cytokines (Dixit *et al.* 2003; Mancuso *et al.* 2002; Shirshev & Orlova 2005; Zarkesh-Esfahani *et al.* 2001). Involvement of leptin in neutrophil chemotaxis and release of oxygen radicals has also been described (Caldefie-Chezet *et al.* 2001; Caldefie-Chezet, Poulin & Vasson 2003; Zarkesh-Esfahani *et al.* 2004), along with NK cell development and maintenance (Tian *et al.* 2002). Leptin can also regulate the development of adaptive immune responses, promoting the antigen-presenting function of DC (Macia *et al.* 2006) and acting as a survival factor for T cells,

particularly promoting the development of $CD4^+$ T_H1 cell responses (La Cava & Matarese 2004; Lord *et al.* 1998). Ultimately, the increased susceptibility of diabetic mice cannot simply be ascribed to hyperglycaemia and insulin resistance due to the integral involvement of leptin in many immune responses. It is feasible that impaired immune function caused by leptin signalling deficiency and not diabetes *per se* could be responsible for the increased growth of *B. pseudomallei* observed in diabetic mice and the poor disease outcome.

In parallel with rapid mortality in diabetic mice, a significant decrease in blood glucose was observed following subcutaneous infection with *B. pseudomallei*. Terminal hypoglycaemia has been widely observed both clinically and in a variety of animal models of bacterial sepsis (Alamgir, Volkova & Peterson 2006; Filkins & Cornell 1974). Typically, inflammatory stress is characterised by hyperglycaemia due to increased hepatic glycogenolysis and glucose production, combined with increased peripheral insulin resistance (Khovidhunkit *et al.* 2004). This process is essential for supporting increased glucose utilisation by immune cells during the host immune response (Lang *et al.* 1993). A recent clinical observation that improved glycaemic control of T2D confers a survival advantage in patients with melioidosis supports the importance of glucoregulation in improving disease outcome (Koh *et al.* 2011). Nevertheless, it is difficult to conclude that glycaemic variability *per se* is the cause of fatality in diabetic mice infected with *B. pseudomallei*. Further research is necessary to explore the clinical significance of hypoglycaemia as a key factor influencing mortality from *B. pseudomallei* infection in individuals with T2D and the therapeutic potential of glycaemic modulators for improving outcome.

A monogenic model of T2D is arguably more useful than the previous streptozotocin-induced model of T1D to investigate comorbid *B. pseudomallei* infection. However, there are important limitations that should be considered. In particular, the large degree of obesity observed in both C57BL/6 and BKS diabetic mice may not be reflective of the clinical parameters documented in the Southeast Asia region where T2D develops at lower BMI (Ma & Chan 2013; Ramachandran, Ma & Snehalatha 2010; Ranasinghe, Jayawardena & Katulanda 2013). Furthermore, since metabolic complications and impaired immune responses occur in parallel with leptin deficiency, it is difficult to definitively determine the mechanisms responsible

for the increased susceptibility to *B. pseudomallei* infection using the *Lept*^{db} model. It is also noteworthy that T2D in humans is rarely caused by leptin deficiency and other single gene mutations. Therefore, development of a polygenic diet-induced murine model of T2D would better reflect the aetiopathology in humans and could provide a more comprehensive understanding of the immunological basis for the increased susceptibility of T2D to melioidosis.

Importantly, this was the first description of a murine model of T2D and comorbid melioidosis. Weight gain, glucose intolerance and fasting hyperglycaemia caused by hyperphagia from the *db/db* mutation in diabetic mice, was associated with increased susceptibility to *B. pseudomallei* infection compared to non-diabetic littermates. Following infection, hyperglycaemia in diabetic mice was reversed by decreasing blood glucose, with development of hypoglycaemia prior to death as has been frequently described in sepsis. Despite evidence of contrasting susceptibility to *B. pseudomallei* infection in diabetic and non-diabetic mice, the aforementioned limitations associated with this monogenic model of T2D provided impetus for the subsequent development of a polygenic murine model of T2D to enable more detailed characterisation of host-pathogen interactions.

CHAPTER 5
GENDER DIFFERENCES IN SUSCEPTIBILITY OF MICE TO DIET-INDUCED TYPE 2 DIABETES

5.1 Introduction

T2D is a multifactorial disease, caused by a combination of genetic susceptibility and environmental risk factors (Malecki 2005; Parillo & Riccardi 2004). The rapid rise in global incidence of T2D during the last half century is specifically attributed to changes in the latter (WHO 2003). One of the most significant modifiable determinants of T2D is the consumption of energy dense diets conducive to weight gain (Hu, van Dam & Liu 2001; Parillo & Riccardi 2004; WHO 2003). It is recognised that up to 90% of cases of T2D are potentially avoidable by improving lifestyle factors (Hu *et al.* 2001).

Industrialisation, urbanisation and economic development have resulted in rapid obesogenic dietary and lifestyle changes, a concept described as the nutrition transition (WHO 2003). The global trend for energy consumption per capita per day has been increasing since the mid 1960's (FAO 2002). This is specifically attributed to the marked rise in the intake of dietary fats over the last 3 decades due to the higher energy density afforded by diets high in fat. During this time, consumption of fat has increased by approximately 40% globally and up to 200% in some regions undergoing rapid economic development, such as China (WHO 2003). In addition to the nutrition transition, many regions are experiencing a demographic shift as average life expectancy increases. The aging population is also contributing to the significant burden of chronic diseases like T2D, which are also age-related.

Genetically altered mouse models, such as the *Lept*^{db} mice described in Chapter 4, fail to incorporate the significant nutritional and polygenic determinants involved in the aetiology of T2D in humans. This could have important clinical implications for the diverse multifactorial pathogenesis of T2D and related metabolic complications in humans. As previously discussed in Chapter 4, deficiency in leptin signalling also confers a variety of complications independent of T2D, including defects in immune

function, which limits the application of this model for research on T2D-related immune abnormalities.

A diet-induced model of T2D that resembles the clinical aetiopathology of the disease in humans is a vital tool for use in research to understand the immune complications of T2D and comorbid melioidosis. Several diet-induced murine models of T2D have previously been documented (Kanasaki & Koya 2011). However, there is considerable discordance between studies and reported metabolic phenotypes (Kanasaki & Koya 2011; Karasawa *et al.* 2009). Inconsistencies between studies are likely complicated by differences in dietary composition and fat content, age of mice, duration of feeding, the rodent strain and gender (Scroyen, Hemmeryckx & Lijnen 2013). Surwit *et al.* (1988) were the first to describe a diet-induced model of T2D in C57BL/6 mice fed a very HFD diet (60% energy from fat). However, the progression to overt hyperglycaemia after HFD feeding is controversial, arguably complicated by the inherently high capacity for insulin secretion in C57BL/6 mice (Karasawa *et al.* 2009; Kooptiwut *et al.* 2002).

To overcome this limitation, Karasawa *et al.* (2009) described a diet-induced model of T2D using B6D2F1 mice (the first generation [F1] cross of C57BL/6 and DBA/2 mice described in detail in Chapter 6). B6D2F1 mice fed a very HFD (52.1% energy from fat derived from lard) developed hyperglycaemia after 8 weeks of feeding, peaking at 18.6mmol/L by 14 weeks. However, this fat content markedly exceeds typical dietary intakes in developed nations (34% of energy) and consists predominantly of saturated fats (Ludwig *et al.* 1999). Furthermore, there were marked differences in diet composition, since all other nutrients except fat were diluted in the HFD compared to the control diet, including proteins, vitamins and minerals, which may have been a confounding factor influencing metabolic alterations. Therefore, the experiments described in this chapter involved a moderately HFD (#SF00-219, Specialty Feeds, Australia), with 40% of energy from fat and a mix of saturated, monounsaturated and polyunsaturated fats, formulated to resemble typical human dietary intake (described in further detail in Chapter 7).

T2D has been described as a sexually dimorphic disease (Franconi *et al.* 2008). It has been suggested that sex hormones play a role in determining the susceptibility to

and progression of T2D (Franconi *et al.* 2008). In Australia, the AIHW has consistently documented a slightly higher prevalence of T2D in males compared to females, particularly aged greater than 45 years of age (AIHW 2008, 2009). However, the global prevalence of T2D is comparable overall between genders (IDF 2011; King, Aubert & Herman 1998). The importance of gender in determining susceptibility to the development of T2D is undoubtedly complex and probably confounded by the multitude of other risk factors for T2D.

Gender differences in susceptibility to T2D have been documented in many rodent models (Rees & Alcolado 2005). Importantly however, there are still inconsistencies as to which sex is more susceptible to insulin resistance and subsequent hyperglycaemia (Pettersson *et al.* 2012; Vital, Larrieta & Hiriart 2006). Most dietary studies have been restricted to male mice, with relatively limited descriptions of the implications in female mice until recently (Gallou-Kabani *et al.* 2007). Therefore, the focus of the current study was to investigate whether a moderately HFD feeding regime was sufficient to induce metabolic complications in B6D2F1 mice and to compare the metabolic profiles of males and females. The specific aims of Chapter 5 were to:

i) determine if B6D2F1 mice fed a moderately HFD develop glucose intolerance, hyperlipidaemia and hyperglycaemia
ii) compare metabolic parameters of diet-induced T2D in male and female B6D2F1 mice fed a moderately HFD

5.2 Materials and Methods

5.2.1 Mice

Six-week old female and male B6D2F1 mice were allocated into dietary groups. One group received *ad libitum* access to a high fat diet (HFD; #SF00-219 Western Diet, Specialty Feeds, Australia), with 40% of energy from fat. Paired control mice received *ad libitum* or isometric quantities of standard rodent chow as specified (control; Specialty Feeds, Australia), with 12% energy from fat. Body mass, blood

glucose, glucose tolerance and blood lipid levels were measured according to Chapter 3.

5.2.2 Food intake

Food consumption was determined by subtracting the amount of food remaining or spilled in the cage after 24 hours from the initial amount of food supplied. Energy intakes were calculated on the basis of 19.4kJ/g of HFD and 14kJ/g of control diet, as determined by the manufacturer. During initial experiments, mice were given free access to HFD and control food. However, after it was determined that food consumption was significantly different between dietary groups, food intake was isometrically controlled as described in Chapter 3. Diet intervention was maintained for 10 to 20 weeks as described.

5.2.3 Statistical analysis

Statistical analysis was performed using Graphpad Prism Software Version 6.0b. Metabolic parameters between genders or dietary groups were compared by unpaired Student's T-tests with or without Welch's correction for unequal standard deviations. Kinetics of weight gain and glucose tolerance were compared by two-way ANOVA with Bonferroni post hoc tests. The trapezoidal rule was used to determine the AUC for the GTT. Statistical significance was determined by $P \leq 0.05$ and data was expressed as mean ± SEM.

5.3 Results

5.3.1 Body mass kinetics in mice fed a high fat diet

Changes in body mass in female and male B6D2F1 mice fed a control or HFD were compared to determine gender differences in susceptibility to weight gain. Body mass of mice was comparable between dietary groups prior to diet intervention. Gain in body mass during the 10 weeks of diet intervention was comparable between dietary groups in female (Figure 5.1a) and male (Figure 5.1b) mice. Overall, body mass was higher in male compared to female mice ($P < 0.05$; Figure 5.1c).

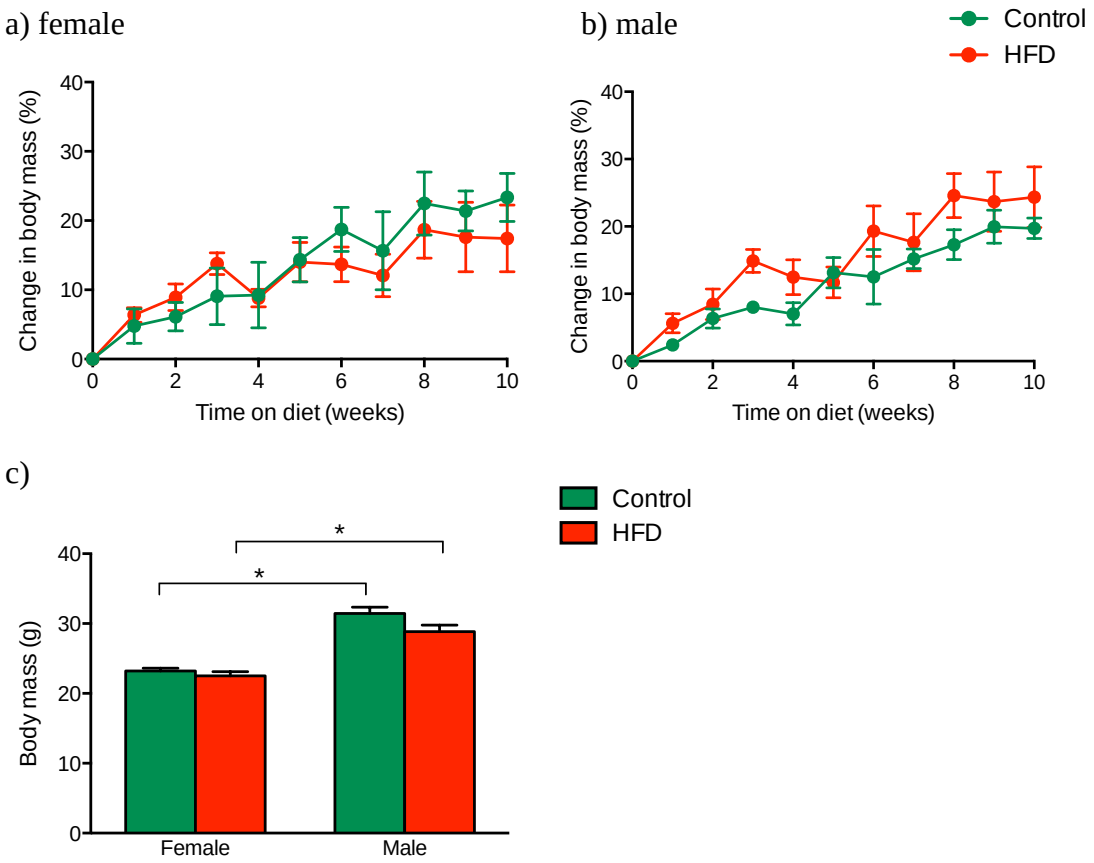

Figure 5.1 Body mass kinetics of female and male B6D2F1 mice after diet intervention
At 6 weeks of age, mice were fed either a high fat diet (HFD) or standard rodent chow (control) for 10 weeks with body mass monitored weekly. Changes in body mass were comparable in a) female (n = 12 per group) and b) male (n = 9 per group) mice consuming a HFD or control diet. c) Overall body mass was comparable between mice consuming a HFD or control diet regardless of gender. Male mice weighed significantly more than female mice on both dietary regimes. * $P < 0.05$; mean ± SEM.

5.3.2 Blood glucose and glucose tolerance kinetics in mice fed a high fat diet

Blood glucose and glucose tolerance kinetics in female and male B6D2F1 mice fed a control or HFD were compared to determine gender differences in susceptibility to diet-induced T2D. Baseline glucose tolerance, prior to diet intervention, was comparable in female and male mice ($P > 0.261$). Glucose tolerance in female mice was comparable to control mice after 5 and 10 weeks consuming a HFD, indicated by comparable AUC ($P > 0.948$; Figure 5.2a). Similarly, glucose tolerance was comparable between dietary groups in male mice ($P > 0.274$; Fig 5.3b). However, glucose tolerance in male mice progressively worsened with increasing age.

Compared to baseline levels, glucose tolerance was significantly impaired in male mice after 5 and 10 weeks consuming a HFD and after 10 weeks in control mice ($P < 0.05$; Figure 5.2b). Individual glucose tolerance curves are provided in Appendix 2.2 and 2.3. Fasting blood glucose levels were also comparable in female and male mice prior to diet intervention and after 5 and 10 weeks of a HFD ($P > 0.102$; Figure 5.3).

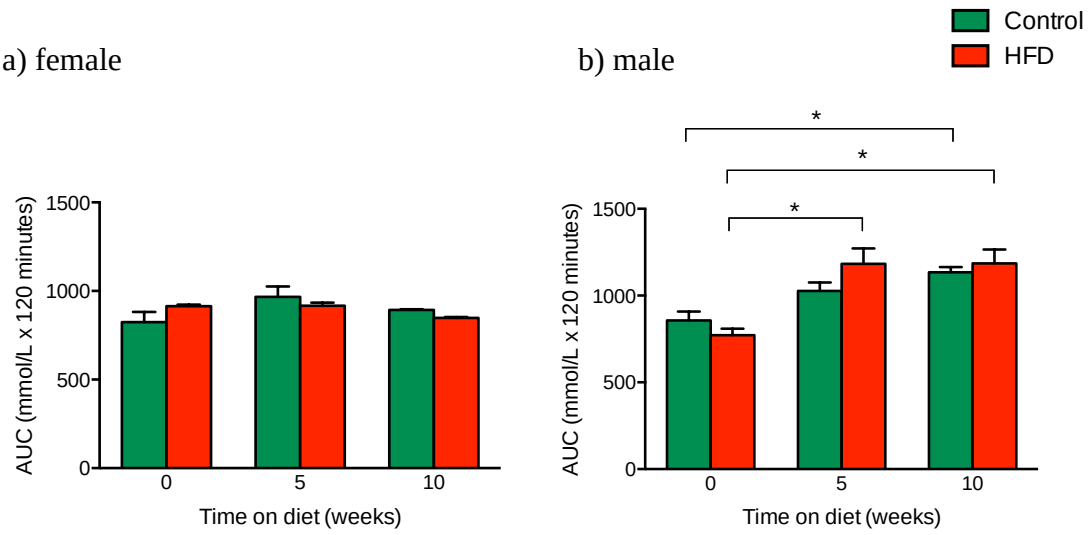

Figure 5.2 Glucose tolerance in female and male B6D2F1 mice after diet intervention
Area under the glucose curve (AUC) was calculated from glucose tolerance tests (GTT) conducted at 0, 5 and 10 weeks post-diet intervention (n = 5 per group). Consumption of a high fat diet (HFD) did not alter AUC in a) female mice, which was comparable to control littermates. In b) male mice, AUC was higher in HFD mice at 5 and 10 weeks compared to baseline levels. Similarly, AUC was higher in control mice after 10 weeks, compared to baseline levels. AUC in male mice was comparable between dietary groups. * $P < 0.05$; mean ± SEM.

5.3.3 Food and energy intake of mice fed a high fat diet

Interestingly, despite the higher energy density of the HFD, comparable gains in body mass were observed between HFD and control mice. This could be explained by the differences in food consumption between dietary groups ($P < 0.05$; Figure 5.4a and b). Control mice consumed significantly more food compared to mice consuming a HFD, which equated to a significantly greater energy intake to the control group despite the higher energy density of the HFD ($P < 0.05$).

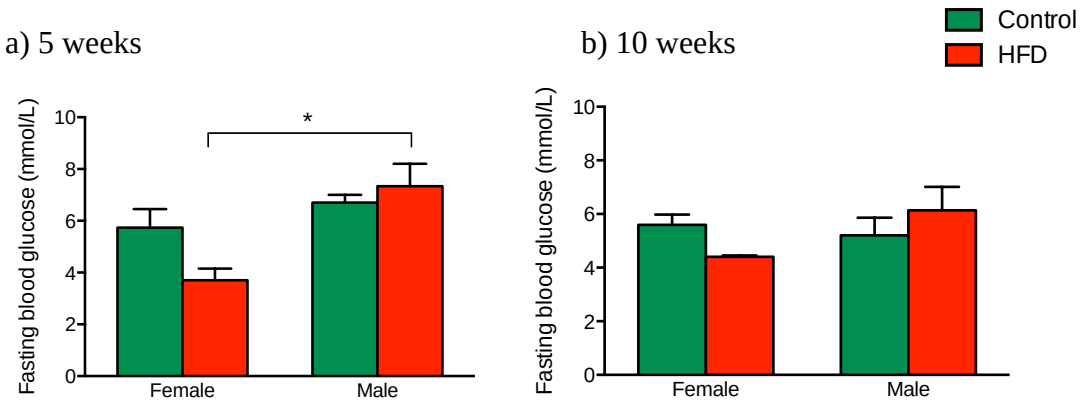

Figure 5.3 Blood glucose in female and male B6D2F1 mice after 10 weeks of diet intervention

Fasting blood glucose was measured after a) 5 and b) 10 weeks following diet intervention (n = 5 per group). Fasting blood glucose was comparable between dietary groups after 5 weeks but was significantly higher in male compared to female mice consuming a HFD ($P < 0.05$). Fasting blood glucose was comparable between female and male mice and between dietary groups after 10 weeks of diet intervention. * $P < 0.05$; mean ± SEM.

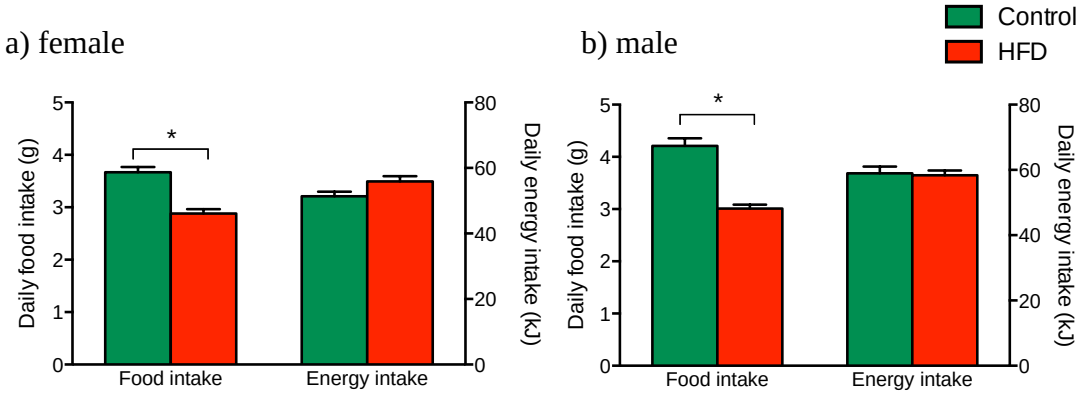

Figure 5.4 Daily dietary intakes of female and male B6D2F1 mice

Food intake of a) female (n = 12 per group) and b) male (n = 9 per group) mice was measured twice a week for the duration of diet intervention. Food intake was significantly higher in control mice compared to littermates consuming a high fat diet (HFD). This equated to comparable energy intake between HFD and control mice. * $P < 0.05$; mean ± SEM.

Whilst mice on the HFD regime consumed significantly more fat compared to control mice due to the higher fat content of the diet ($P < 0.05$; Figure 5.5a and b), the latter group consumed significantly more protein and carbohydrates ($P < 0.05$; Figure 5.5a and b). To account for the differences in palatability and food intake

between diets, all subsequent experiments were performed using an isometrically controlled dietary regime based on HFD food consumption of pair-fed mice. Following the introduction of isometrically controlled diets, protein consumption was comparable between dietary groups ($P > 0.999$), with the only difference being the proportion of carbohydrates and fat consumed ($P < 0.05$; Figure 5.5a and b). Overall, mice consuming the HFD had a higher daily energy intake compared to control mice, due to the higher energy density of the HFD.

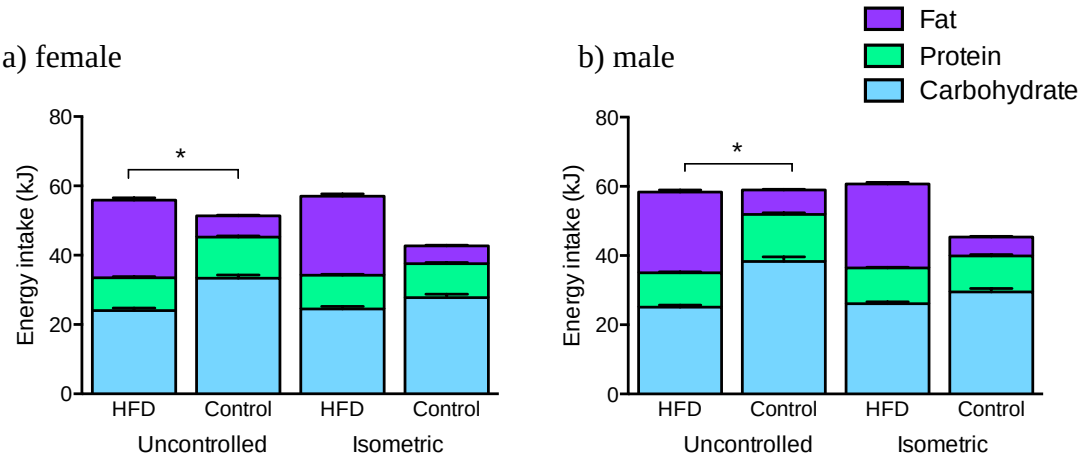

Figure 5.5 Proportion of macronutrients consumed by female and male B6D2F1 mice
Macronutrient consumption was compared between dietary groups prior to (uncontrolled) and following isometric dietary regimes. Protein consumption was higher in a) female and b) male control mice with uncontrolled food intake ($P < 0.05$). After isometric food intake, protein consumption was comparable between HFD and control mice and the only difference in macronutrient intake consisted of fat and carbohydrates. * $P < 0.05$; mean ± SEM.

5.3.4 Metabolic profile of mice on an isometrically controlled high fat diet

To maximise the potential for development of chronic complications from consuming a HFD, the duration of diet intervention was extended to 20 weeks. Differences in body mass between dietary groups were significant between HFD and control mice consuming isometric quantities of the respective diets for 20 weeks ($P < 0.05$; Table 5.1). Female mice consuming a HFD were 30% heavier compared to control mice, while male mice consuming a HFD were 50% heavier than control mice (Table 5.1). Plasma triglyceride levels were not altered by the HFD, however

cholesterol levels were significantly higher in mice consuming a HFD compared to control mice ($P < 0.05$; Table 5.1). Although fasting blood glucose levels remained comparable in female mice, blood glucose was significantly higher in male mice fed a HFD compared to control mice ($P < 0.05$; Table 5.1). Similarly, while there was no difference in glucose tolerance observed in female mice (Figure 5.6a), glucose tolerance was significantly impaired in male mice fed a HFD compared to control mice ($P < 0.05$; Figure 5.6b) and female littermates ($P < 0.05$; Figure 5.6c). Impaired glucose tolerance became apparent in male mice by week 15 of consuming a HFD (Appendix 2.5).

Table 5.1 Metabolic profiles of female and male B6D2F1 mice after 20 weeks of diet intervention

Metabolic Parameter	Female		Significance	Male		Significance
	Control	HFD		Control	HFD	
Body mass (g)	25.1 ± 0.5	32.8 ± 1.3	* $P = 0.001$	27.2 ± 0.2	40.8 ± 2.6	* $P < 0.001$
Daily energy intake (kJ)	42.7 ± 1.5	57.0 ± 1.7	* $P < 0.001$	45.4 ± 1.6	60.7 ± 1.2	* $P < 0.001$
Energy from fat (kJ)	5.1 ± 0.2	22.8 ± 0.7	* $P < 0.001$	5.4 ± 0.2	24.3 ± 0.5	* $P < 0.001$
Energy from protein (kJ)	9.8 ± 0.4	9.7 ± 0.3	$P > 1.000$	10.4 ± 0.4	10.3 ± 0.2	$P > 1.000$
Glucose tolerance (AUC)	724 ± 16	754 ± 26	$P = 0.967$	618 ± 15	974 ± 46	* $P < 0.001$
Blood glucose (mmol/L)	4.4 ± 0.3	4.3 ± 0.6	$P > 1.000$	5.2 ± 0.2	7.5 ± 0.4	* $P = 0.006$
Triglycerides (mmol/L)	1.0 ± 0.1	1.5 ± 0.3	$P = 0.077$	1.1 ± 0.1	1.5 ± 0.2	$P = 0.118$
Cholesterol (mmol/L)	2.4 ± 0.1	4.6 ± 0.3	* $P < 0.001$	2.4 ± 0.0	4.7 ± 0.3	* $P < 0.001$

AUC – area under the glucose curve; HFD – high fat diet; mean ± SEM.

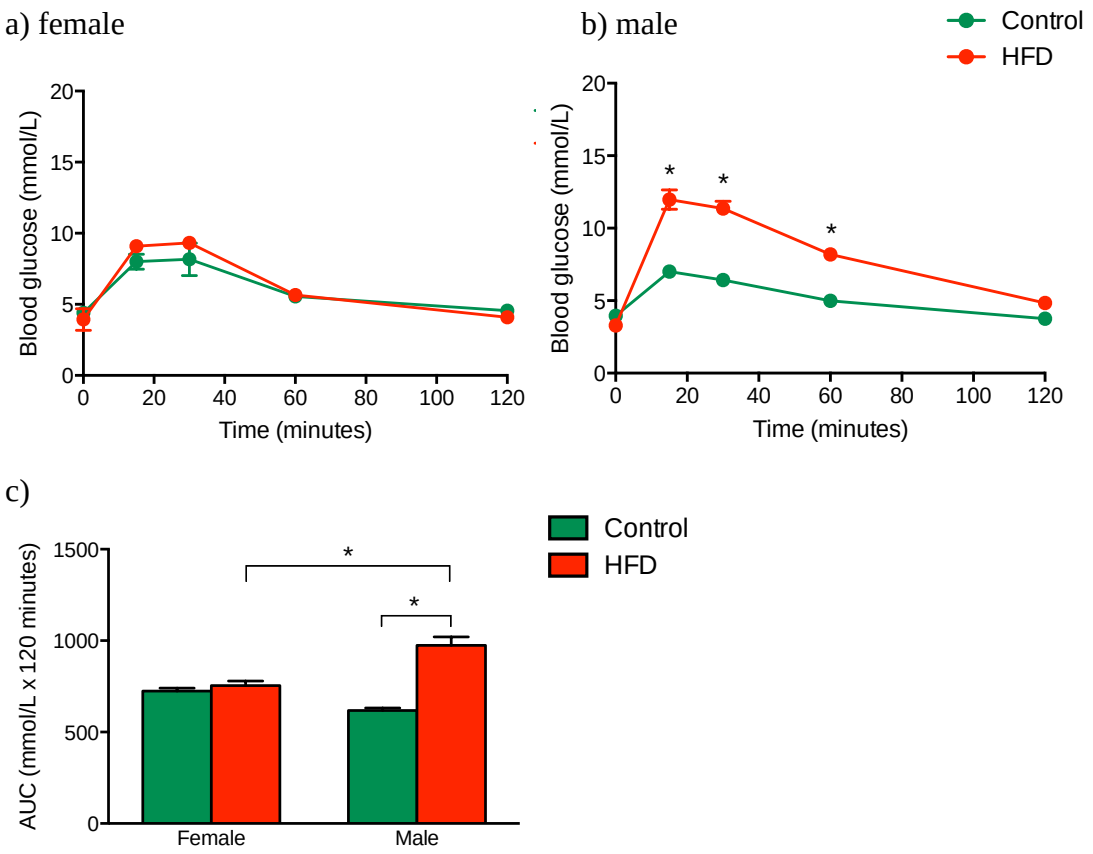

Figure 5.6 Glucose tolerance in B6D2F1 mice after 20 weeks of diet intervention
Glucose tolerance in a) female mice (n = 5 per group) was comparable after 20 weeks of consuming a HFD. In contrast, glucose tolerance in b) male mice (n = 5 per group) consuming a HFD was significantly impaired after 20 weeks compared to isometrically controlled littermates. Glucose clearance was significantly impaired in male mice consuming a HFD indicated by the greater c) area under the curve (AUC) compared to control mice. * $P < 0.05$; mean ± SEM.

5.4 Discussion

The increased incidence of T2D has been attributed to the nutritional transformation associated with economic advancement and industrialisation. The maximum recommendation of dietary energy supply from fat is 30%, ideally consisting of less than 10% saturated fat and 6-10% mono- and poly-unsaturated fat (WHO 2003). However, in industrialised regions today, approximately 30-46% of energy consumed is derived from dietary fats (WHO 2003). In particular, the intake of saturated fat has increased to unprecedented levels, at or exceeding 10% of energy intake in many developed regions (WHO 2003). Prior to this study, the most common diet used in rodent models of T2D has consisted of diets with 60% of

energy from fat, which is exceptionally high and not an accurate reflection of human dietary intakes (Omar, Pacini & Ahren 2012). Furthermore, saturated fat accounts for greater than 90% of fat content in this diet, in stark contrast to human diets (Omar, Pacini & Ahren 2012). The moderately HFD used in developing the murine model described in this chapter, was selected with the intention of resembling the 'Western diet' characteristic of industrialised regions. Consistent with this, the total energy derived from dietary fat was 40%, with the increased fat content consisting predominantly of saturated fat (13.99% of energy intake) as opposed to mono- and poly-unsaturated fats (7% of energy intake), compared to the control diet (0.74% and 3.77%, respectively).

Male B6D2F1 mice fed a moderately HFD for 20 weeks were shown to develop metabolic characteristics reflective of T2D. This included severe glucose intolerance and increased fasting blood glucose compared to control littermates. In preliminary studies, body mass and glucose tolerance were found to be comparable between dietary groups in female and male B6D2F1 mice, despite consumption of the HFD for 10 weeks. Detailed investigation of food intake and energy consumption subsequently demonstrated that these unexpected findings were related to differences in palatability of the HFD and control diet. Despite consuming significantly more saturated fat, there was no significant difference in glucose tolerance between HFD and control mice when energy intake and body mass was equivalent. Therefore, the importance of overall energy balance and increased adiposity in the pathogenesis of impaired glucose tolerance and subsequent T2D cannot be understated. Since control mice consumed 25% more food compared to littermates consuming a HFD, dietary energy intake and body mass was equivalent despite the higher fat content of the HFD. Furthermore, mice on a control diet were also consuming significantly more protein compared to littermates consuming a HFD, giving rise to another potential confounding factor, due to the increased insulin secretion and hepatic gluconeogenesis stimulated by this macronutrient (Linn *et al.* 2000; Radziuk, Pye & Zhang 1993). Consequently, food consumption was regulated in all subsequent experiments to ensure isometric food intake and protein consumption between dietary groups.

In addition to total dietary energy, it has been argued that the overall macronutrient content, particularly the quantity and quality of dietary fat, is equally if not more important (Petro *et al.* 2004). Dietary fats can be broadly divided into saturated, monounsaturated and polyunsaturated. There is evidence to suggest that both the amount and type of dietary fat plays a role in T2D pathogenesis, independent of obesity, through direct influences on insulin sensitivity by mediating the fatty acid composition of cell membranes (Borkman *et al.* 1993; Hu, van Dam & Liu 2001; Pan *et al.* 1995; Riccardi, Giacco & Rivellese 2004; Storlien *et al.* 1991; Vessby 2000). This concept is still controversial but animal studies have shown that despite equivalent overall fat content, mono- and poly-unsaturated fats can improve insulin action, while saturated fats increase insulin resistance (Fickova *et al.* 1994; Lardinois & Starich 1991). Similar findings have been documented in observational and interventional clinical studies (Bo *et al.* 2001; Feskens *et al.* 1995; Meyer *et al.* 2001; Salmeron *et al.* 2001; Uusitupa *et al.* 1994; Vessby *et al.* 2001). However, inconsistencies remain, possibly since the type of dietary fat appears to be less important then the total energy consumed when fat intake is high (>37% of energy) (Feskens *et al.* 1995; Marshall *et al.* 1994; Vessby *et al.* 2001). Interpretation of diet interventional studies in humans are complicated by difficulties in controlling diets, including fat content and other macronutrient constituents, different measures used to determine insulin sensitivity and often the relatively short-term duration of the intervention. Therefore, mouse models are helpful for understanding the consequences of specific nutritional parameters in the pathology of T2D.

Until recently, the positive energy balance and subsequent weight gain associated with increased consumption of energy dense HFD was believed to be the fundamental mechanism linking dietary fat to the development of insulin resistance and T2D (WHO 2003). Evidence from epidemiological studies suggests that the risk of T2D increases exponentially when BMI exceeds 25kg/m^2 (Chan *et al.* 1994; Colditz *et al.* 1995). Longitudinal studies have shown that increasing body mass index and waist circumference are powerful indicators for the development of T2D (Boyko *et al.* 2000; Chan *et al.* 1994; Colditz *et al.* 1990; Despres 2001; Despres & Lemieux 2006). The risk of T2D varies depending on ethnicity and the duration and distribution of adiposity but is generally increased 2- to 8-fold at BMI of 25kg/m^2, 10- to 40-fold at BMI >30kg/m^2, and more than 40-fold at BMI >35kg/m^2 (Day &

Bailey 2011). The association between T2D and adiposity is further supported by evidence that weight loss reduces the risk of developing T2D (Knowler *et al.* 2002; Tuomilehto *et al.* 2001).

The causative link between obesity, insulin resistance and T2D involves dyslipidaemia, comprising chronically elevated concentrations of FFA and the process of lipotoxicity (Brunzell & Ayyobi 2003; Day & Bailey 2011). The inextricable role of dyslipidaemia in the pathogenesis of T2D was first recognised in the early 1960's (Crepaldi & Tiengo 1976) and has since been confirmed (Raitakari *et al.* 1994; Tan 2000; Twisk *et al.* 1999). Excess adiposity contributes to the release of FFA to the circulation via increased lipolysis (Boden 2011; Day & Bailey 2011; McGarry 2002). This reduces the utilisation of glucose and directly interferes with insulin signalling by facilitating ectopic triglyceride storage (Griffin *et al.* 1999; Itani *et al.* 2002; Olefsky & Glass 2010). In addition, increased FFA stimulates hepatic gluconeogenesis, thus exacerbating insulin resistance and hyperglycaemia (Lam *et al.* 2003). Although insulin resistance is a central pathogenic determinant of T2D, the relative capacity of beta cells for insulin secretion is a principal factor dictating susceptibility to T2D in an obesogenic environment (Astrup & Finer 2000). This accounts for the genetic variability in susceptibility to T2D and explains why not all overweight or obese people develop T2D.

Epidemiological studies have documented gender disparity in the risk of developing T2D. Prevalence data suggests an increased incidence of T2D in men than women, although other studies have disputed this. The influence of gender on development of T2D is probably confounded by involvement of additional risk factors such as weight gain and insulin resistance, which also vary between sexes and ethnicities (Regitz-Zagrosek, Lehmkuhl & Weickert 2006). The increasing prevalence of metabolic complications in young women (20-39 years of age) in the United States has been paralleled to the rise in obesity, which affects more women than men in this age group (Ford, Giles & Mokdad 2004; Steinbaum 2004). Age is also a confounding factor, with risk of T2D being higher in men in younger age groups (<55 years of age), but higher in women in older age groups (>55 years of age), probably influenced by pre- and post-menopausal factors (Must *et al.* 1999). For example, several studies have reported gender-specific differences in adipose tissue

distribution, with men and post-menopausal women predisposed to visceral fat accumulation, perhaps explaining the increased risk of metabolic complications like T2D in these age groups (Armitage *et al.* 2004; Arner 1998; Kissebah & Krakower 1994; Linder *et al.* 2004; Pi-Sunyer 1995; Regitz-Zagrosek, Lehmkuhl & Weickert 2006; Stork *et al.* 2004). It is possible that an increased risk of developing T2D in post-menopausal females is associated with the concomitant decrease in oestrogen, though this is yet to be demonstrated.

Similar to the gender-differences in the development of T2D described in humans, a critical observation in this study was that HFD-feeding resulted in distinct metabolic phenotypes in male and female mice. Male mice gained considerably more body mass compared to female mice despite comparable energy intake and were more glucose intolerant after 20 weeks of HFD-feeding. Increased glucose intolerance in male mice may be due to the higher propensity for weight gain and altered lipid metabolism compared to female mice, as has been demonstrated by others (Macotela *et al.* 2009; Stubbins *et al.* 2012; Yakar *et al.* 2006). There is evidence that oestrogen regulates the expression of genes involved in lipid oxidation, perhaps preferentially stimulating the use of FFA for energy in females (Campbell *et al.* 2003; D'Eon *et al.* 2005; Homma *et al.* 2000; Morise *et al.* 2009; Naugler *et al.* 2007). Furthermore, oestrogen has been shown to reduce overall food consumption whilst promoting energy expenditure, thus favouring a negative energy balance (Brown *et al.* 2010; Eckel 2011; Musatov *et al.* 2007). Oestrogen is also thought to play a role in regulating adipocyte hypertrophy, oxidative stress and inflammation, thereby protecting female mice from obesity induced insulin resistance (Stubbins *et al.* 2012). Similarly, it has been firmly established that oestrogen is involved in decreasing ROS and inflammation in endothelial cells to help protect against atherosclerosis (Gao *et al.* 2006; Wagner, Schroeter & Hecker 2001).

Gender-related differences in insulin resistance may also be mediated through regulation of adiponectin. Levels of adiponectin are more than 2-fold higher in female compared to male mice (Medina-Gomez *et al.* 2005). Cross sectional studies have also shown that gender is a strong determinant of adiponectin concentrations, with levels being significantly higher in women than in men, independent of age and body fat distribution (Arita *et al.* 1999; Cnop *et al.* 2003; Nishizawa *et al.* 2002;

Zoccali *et al.* 2002). This may be due to differences in adipocyte number and size between sexes, the main source of adiponectin, or the inhibitory effects of androgens on adiponectin secretion (Nishizawa *et al.* 2002). Adiponectin is believed to be an important mediator linking adiposity and insulin sensitivity. One of the mechanisms by which adiponectin is postulated to exert its positive effects on insulin sensitivity is by enhancing lipid oxidation thereby reducing circulating and ectopic triglyceride content (Fruebis *et al.* 2001). Elucidating the mechanisms responsible for the gender-related differences in susceptibility to T2D remains an active area of research (Hao *et al.* 2010; Heine *et al.* 2000; Riant *et al.* 2009; Straub 2007; Stubbins *et al.* 2012).

It should be noted that some studies have found female mice fed a HFD do develop insulin resistance, impaired glucose tolerance and T2D (Winzell & Ahren 2004). The inconsistencies between studies could be due to differences in diet composition or duration of diet intervention. The diet used by Winzell and Ahren (2004) consisted of 60% of energy from fat and mice were fed for 12 months. It is possible that a longer period of diet intervention may lead to development of glucose intolerance in female mice since the risk of T2D increases with age and as sex hormones wane. However, this avenue was not pursued in subsequent work outlined in this thesis due to time constraints associated with increasing the duration of diet intervention.

T2D has been described as an age-related disease due to the increased incidence associated with aging. T2D most commonly develops at or above 45 years of age, although the incidence of T2D is rising in younger age groups in parallel with the growing prevalence of obesity (Mokdad *et al.* 2001). In both sexes, aging is associated with reduction in insulin sensitivity and increased accumulation of visceral fat (Chen *et al.* 1985; Colman *et al.* 1995; Defronzo 1979). Consistent with this, glucose intolerance was found to increase with age in male mice in this study, regardless of the diet consumed. The diet interventional period in the current study was increased from 10 to 20 weeks since this is more reflective of the comparative age of T2D development in humans and enables chronic complications to become established.

T2D is characterised by glucose intolerance and elevated fasting or post-prandial blood glucose. We investigated the effect of a moderately HFD, reflective of a

typical ‘Western diet’, on physical and metabolic parameters in B6D2F1 mice to assess the usefulness of this feeding regime in the development of a model of T2D. A significant gender disparity in the metabolic phenotype was observed, with male mice significantly more susceptible to weight gain, impaired glucose tolerance and hyperglycaemia compared to female mice. However, although significantly higher compared to control littermates, blood glucose was only moderately elevated in male mice consuming a HFD compared to other models of T2D, such as the monogenic model previously described in Chapter 4. Therefore, to satisfy the criteria of T2D, further optimisation of this model to increase the level of hyperglycaemia was necessary.

CHAPTER 6
COMPARISON OF METABOLIC PHENOTYPE AND *B. pseudomallei* INFECTION AFTER DIET INTERVENTION IN B6D2F1 AND C57BL/6 MICE

6.1 Introduction

Murine models of diet-induced T2D are complicated by many factors. In addition to the importance of gender, susceptibility to diet-induced T2D is also highly dependent on the genetic background (Alexander *et al.* 2006). Without a genetic predisposition to insulin resistance, diet and weight gain alone is often not sufficient to induce T2D in mice (Chalkley *et al.* 2002). This has been emphasised extensively in A/J mice, which are resistant to diet-induced obesity, hyperglycaemia and insulin resistance (Gallou-Kabani *et al.* 2007). The distinct phenotypes observed between murine strains after HFD-feeding have been attributed to marked differences in insulin secretory capacity, insulin sensitivity and overall glucose homeostasis (Rossmeisl *et al.* 2003). As in mice, there is a strong genetic complexity to T2D in humans (Leiter 1989). A number of clinical studies indicate the importance of genetic determinants of insulin secretion to overall development and progression of T2D (Kahn 2001; Levy *et al.* 1998). Particular differences between ethnicities have been described, in which individuals may be more susceptible to insulin resistance and T2D despite similar or even lower body weights (Sims 2001).

The use of B6D2F1 mice as a novel model of diet-induced obesity and T2D was first described in 2009 (Karasawa *et al.* 2009). Consistent with this, the results presented in Chapter 5 indicate that male B6D2F1 mice were more susceptible to HFD-induced glucose intolerance than female mice. However, it was evident that the development of hyperglycaemia was variable and relatively modest compared to the monogenic model of T2D discussed in Chapter 4. This discrepancy between studies is likely attributed to the marked differences in HFD, since previous findings involved the use of a very HFD (60% energy from fat) (Karasawa *et al.* 2009). In contrast, the diet evaluated in the studies described in the current chapter was a moderate HFD (40% energy from fat), which is more reflective of human dietary intakes as previously described in Chapter 5.

Diet-induced obesity has previously been described in C57BL/6 mice, however there is disagreement as to the degree of metabolic complications that develop. While it is widely documented that feeding C57BL/6 mice a very HFD (60% energy from fat) induces obesity, insulin resistance and glucose intolerance, the progression to overt hyperglycaemia is controversial (Gallou-Kabani *et al.* 2007; Karasawa *et al.* 2009; Surwit *et al.* 1988). Moreover, studies using diets with less than 60% energy from fat are limited. It has been reported that C57BL/6 mice are genetically resistant to T2D due to the high capacity of pancreatic beta cells to compensate with increased insulin secretion (Leiter, Coleman & Hummel 1981).

An added incentive to assess the development of diet-induced T2D in C57BL/6 mice in the current study is the widespread use of this strain as a model of chronic melioidosis. Immune responses to *B. pseudomallei* infection in this strain have already been extensively characterised, in contrast to B6D2F1 mice, which have not previously been used in melioidosis research. The development of effective T_H1-type immune responses against *B. pseudomallei* is believed to account for the greater resistance and improved outcome of C57BL/6 mice to melioidosis compared to other susceptible strains, such as BALB/c mice. Therefore, the focus of the work described in this chapter was to determine if HFD-feeding would lead to development of T2D in C57BL/6 mice and how this would influence the susceptibility of this innately resistant strain to *B. pseudomallei* infection. The aims of Chapter 6 were to:

i) compare the metabolic profiles of male B6D2F1 and C57BL/6 mice after consumption of a HFD
ii) determine if consumption of a HFD increases susceptibility of B6D2F1 and C57BL/6 mice to subcutaneous *B. pseudomallei* infection

6.2 Materials and Methods

6.2.1 Mice

Male, 6-week old B6D2F1 and C57BL/6 mice were allocated into dietary groups. One group received *ad libitum* access to a HFD (40% energy from fat; #SF00-219,

Specialty Feeds, Australia) for 20 weeks, while paired control mice received isometric quantities of standard rodent chow (12% of energy from fat; Specialty Feeds, Australia). Body mass, blood glucose, glucose tolerance and blood lipid levels were measured as previously described in Chapter 3.

6.2.2 Infection with *B. pseudomallei*

Mice (n = 15 per group) were subcutaneously infected with 4.9×10^5 CFU of *B. pseudomallei* as previously described in Chapter 3. Survival was monitored in 5 mice per group for 10 days. At 24 and 72 hours post-infection, 5 mice were euthanased to determine bacterial loads in the liver, lung, spleen, blood and SAT at the site of infection, according to protocols described in Chapter 3.

6.2.3 Statistical analysis

Statistical analysis was performed using Graphpad Prism Software Version 6.0b. Susceptibility to subcutaneous infection with *B. pseudomallei* was compared between HFD and control mice by Kaplan–Meier survival analysis. Body weight gain and glucose tolerance were compared by two-way ANOVA with Bonferroni post hoc tests. The trapezoidal rule was used to determine AUC for the GTT. All other data was compared by unpaired Student's T-tests between HFD and control mice. Comparisons were considered to be significant at $P \leq 0.05$ and data was expressed as mean ± SEM.

6.3 Results

6.3.1 Baseline glucose intolerance in C57BL/6 mice relative to B6D2F1 mice

Physical and metabolic parameters were compared in B6D2F1 and C57BL/6 mice fed a HFD to evaluate genetic differences in susceptibility to diet-induced complications and determine the most representative model of T2D. Prior to diet intervention, C57BL/6 mice were already less glucose tolerant than weight-matched B6D2F1 mice, indicated by the significantly higher peak in blood glucose 15 minutes after a glucose challenge ($P < 0.05$; Figure 6.1a). Blood glucose clearance

was also delayed, leading to a higher AUC compared to B6D2F1 mice ($P < 0.05$; Figure 6.1b).

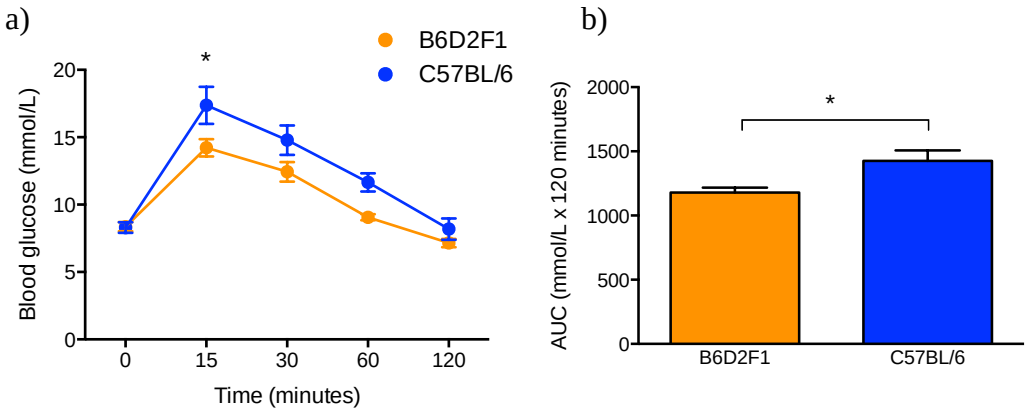

Figure 6.1 Baseline glucose tolerance in B6D2F1 and C57BL/6 mice
Prior to diet intervention, baseline glucose tolerance was compared between B6D2F1 and C57BL/6 mice ($n = 5$ per group). a) C57BL/6 mice had a higher peak in blood glucose at 15 minutes post-glucose tolerance test and b) greater area under the glucose curve (AUC) indicating impaired glucose tolerance compared to B6D2F1 mice. * $P < 0.05$; mean ± SEM.

6.3.2 Weight gain in B6D2F1 and C57BL/6 mice after diet intervention

During the course of the study, B6D2F1 (Figure 6.2a) and C57BL/6 (Figure 6.2b) mice gained significantly more body mass when fed a HFD compared to control mice. B6D2F1 mice fed a HFD gained significantly more body mass than control mice within two weeks of diet intervention, with weight continuing to increase for the duration of the study ($P < 0.05$). Over the 20-week study period, B6D2F1 mice fed a HFD gained approximately double the body mass per week compared to control mice. In contrast to B6D2F1 mice, C57BL/6 mice exhibited only modest gains in body mass per week when fed a HFD, although this was still significantly greater than control littermates ($P < 0.05$). A significant increase in body mass in C57BL/6 mice fed a HFD was observed after 3 weeks of diet intervention, with weight remaining elevated compared to control littermates for the duration of the study. At the end of the 20-week study period, final body mass was significantly higher in HFD compared to control mice and in B6D2F1 mice compared to C57BL/6 mice ($P < 0.05$; Figure 6.2c).

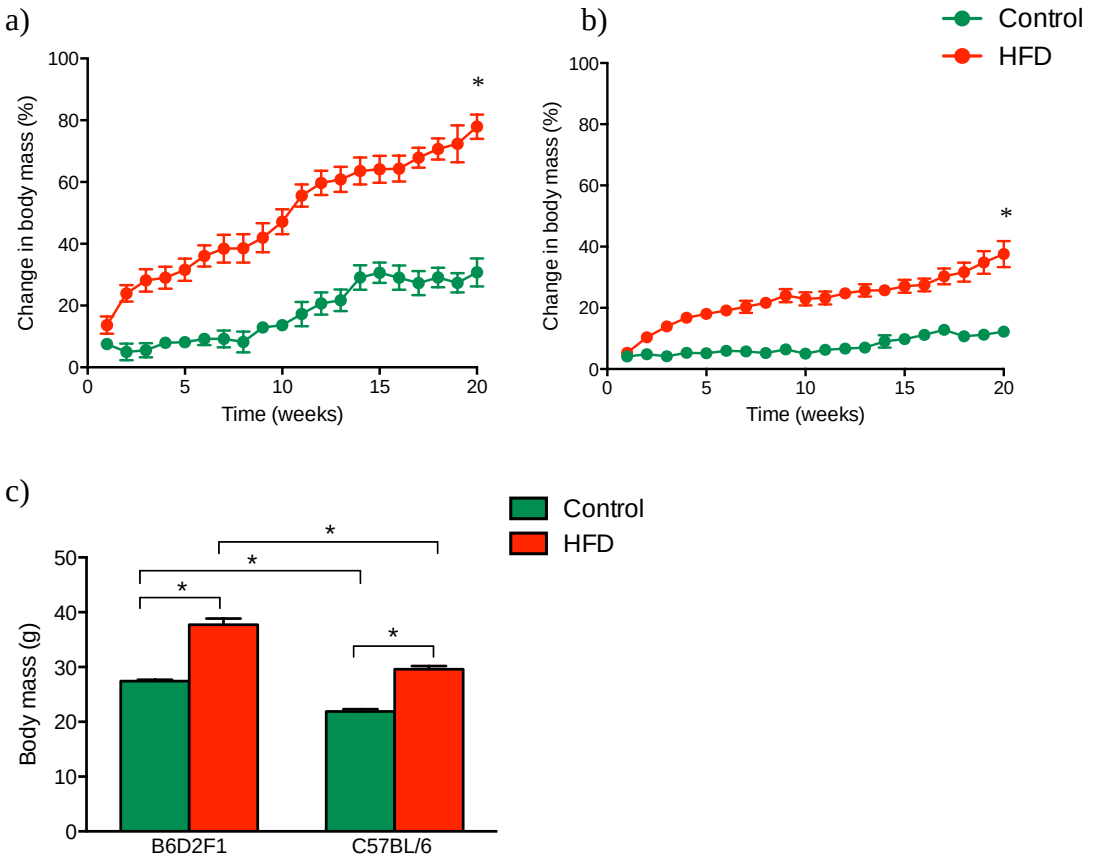

Figure 6.2 Body mass kinetics in B6D2F1 and C57BL/6 mice after diet intervention
Body mass of B6D2F1 and C57BL/6 mice was measured weekly to calculate the change in body mass between dietary groups (n = 20 per group). a) Body mass gain was significantly higher in B6D2F1 mice fed a high fat diet (HFD) compared to control mice. b) Body mass gain was also significantly higher in C57BL/6 mice fed a HFD compared to control mice. c) In both dietary groups, final body mass was significantly higher in B6D2F1 mice compared to C57BL/6 mice. * $P < 0.05$; mean ± SEM.

The higher body mass in B6D2F1 mice compared to C57BL/6 mice was associated with significantly greater size of adipose tissue depots (Figure 6.3). In particular, SAT was increased by almost 40% in B6D2F1 mice compared to C57BL/6 mice fed a HFD ($P < 0.05$). In the control dietary group, SAT was increased by approximately 30% in B6D2F1 mice compared to C57BL/6 mice ($P < 0.05$). In mice fed a HFD, VAT was increased by 50% in B6D2F1 compared to C57BL/6 mice ($P < 0.05$). While both SAT and VAT depots were significantly greater in B6D2F1 mice fed a HFD compared to control littermates, only VAT depots were increased in C57BL/6

mice fed a HFD compared to control littermates (Table 6.1). Blood lipid profiles were comparable between B6D2F1 and C57BL/6 mice, with hypercholestorelaemia and hypertriglyceridaemia observed in mice after consumption of a HFD ($P < 0.05$; Table 6.1).

6.3.3 Glucose intolerance in B6D2F1 and C57BL/6 mice after diet intervention

Within 10 weeks of diet intervention, glucose intolerance was impaired in both strains of HFD mice compared to control mice ($P < 0.05$; Appendix 2.6). This persisted for the duration of the diet intervention. Interestingly however, after 10 weeks of feeding, glucose intolerance of C57BL/6 mice became significantly greater than B6D2F1 in both HFD and control dietary groups ($P < 0.05$; Appendix 2.6). The increased susceptibility of C57BL/6 mice to glucose intolerance was maintained for the duration of the experimental period (Figure 6.4). While the blood glucose peak in B6D2F1 mice fed a HFD for 20 weeks was higher than control littermates at 15 minutes post-glucose challenge and remained higher for the duration of the test, the rate of glucose clearance was comparable between dietary groups (Figure 6.4a). In contrast, glucose clearance was delayed in C57BL/6 mice fed a HFD between 15 and 30 minutes post-glucose challenge compared to control littermates, indicative of increased insulin resistance (Figure 6.4b). This coincided with significantly higher fasting blood glucose levels compared to control littermates and B6D2F1 mice fed a HFD ($P < 0.05$; Figure 6.4d).

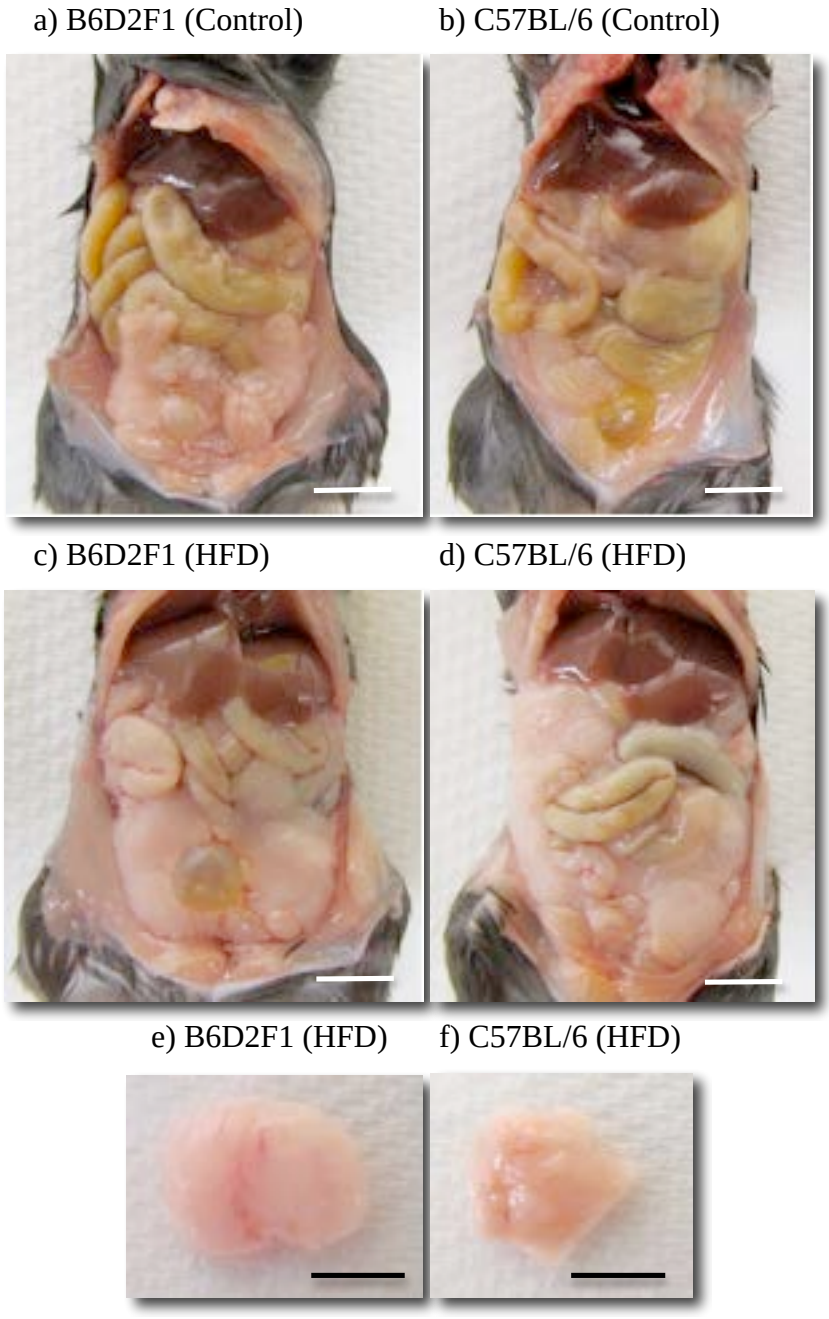

Figure 6.3 Abdominal visceral fat deposits of B6D2F1 and C57BL/6 mice after 20 weeks of diet intervention

Gross anatomy of mice was observed after 20 weeks of diet intervention. Visceral adipose tissue (VAT) depots are relatively small in a) B6D2F1 and b) C57BL/6 mice fed a control diet. In comparison, VAT depots are much larger in c) B6D2F1 and d) C57BL/6 mice fed a high fat diet (HFD). In particular, VAT depots in mice fed a HFD were larger in e) B6D2F1 mice compared to f) C57BL/6 mice. Scale = 1cm.

Table 6.1 Metabolic profiles of B6D2F1 and C57BL/6 mice after 20 weeks of diet intervention

Metabolic Parameter	B6D2F1		Significance	C57BL/6		Significance
	Control	HFD		Control	HFD	
Body mass (g)	27.4 ± 0.2	37.7 ± 1.1	* $P < 0.001$	21.9 ± 0.4	29.6 ± 0.6	* $P < 0.001$
Change in body mass (%)	30.7 ± 4.5	77.9 ± 3.9	* $P < 0.001$	11.2 ± 0.8	37.6 ± 4.3	* $P < 0.001$
Liver (g)	1.37 ± 0.02	1.66 ± 0.20	$P = 0.205$	0.93 ± 0.07	1.32 ± 0.11	$P = 0.074$
SAT (g)	0.30 ± 0.01	0.73 ± 0.10	* $P < 0.001$	0.10 ± 0.01	0.27 ± 0.04	$P = 0.094$
VAT (g)	0.44 ± 0.04	1.59 ± 0.13	* $P < 0.001$	0.16 ± 0.02	0.82 ± 0.16	* $P < 0.001$
Daily energy intake (kJ)	46.48 ± 0.84	63.83 ± 0.93	* $P < 0.001$	37.94 ± 0.65	51.70 ± 0.41	* $P < 0.001$
Glucose tolerance (AUC)	633 ± 33	1061 ± 45	* $P = 0.004$	880 ± 32	1685 ± 117	* $P = 0.001$
Blood glucose (mmol/L)	5.3 ± 0.5	6.6 ± 0.4	$P = 0.122$	6.1 ± 0.4	9.2 ± 0.3	* $P = 0.002$
Triglyceride (mmol/L)	1.0 ± 0.05	2.2 ± 0.2	* $P = 0.005$	0.6 ± 0.04	1.7 ± 0.4	* $P = 0.009$
Cholesterol (mmol/L)	2.6 ± 0.06	4.8 ± 0.5	* $P < 0.001$	2.1 ± 0.1	4.1 ± 0.4	* $P < 0.001$

AUC – area under the glucose curve; HFD – high fat diet; SAT – subcutaneous adipose tissue; VAT – visceral adipose tissue; mean ± SEM.

6.3.4 Susceptibility of B6D2F1 and C57BL/6 mice after diet intervention to *B. pseudomallei* infection

Following 20 weeks of diet intervention, B6D2F1 and C57BL/6 mice were subcutaneously infected with *B. pseudomallei* to compare susceptibility to infection. B6D2F1 mice fed a HFD were significantly more susceptible to *B. pseudomallei* infection compared to control littermates ($P < 0.05$; Figure 6.5a). B6D2F1 mice fed a HFD had a median survival of 3 days, with all mice succumbing to *B. pseudomallei* infection within five days compared to a mortality rate of 20% in control littermates

at 10 days post-infection (Figure 6.5a). C57BL/6 mice fed a HFD were also significantly more susceptible to *B. pseudomallei* infection compared to control littermates ($P < 0.05$; Figure 6.5b). While C57BL/6 mice fed a control diet had a 100% survival rate at 10 days post-infection, this was reduced to 40% in littermates consuming a HFD, with a median survival of 3 days (Figure 6.5b). Splenic enlargement and abscesses were observed in all surviving mice at necropsy and *B. pseudomallei* was detectable in the spleen, liver and SAT, indicating incomplete resistance to *B. pseudomallei* infection.

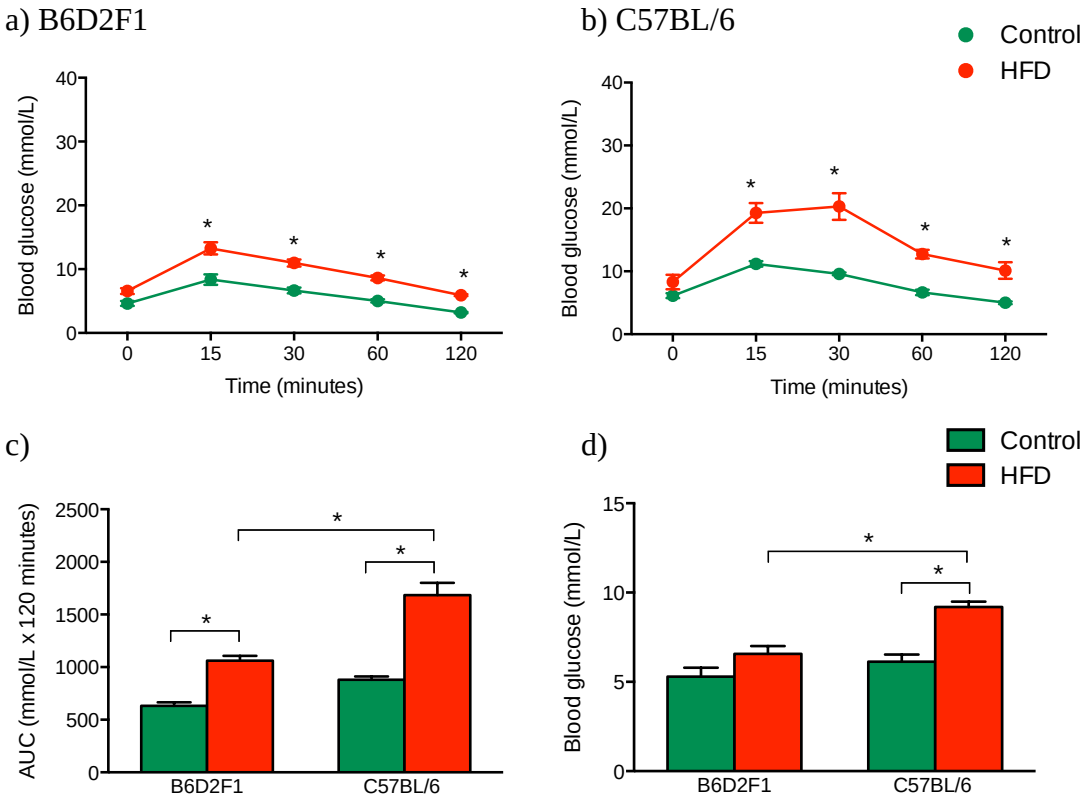

Figure 6.4 Glucose tolerance in B6D2F1 and C57BL/6 mice after 20 weeks of diet intervention

Glucose tolerance tests (GTT) were performed on mice (n = 5 per group) after 20 weeks of dietary intervention. a) Blood glucose levels were higher in B6D2F1 mice fed a high fat diet (HFD) compared to control mice at 15 minutes post-glucose challenge and remained elevated for the duration of the test. b) Similarly, this was observed in C57BL/6 mice fed a HFD, although the peak in blood glucose was higher compared to B6D2F1 mice. c) Glucose intolerance was more severe in C57BL/6 mice compared to B6D2F1 mice fed a HFD as shown by the greater area under the glucose curve (AUC). d) This coincided with significantly higher blood glucose levels in C57BL/6 mice fed a HFD compared to B6D2F1 mice. * $P < 0.05$; mean ± SEM.

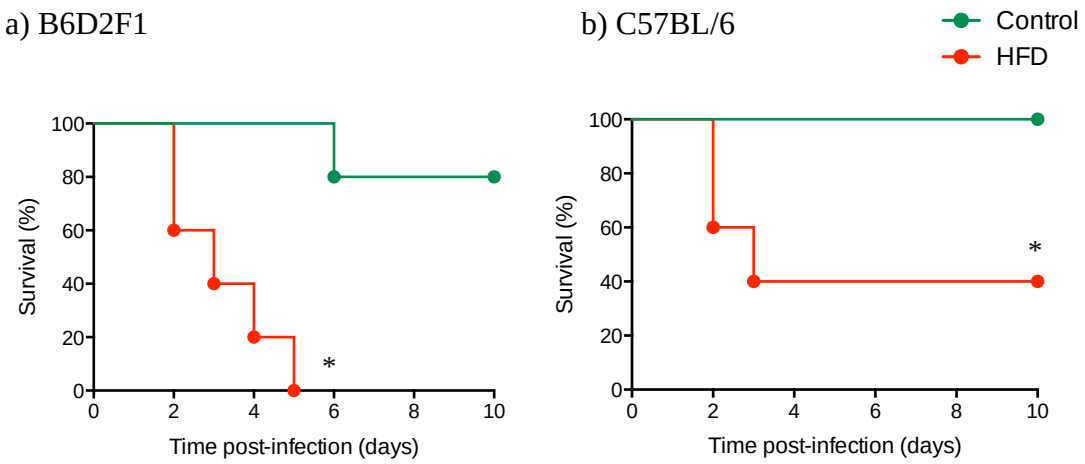

Figure 6.5 Influence of diet on survival of B6D2F1 and C57BL/6 mice after subcutaneous *B. pseudomallei* infection
After 20 weeks of diet intervention, mice (n = 5 per group) were infected with 4.9×10^5 CFU of *B. pseudomallei*. Both a) B6D2F1 ($P < 0.05$) and b) C57BL/6 ($P < 0.05$) mice fed a HFD were significantly more susceptible to *B. pseudomallei* infection, with a median survival of 3 days post-infection, compared to control littermates. While 100% of B6D2F1 mice fed a HFD succumbed to infection, 60% mortality was observed in C57BL/6 mice fed a HFD at the end of the 10-day experimental period. * $P < 0.05$.

Dissemination of *B. pseudomallei* from the site of infection was evident by 24 hours post-infection. Levels of *B. pseudomallei* in the spleen of B6D2F1 mice fed a HFD tended to be higher compared to control littermates at 24 hours post-infection ($P = 0.099$; Figure 6.6a). Growth of *B. pseudomallei* between 24 and 72 hours post-infection was significantly higher in the liver and lung of B6D2F1 mice fed a HFD compared to control littermates ($P < 0.05$; Figure 6.6c and e). Similarly, growth of *B. pseudomallei* in the spleen and liver between 24 and 72 hours post-infection was significantly greater in C57BL/6 mice fed a HFD compared to control littermates ($P < 0.05$; Figure 6.6b and d). Compared to control littermates, levels of *B. pseudomallei* also tended to be higher in the lung at 24 hours post-infection in C57BL/6 mice fed a HFD ($P = 0.066$), but were comparable at 72 hours post-infection ($P = 0.104$; Figure 6.6f). B6D2F1 mice fed a HFD also developed severe bacteraemia by 72 hours post-infection, with a significant increase in levels of *B. pseudomallei* in the blood compared to control littermates ($P < 0.05$).

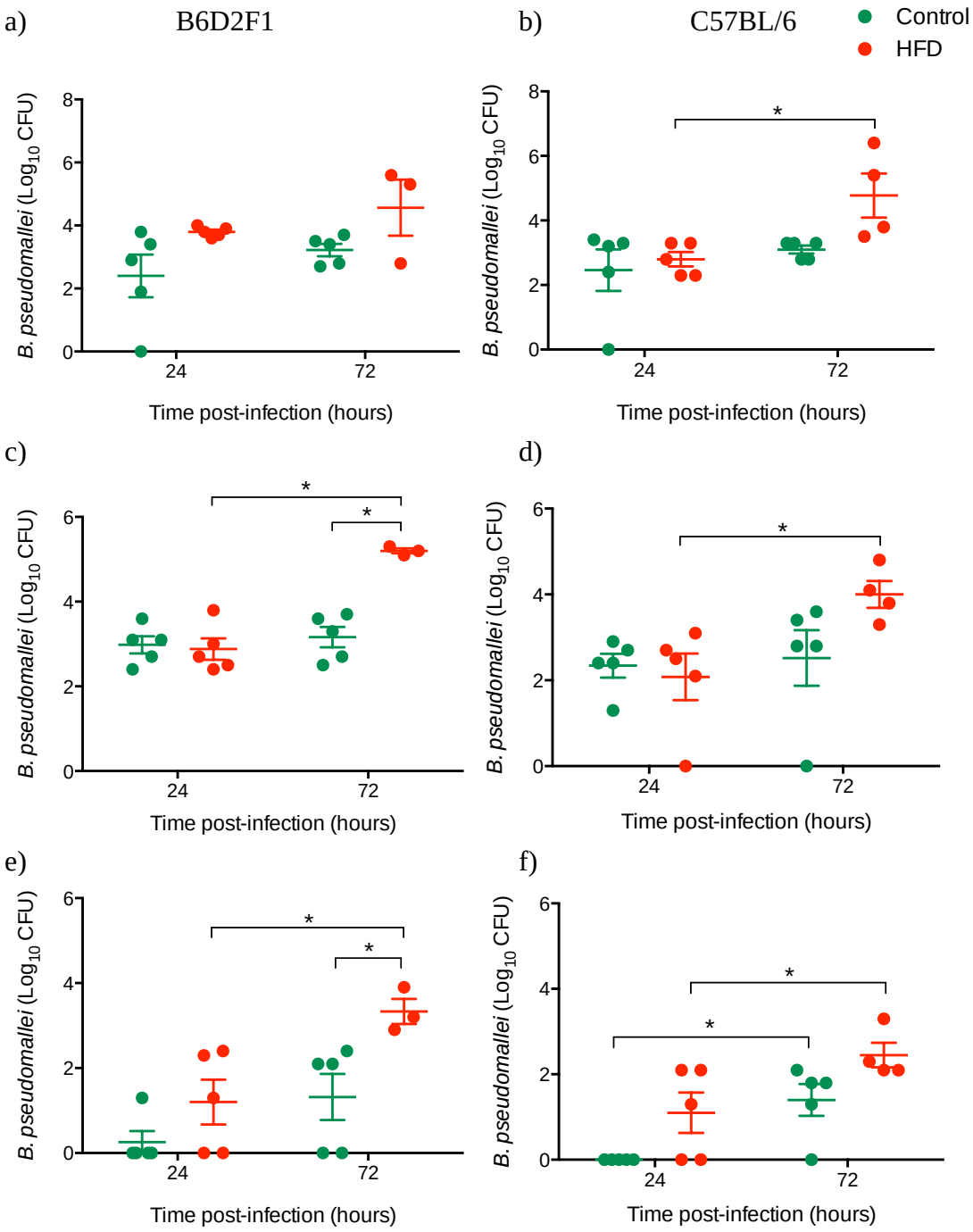

Figure 6.6 Dissemination of *B. pseudomallei* following subcutaneous infection of B6D2F1 and C57BL/6 mice

After 20 weeks of diet intervention, mice (n = 5 per group) were infected with 4.9×10^5 CFU of *B. pseudomallei*. At 24 hours post-infection, levels of *B. pseudomallei* tended to be higher in the a) spleen of B6D2F1 mice fed a high fat diet (HFD; $P = 0.099$) and were significantly higher in the c) liver and e) lung by 72 hours post-infection compared to control littermates ($P < 0.05$). Growth of *B. pseudomallei* in the b) spleen and d) liver of C57BL/6 mice fed a HFD was also significantly greater by 72 hours post-infection compared to control littermates ($P < 0.05$) and tended to be higher in the lung at 24 ($P = 0.066$) hours post-infection. Loads at 72 hours could only be determined in $n = 3$ B6DF1 mice and $n = 4$ C57BL/6 mice fed a HFD due to mortality. * $P < 0.05$; mean Log_{10} CFU ± SEM.

6.4 Discussion

The major finding of this chapter was the significant differences observed in weight gain and glucose tolerance between B6D2F1 and C57BL/6 mice. While B6D2F1 mice gained significantly more body weight when fed a HFD compared to C57BL/6 mice consuming a HFD, glucose intolerance was more severe in C57BL/6 mice compared to B6D2F1 mice. This was unexpected, since it has previously been reported that B6D2F1 mice fed a HFD develop severe glucose intolerance and hyperglycaemia compared to C57BL/6 mice (Karasawa *et al.* 2009). The apparent disparity between this study and the findings reported in this chapter are likely a result of the differences in diet composition. Karasawa *et al.* (2009) used a very HFD with 60% energy from fat, compared to the HFD used in the current study of 40% energy from fat. To our knowledge, the only other study to investigate the metabolic profile of B6D2F1 mice conducted by Hull *et al.* (2005) also found that B6D2F1 mice fed a HFD (45% energy from fat) did not develop hyperglycaemia, consistent with our findings.

Differences in the propensity for weight gain and glucose homeostasis have been described in inbred mouse strains, particularly between DBA/2 and C57BL/6 mice, the parent strains of the B6D2F1 hybrid (Alexander *et al.* 2006; West *et al.* 1992; West, Waguespack & McCollister 1995). Consistent with the findings reported in this chapter, despite increased adiposity in DBA/2 mice fed a HFD compared to C57BL/6 mice, C57BL/6 mice are more hyperglycaemic and exhibit increased gluconeogenesis (Alexander *et al.* 2006; Rossmeisl *et al.* 2003; Tortoriello, McMinn & Chua 2004). This has been attributed to differences in the behavioural profile of the strains and may also involve differences in the underlying metabolic and endocrine pathways, although the exact mechanisms remain to be defined (Alexander *et al.* 2006).

Intracellular accumulation of ectopic lipids is widely agreed to be one of the major pathogenic determinants of insulin resistance (McGarry & Dobbins 1999; Olefsky & Glass 2010; Unger 2002). It has thus been proposed that an increased ability to accumulate triglycerides in adipose tissue reduces susceptibility to insulin resistance caused by ectopic deposition of lipids in non-adipose tissue (Danforth 2000).

Importantly, adipose tissue dysfunction as opposed to total adipose tissue mass is the key factor for development of insulin resistance and T2D (Chandalia & Abate 2007). In the current study, the increased weight gain in B6D2F1 mice fed a HFD was associated with significant expansion of SAT and VAT depots, which may account for the decreased susceptibility to glucose intolerance and hyperglycaemia from HFD-feeding compared to C57BL/6 mice.

Obesogenic environments involving increased dietary fat intake are a major factor determining development of T2D, as previously described in Chapter 5. However, not all people exposed to this environment develop the disease, supporting the role of genetic predisposition in determining susceptibility to T2D (Abbasi *et al.* 2004; Astrup & Finer 2000). The complex interplay between nutrient intake and gene expression is not well understood, complicated further by significant genetic variability in metabolic responses to dietary components in the human population (WHO 2003). Genetic factors determining the functional capacity of pancreatic beta cells are thought to account for some of this variability in the development of T2D.

Despite the strong link between obesity and T2D, it is widely recognised that some 'normal' weight individuals also develop T2D (Lee *et al.* 2007). This is particularly relevant since the Southeast Asia region, where the incidence of melioidosis is high, is generally regarded as having a higher prevalence of T2D at a lower level of adiposity (He *et al.* 2002; Huxley *et al.* 2008; Yoon *et al.* 2006). This is attributed to the genetic susceptibility to insulin resistance and impaired pancreatic beta cell function, referred to as a 'metabolically obese' phenotype, that has been described in particular ethnicities, including Asians and Australian Aboriginals (Aekplakorn *et al.* 2006; Fukushima, Suzuki & Seino 2004; McDermott, Li & Campbell 2010; Ramachandran, Ma & Snehalatha 2010; Ruderman *et al.* 1998). This enhances the suitability of C57BL/6 mice fed a HFD as a model of T2D and melioidosis, since impaired glucose tolerance develops with only minimal weight gain, which is characteristic of the 'metabolically obese' phenotype of most patients with melioidosis and T2D.

Whether the association between T2D and melioidosis is directly related or a result of confounding metabolic alterations such as increased adiposity and dyslipidaemia

is not easily answered. There have been no investigations into associations between other metabolic risk factors of T2D and *B. pseudomallei* infection. Thailand, which has the highest incidence of melioidosis, also has the highest rates of obesity in Asia (Ramachandran & Snehalatha 2010). Interestingly, although B6D2F1 mice fed a HFD were not hyperglycaemic, they exhibited severe weight gain and were significantly more susceptible to *B. pseudomallei* infection compared to control littermates. Increased mortality of B6D2F1 mice fed a HFD coincided with increased bacterial growth in the spleen, liver and lung and severe bacteraemia by 72 hours post-infection.

Preliminary clinical and epidemiological studies indicate that obesity increases the incidence and severity of infections, independently of blood glucose (Canturk *et al.* 2003; Jubber 2004). An increased risk of infection, bacteraemia and poor wound healing has been reported in obese patients (Gottschlich *et al.* 1993; Lamas, Marti & Martinez 2002) and a large prospective study in Australia found that obesity conferred an even greater risk of surgical site infection than T2D (Harrington *et al.* 2004). It has been argued that results are biased by the lengthened operative time, hospital stay and surgical site trauma in obese patients however studies documenting a heightened susceptibility to community-acquired infections have also emerged. In one of the largest prospective studies of its kind, overweight and obesity was positively correlated with the incidence of community-acquired pneumonia, which was increased more than 2-fold (Baik *et al.* 2000). How the immune modulating effects of obesity contribute to this enhanced susceptibility to infections remains to be investigated and could have important implications for a respiratory pathogen, such as *B. pseudomallei*.

From the findings described in this chapter, it is apparent that the metabolic phenotype induced by a HFD has a high degree of genetic variability depending on the mouse strain. The increased susceptibility of B6D2F1 mice to weight gain after HFD-feeding make this strain more suitable as model of obesity. In contrast, C57BL/6 mice fed a HFD exhibit greater alterations in glucose homeostasis at a lower degree of adiposity, enhancing their suitability as a model of T2D, particularly for research investigating *B. pseudomallei* infection. However, despite being more glucose intolerant than B6D2F1 mice fed a HFD, the degree of hyperglycaemia in

C57BL/6 mice after HFD-feeding was still modest, relative to the threshold of hyperglycaemia characteristic of T2D in humans. As such, our subsequent studies focused on further optimisation of diet composition for the development of an improved animal model of T2D.

CHAPTER 7
OPTIMISATION OF A DIET-INDUCED MODEL OF TYPE 2 DIABETES IN C57BL/6 MICE AND SUSCEPTIBILITY TO *B. pseudomallei* INFECTION

7.1 Introduction

While it is widely recognised that obesogenic environments involving increased dietary energy intake contribute to the development of T2D, the independent risk of individual dietary components in the development of T2D remains unclear. Over the past decade, the importance of refined carbohydrates in the development of T2D has been recognised (WHO 2003; Willett, Manson & Liu 2002). It has become increasingly apparent that the increase in proportion of daily energy derived from refined carbohydrates, which increases the GI of foods, is arguably equally as important as saturated fat content in inducing T2D (WHO 2003).

The description of GI was introduced by Jenkins *et al.* (1981) to classify carbohydrates based on the glycaemic level produced after consumption. Since then, clinical trials and epidemiological studies have shown that low GI foods can improve the control of glycaemia in subjects with T2D (Brand-Miller *et al.* 2009; Wolever *et al.* 1992) and could even prevent the development of T2D (Salmeron *et al.* 1997a; Salmeron *et al.* 1997b). GI is also believed to influence the relative degree of weight gain, possibly indirectly contributing to the association with T2D. Low GI foods are associated with a lower insulin response, which favours lipid oxidation as opposed to storage as triglycerides (Ludwig *et al.* 1999).

Diet-induced animal models of T2D, rather than monogenic models, are being increasingly used for T2D-related research since they more closely reflect the aetiopathogenesis of the disease. Surwit *et al.* (1988) were the first to describe a diet-induced model of T2D in C57BL/6 mice. However, the fat content of the diet (60% of energy) used in HFD fed models often markedly exceeds typical human dietary intake in developed nations (34% of energy) (WHO 2003). The results presented in Chapter 6 indicated that while C57BL/6 mice developed glucose intolerance after being fed a HFD for 20 weeks, development of overt hyperglycaemia was modest. C57BL/6 mice have previously been demonstrated to have an inherently high

capacity for insulin secretion, which may explain the maintenance of moderate blood glucose levels despite impaired glucose tolerance observed in the studies described in Chapter 6 (Kooptiwut *et al.* 2002).

To satisfy the key diagnostic criteria for T2D, animal models should develop glucose intolerance and overt hyperglycaemia. Several studies have combined a diet-induced C57BL/6 model with single or multiple doses of streptozotocin, which is selectively toxic to pancreatic beta cells (Gilbert, Fu & Liu 2011). However, despite rapid onset of hyperglycaemia, the micro- and macro-vascular complications of T2D require significant time to become established and cannot be achieved in a short-term treatment regime. In addition, such models have limited utility due to inadvertent toxicity of streptozotocin, as previously discussed in Chapter 4.

Over the past decade, evidence has emerged supporting a link between high GI diets and the development of T2D (Willett, Manson & Liu 2002). Several rodent studies have documented impaired insulin release and severe destruction of pancreatic islets after consumption of a high fat, high glycaemic index diet (HF/HGD) (Andersson *et al.* 2010; Pawlak, Kushner & Ludwig 2004). However, the role of GI in the development of T2D is still a matter of contention, due to limitations in current epidemiological and longitudinal studies (Pawlak, Kushner & Ludwig 2004; Willett, Manson & Liu 2002). The purpose of the this study was to determine if dietary GI influences the development of hyperglycaemia in C57BL/6 mice and could therefore be used to improve the current model for T2D and melioidosis research. Therefore, the specific aims of Chapter 7 were to:

i) compare metabolic profiles of C57BL/6 mice after consuming a HFD or HF/HGD
ii) compare susceptibility of C57BL/6 mice consuming a HF/HGD and control mice to *B. pseudomallei* infection

7.2 Materials and Methods

7.2.1 Animals and study design

Male C57BL/6 mice (6 weeks of age; $n = 30$) were randomly divided into 3 dietary groups. The control group received standard rodent chow (Specialty Feeds, Australia), while the other groups received a HFD (#SF00-219, Specialty Feeds, Australia) as previously described in Chapter 5 or a HF/HGD with equivalent fat content but increased refined carbohydrates (50.5% dextrose; #SF03-030, Specialty Feeds, Australia), *ad libitum* for 10 or 15 weeks as indicated. The duration of the diet intervention was reduced from 20 weeks due to the rapid onset of hyperglycaemia compared to previous experiments with the HFD. Food consumption, body weight, blood glucose and lipid profiles were measured as previously described in Chapter 3. Urine albumin/creatinine ratio (ACR; $n = 5$ per group) was measured using the DCA Vantage Analyser (Siemens, Australia) and serum cytokine levels determined by cytometric bead array (CBA; Mouse Inflammation Kit, BD Biosciences, Australia; $n = 5$ per group). Samples were analysed in duplicate according to the manufacturer's instructions. Data was acquired on a FACS Calibur flow cytometer using CellQuest Pro (BD Biosciences, Australia).

7.2.2 *B. pseudomallei* infection

After diet intervention, C57BL/6 mice were subcutaneously infected with *B. pseudomallei* as previously described in Chapter 3. Survival was monitored for 10 days at which time any surviving animals were euthanased and necropsied to investigate evidence of persistent infection.

7.2.3 Statistical analysis

Statistical analysis was performed using GraphPad Prism Software Version 6.0b. Survival was compared by Kaplan-Meier curves. All other measures were compared by analysis of variance (ANOVA) using Bonferroni post hoc tests. The trapezoidal rule was used to determine AUC for the GTT. Significance was determined by $P \leq 0.05$ and data presented as mean ± SEM.

7.3 Results

7.3.1 Food intake and weight gain following 10 weeks of diet intervention

Prior to diet intervention, body mass was comparable between groups. After 10 weeks of the diet intervention, body mass increased by approximately 20% in mice consuming the HFD and HF/HGD ($P < 0.05$; Table 7.1). Both HFD and HF/HGD mice were significantly heavier than control mice ($P < 0.05$; Table 7.1). SAT and VAT depots were comparably higher in mice consuming high fat diets compared to control mice ($P < 0.05$; Table 7.1). The increased body mass, specifically adipose tissue, in mice consuming high fat diets corresponded with a 40% higher daily energy intake compared to control mice ($P < 0.05$; Table 7.1). Increased body mass and energy intake was associated with higher circulating cholesterol levels in HFD ($P < 0.05$) and HF/HGD ($P < 0.05$) mice compared to control mice (Table 7.1). Circulating triglyceride levels tended to be higher in HF/HGD mice compared to HFD mice ($P = 0.067$), while both groups were higher than control mice ($P < 0.05$; Table 7.1).

7.3.2 Glucose tolerance of mice following 10 weeks of diet intervention

Glucose intolerance was apparent in mice fed both HF/HGD and HFD compared to control mice after 4 weeks of diet intervention ($P < 0.05$). Furthermore, fasting blood glucose in HF/HGD mice remained elevated at the end of the GTT compared to both HFD and control mice ($P < 0.05$; Figure 7.1a). After 10 weeks of diet intervention, glucose intolerance was comparable between HF/HGD and HFD mice ($P = 0.690$) and was significantly greater than control mice ($P < 0.05$; Figure 7.1b).

Table 7.1 Metabolic profiles of C57BL/6 mice following 10 weeks of diet intervention

Metabolic Parameter	Control	HFD	HF/HGD
Daily food intake (g/mouse)	2.90 ± 0.05	2.89 ± 0.05	2.91 ± 0.06
Daily energy intake (kcal/mouse)	9.4 ± 0.2	13.4* ± 1.3	13.5* ± 0.3
Body mass (g)	21.9 ± 0.4	27.0* ± 0.5	27.4* ± 0.4
SAT mass (mg)	92.6 ± 10.1	197.0* ± 29.9	201.4* ± 11.2
VAT mass (mg)	56.0 ± 9.4	476.0* ± 14.0	469.6* ± 65.6
Blood glucose (mmol/L)	**6.2 ± 0.3**	**10.2*† ± 0.9**	**14.1*† ± 1.3**
Insulin resistance (AUC)	1003.5 ± 79.5	1818.7* ± 167.8	2057. 7* ± 109.5
Cholesterol (mmol/L)	3.98 ± 0.05	4.37* ± 0.07	4.2* ± 0.02
Triglycerides (mmol/L)	1.03 ± 0.07	1.56* ± 0.15	2.03* ± 0.14

AUC – area under the glucose curve; HFD – high fat diet (low glycaemic index); HF/HGD – high fat, high glycaemic index diet; mean ± SEM; * $P < 0.05$ vs control; † $P < 0.05$ HF/HGD vs HFD.

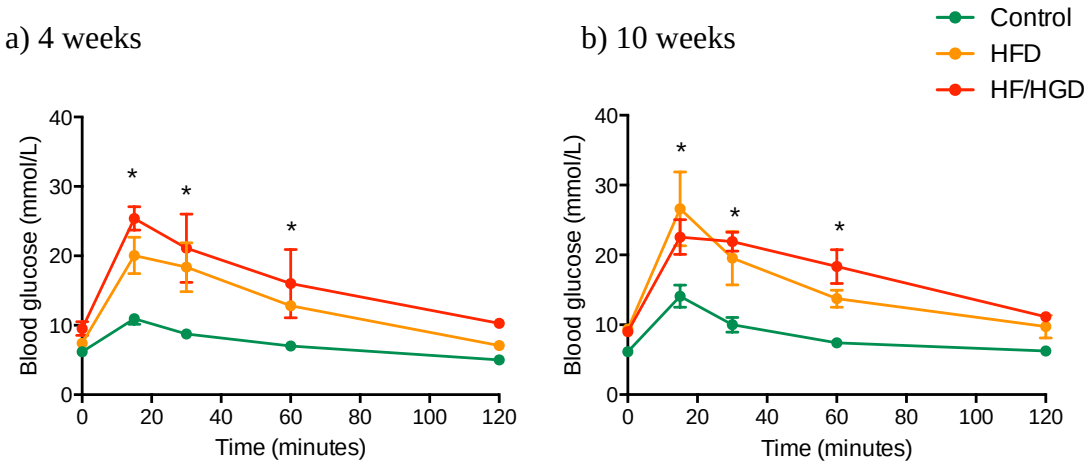

Figure 7.1 Kinetics of glucose tolerance following diet intervention
Glucose tolerance was impaired in mice fed a HFD and HF/HGD compared to control mice (n = 5 per group) after a) 4 and b) 10 weeks of diet intervention. Glucose intolerance tended to be more severe in HF/HGD mice, with delayed glucose clearance at 60 minutes post-glucose challenge. * $P < 0.05$; mean ± SEM.

Prior to the diet intervention, blood glucose was similar between all groups ($P > 0.999$; Figure 7.2). In response to HF/HGD feeding, hyperglycaemia developed after 4 weeks, with blood glucose increasing by approximately 66% ($P < 0.05$; Figure 7.2). This was significantly higher than blood glucose in both mice fed a HFD and control mice ($P < 0.05$; Figure 7.2). In contrast, mice consuming the HFD were normoglycaemic ($P > 0.999$ vs control mice; Figure 7.2). After 10 weeks of feeding, hyperglycaemia had increased to more than 90% of baseline levels ($P < 0.05$) in HF/HGD mice and was higher than both HFD and control mice ($P < 0.05$; Figure 7.2).

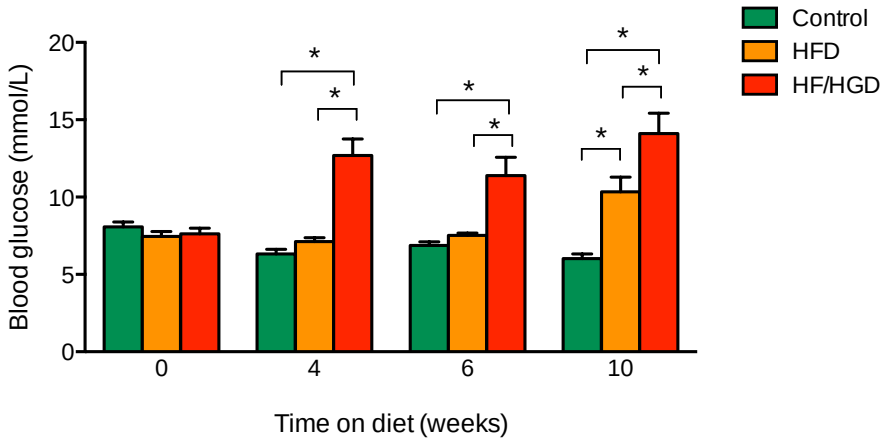

Figure 7.2 Kinetics of blood glucose following diet intervention
Blood glucose in mice (n = 10 per group) consuming a high fat, high glycaemic index diet (HF/HGD) was significantly higher compared to mice consuming a high fat diet (HFD) and control diet after 4, 6 and 10 weeks of diet intervention. * $P < 0.05$; mean ± SEM.

7.3.3 Metabolic profile of mice following 15 weeks of diet intervention

Given the rapid and severe development of hyperglycaemia in mice consuming HF/HGD, this diet was selected for all further experiments, with mice herein referred to as diabetic and non-diabetic. The duration of the diet intervention was increased from 10 to 15 weeks to maximise the time for development of chronic pathophysiological complications. After 15 weeks of diet intervention, body mass (Figure 7.3a), circulating blood glucose (Figure 7.3b) and insulin levels (Figure 7.3c) were significantly higher in diabetic mice compared to non-diabetic mice.

Microalbuminuria was also observed in diabetic mice, indicative of early stage kidney complications (Figure 7.3d).

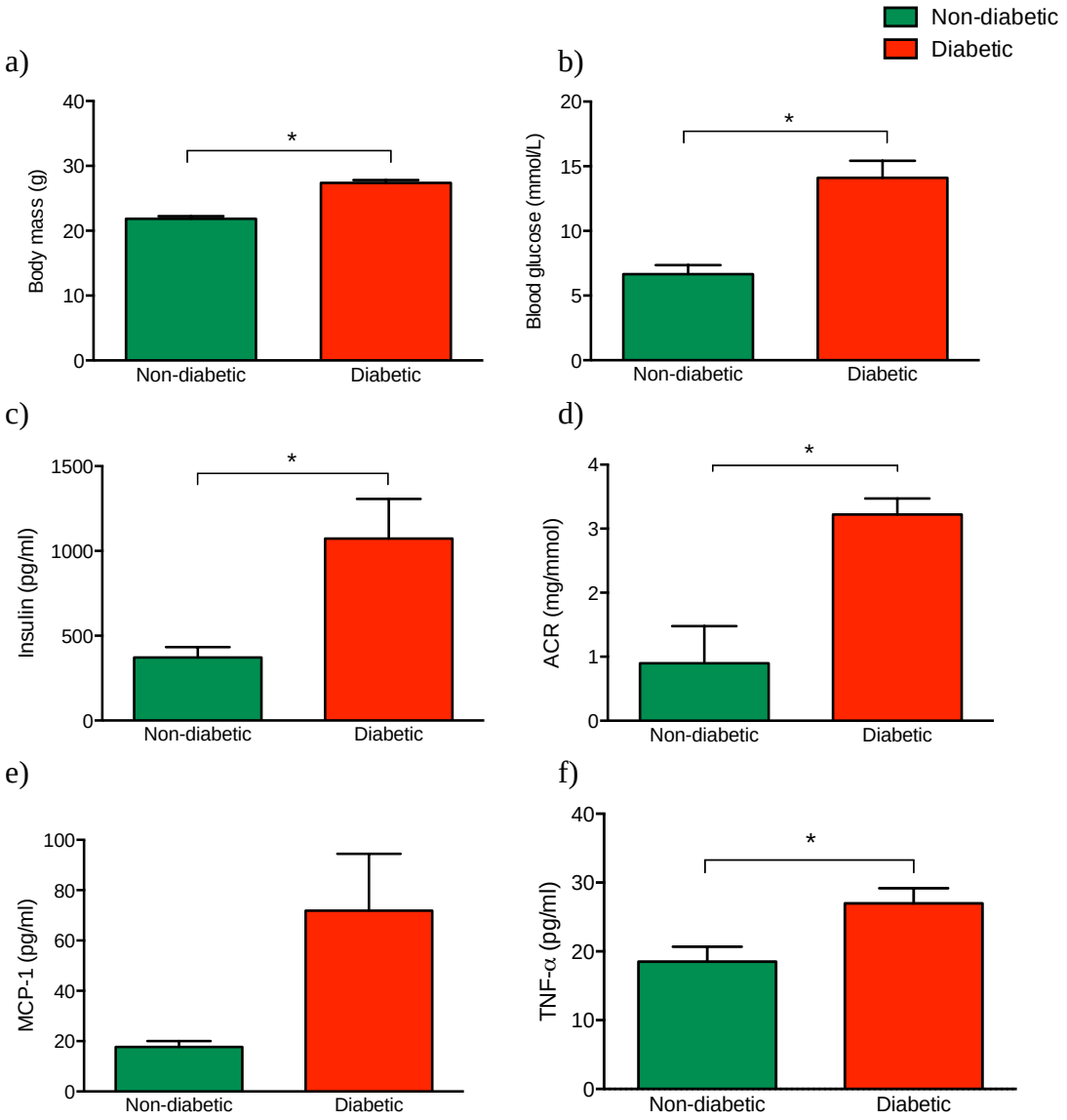

Figure 7.3 Baseline metabolic profile of diabetic and non-diabetic mice
After 15 weeks of diet intervention a) body weight (n = 10 per group), b) blood glucose (n = 10 per group), c) insulin (n = 5 per group) and urine albumin/creatinine ratio (ACR; n = 5 per group) were significantly higher in diabetic mice compared to non-diabetic mice (* $P < 0.05$). Circulating levels of e) MCP-1 tended to be higher in diabetic mice ($P = 0.066$) while baseline levels of f) TNF-α were significantly higher compared to non-diabetic mice. * $P < 0.05$; mean ± SEM.

7.3.4 Susceptibility of diabetic mice to subcutaneous infection with *B. pseudomallei*

Diabetic mice were significantly more susceptible to subcutaneous infection with *B. pseudomallei* compared to their non-diabetic littermates ($P < 0.05$; Figure 7.4). Diabetic mice succumbed rapidly to acute *B. pseudomallei* infection, with a median survival of 4 days and 90% mortality by 6 days post-infection (Figure 7.4). In contrast, non-diabetic mice were relatively resistant to acute infection, with 20% mortality occurring after 10 days post-infection (Figure 7.4). Gross abscessation was observed at the site of infection and in the spleen and liver of mice that survived the experimental period, indicative of persistent *B. pseudomallei* infection.

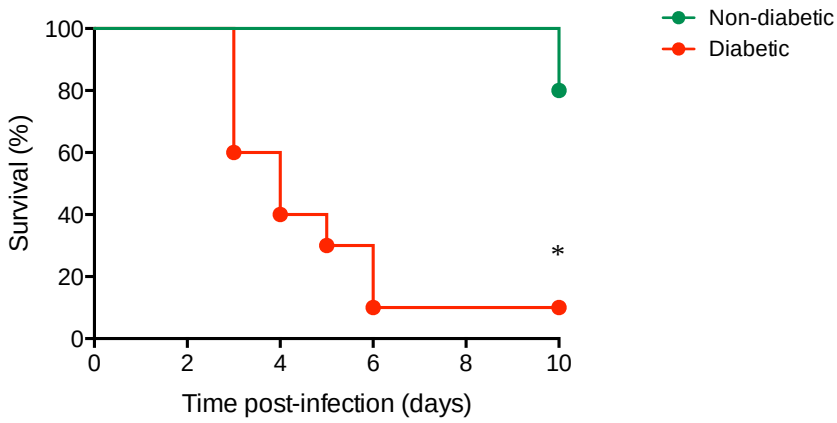

Figure 7.4 Susceptibility of diabetic and non-diabetic mice to subcutaneous infection with *B. pseudomallei*
Following 15 weeks of diet intervention, mice were infected subcutaneously with 9×10^5 CFU of *B. pseudomallei*. Diabetic mice ($n = 10$) were significantly more susceptible to infection with a median survival of 4 days compared to non-diabetic mice. $n = 10$ per group; * $P < 0.05$.

7.4 Discussion

Murine models are complicated by the intrinsic resistance of many strains to the development of T2D and associated micro- and macro-vascular complications, as well as the complex disease pathogenesis of T2D. The results of this study emphasise the importance of dietary GI in the pathogenesis of T2D in C57BL/6 mice, as determined by overt hyperglycaemia. It is noteworthy that weight gain was

similar between HF/HGD and HFD mice, providing support that obesity *per se* does not culminate in T2D (Andersson *et al.* 2010; Pawlak, Kushner & Ludwig 2004). Impaired glucose tolerance and hyperglycaemia in HF/HGD mice relative to littermate controls preceded that in HFD mice and was associated with increased severity of hyperglycaemia at 10 weeks following diet intervention.

Increased consumption of refined carbohydrates predisposes to hyperinsulinaemia due to increased demand for insulin to control rapid spikes in postprandial blood glucose. This is thought to result in pancreatic beta cell exhaustion and subsequent relative insulin deficiency (Bergman 2013). Therefore, it is plausible that the relatively high insulin secretory capacity of C57BL/6 mice (Kooptiwut *et al.* 2002) is able to compensate for HFD-induced insulin resistance to maintain blood glucose homeostasis in HFD mice. On the other hand, pancreatic beta cell exhaustion in HF/HGD mice may lead to rapid hyperglycaemia and worsening glucose tolerance. Consistent with this, several murine studies have documented impaired insulin release and severe destruction of pancreatic islets after consumption of a high GI diet (Andersson *et al.* 2010; Pawlak, Kushner & Ludwig 2004). In addition, the combination of elevated glucose and fatty acid levels in mice fed a HF/HGD could further impair pancreatic beta cell function due to the effects of glucolipotoxicity (Bergman 2013). Although a similar study by Andersson *et al.* (2010) found no significant differences in blood glucose or pancreatic beta cell function in female C57BL/6 mice fed a high GI diet, the disconcordant results may be attributed to confounding effects of gender differences as previously described in Chapter 5 (Stubbins *et al.* 2012).

In this study, C57BL/6 mice consuming a moderately HFD became overweight and insulin resistant but only developed hyperglycaemia when fed a HF/HGD. This has significant clinical implications for nutritional guidelines, which presently advocate low fat, high carbohydrate diets and therefore may actually promote insulin resistance and development of T2D (Acheson 2004). Recent studies have also indicated that in addition to improved insulin sensitivity, increased weight loss (Samaha *et al.* 2003) and reduced biomarkers of CVD (Velazquez-Lopez *et al.* 2013) can be achieved by low carbohydrate diets. The findings from this chapter have significant clinical implications for the re-evaluation of dietary recommendations,

providing impetus for future research on the role of dietary carbohydrates in the pathophysiology of T2D and associated complications.

Findings from this study indicate that increased consumption of refined carbohydrates in combination with dietary fat intake exacerbates glucose intolerance and further increases blood glucose levels in C57BL/6 mice. Male C57BL/6 mice fed a HF/HGD satisfy criteria for diagnosis of clinical T2D according to the development of severe hyperglycaemia. Insulin resistance is evident in T2D mice indicated by hyperinsulinaemia and impaired glucose tolerance. In addition, C57BL/6 mice fed a HF/HGD also exhibit evidence of microalbuminuria, indicating abnormally high permeability of the renal glomerulus, an important prognostic marker of kidney complications in T2D. Therefore, based on these findings, HF/HGD-fed C57BL/6 mice were selected as the most reflective model of T2D for subsequent mechanistic studies investigating the pathogenesis of co-morbid *B. pseudomallei* infection.

CHAPTER 8
DELAYED CYTOKINE RESPONSES TO *B. pseudomallei* INFECTION IN A POLYGENIC MURINE MODEL OF TYPE 2 DIABETES

8.1 Introduction

The use of an *in vivo* animal model has distinct advantages over *in vitro* and *ex vivo* studies, by facilitating investigation of disease progression and local cellular responses within the host in the early stages of infection, prior to the development of overt clinical signs and symptoms. Murine models of melioidosis have been used extensively to understand disease pathogenesis and host immune responses to *B. pseudomallei*. The first murine model of melioidosis was described half a century ago (Dannenberg & Scott 1958). Since then, detailed pictures of acute and chronic melioidosis have emerged from comprehensive studies in BALB/c and C57BL/6 murine strains, respectively (Barnes & Ketheesan 2005; Barnes *et al.* 2001; Barnes *et al.* 2004; Barnes, Williams & Ketheesan 2008; Hoppe *et al.* 1999; Leakey, Ulett & Hirst 1998; Liu *et al.* 2002; Tan *et al.* 2008; Ulett, Ketheesan & Hirst 2000a, 2000b). Differences in innate immunity contribute to the divergence in presentation of *B. pseudomallei* infection and progression in BALB/c and C57BL/6 murine models (Hoppe *et al.* 1999; Leakey, Ulett & Hirst 1998; Ulett, Ketheesan & Hirst 2000a, 2000b). As a model of T2D and comorbid melioidosis has not yet been described, it is unclear how pre-existing T2D impacts on the host immune response to *B. pseudomallei* infection.

Innate immune responses are critical for host defence against *B. pseudomallei* infection. Early resistance to *B. pseudomallei* infection relies on effective macrophage function and production of the macrophage-activating cytokine, IFN-γ, to enable efficient killing of *B. pseudomallei* (Barnes *et al.* 2001; Breitbach *et al.* 2006; Ekchariyawat *et al.* 2005; Santanirand *et al.* 1999; Ulett, Ketheesan & Hirst 1998, 2000a, 2000b; Utaisincharoen *et al.* 2001). Acute melioidosis, modelled by BALB/c mice, develops from uncontrolled growth of *B. pseudomallei* in the spleen, liver and blood, primarily attributed to the poor antimicrobial activity of macrophages (Breitbach *et al.* 2006; Breitbach *et al.* 2009; Leakey, Ulett & Hirst 1998). This leads to increased neutrophil infiltration, severe inflammation, tissue

necrosis and fulminant sepsis (Gan 2005; Liu *et al.* 2002; Ulett, Ketheesan & Hirst 2000a, 2000b).

The severity and outcome of *B. pseudomallei* infection is highly dependent on the presence of host risk factors, of which T2D is the most common. Patients with melioidosis and T2D have higher rates of bacteraemia and relapse in Australia and increased mortality in Malaysia (Currie, Ward & Cheng 2010; Hassan *et al.* 2010). Recent studies have described functional defects in PBMC responses to *B. pseudomallei* from T2D individuals (Morris *et al.* 2012b). Impaired neutrophil function, including phagocytosis of *B. pseudomallei* and neutrophil migration *in vitro*, has also been documented in individuals with T2D (Chanchamroen *et al.* 2009). However, one of the most notable disadvantages of these studies is that they may not reflect the complexity of cell interactions that occur *in vivo*.

It has been difficult to investigate the mechanisms underlying the increased susceptibility of individuals with T2D to *B. pseudomallei* infection without an established model of comorbidity. Studies using streptozotocin-induced diabetic mice have shown that DC and macrophages from C57BL/6 mice have reduced phagocytic, bactericidal mechanisms and impaired production of IL-1β, TNF-α and IL-12 in response to *B. pseudomallei* (Chin, Monack & Nathan 2012; Williams *et al.* 2011). However, as previously discussed, the use of streptozotocin to induce diabetes has distinct aetiological implications and more closely resembles features of T1D rather than T2D.

In earlier studies, we assessed the susceptibility of a monogenic (Chapter 4) and diet-induced model (Chapter 6) of T2D to *B. pseudomallei* infection by comparing survival and bacterial dissemination. Having now developed and refined the polygenic diet-induced model of T2D (Chapter 7), it is now possible to investigate for the first time the early immune responses to *B. pseudomallei* infection and how these might differ from non-diabetic mice. As discussed in Chapter 4, leptin receptor-deficient $Lept^{db}$ diabetic mice are highly susceptible to *B. pseudomallei* infection, with mortality occurring in the first 4 to 6 days, compared to 100% survival of non-diabetic littermates at 10 days post-infection (Chapter 4). Prior to mortality at 3 days post-infection, excessive expression of IL-1β and TNF-α mRNA

was documented in *Lept*^{db} diabetic mice (Hodgson *et al.* 2011). This is consistent with studies based on non-diabetic models and clinical evidence correlating mortality with over-expression of proinflammatory cytokines in septic melioidosis (Suputtamongkol *et al.* 1992; Ulett, Ketheesan & Hirst 2000a, 2000b; Wiersinga *et al.* 2007a). The purpose of this chapter was to investigate the kinetics of bacterial dissemination within the first 24 hours post-infection and the corresponding host cytokine response by measuring protein levels in tissue homogenates. These early kinetics were of particular interest given that disparate bacterial loads in organs are evident by 24 hours post-infection (Chapter 7) and later immune responses during septic melioidosis have been extensively described. Therefore, the specific aims of Chapter 8 were to:

i) compare kinetics of bacterial dissemination following subcutaneous infection with *B. pseudomallei* in diabetic and non-diabetic mice
ii) compare inflammatory cytokine responses and histological changes following *B. pseudomallei* infection in diabetic and non-diabetic mice

8.2 Materials and Methods

8.2.1 Survival, bacterial organ loads and histology

Male C57BL/6 mice were fed either a HF/HGD or normal rodent chow for 15 weeks. Mice were infected subcutaneously with 9×10^5 CFU of *B. pseudomallei* (Chapter 3). Survival of diabetic and non-diabetic mice (n = 10 per group) was monitored daily for 14 days. At 1, 6, 12, 24 and 72 hours post-infection, mice (n = 5 per group and time point) were euthanased to compare bacterial burden in the spleen, liver, lung, inguinal LN, SAT and blood using methods described previously (Chapter 3). Mice were also euthanased at 1 and 12 hours post-infection (n = 3 mice per group and time point) and the spleen, liver and lung were fixed for histological analysis, as described in Chapter 3. To compare inflammatory cell infiltration and necrosis, sections were stained with H & E (5 sections per organ at 10μm intervals).

8.2.2 Cytokine kinetics

At 0, 6, 12 and 24 hours post-infection, mice were euthanased (n = 5 per group and time point) and spleen was collected and homogenised in 1ml of PBS. Cell free homogenates were frozen at -80°C until analysed. Concentrations of MCP-1, TNF-α, IL-12, IFN-γ, IL-10 and IL-6 were determined by CBA (Mouse Inflammation Kit, BD Biosciences, Australia) in duplicate according to the manufacturer's instructions. The theoretical limit of detection of the assay was 20pg/ml to 5,000pg/ml. Data was acquired on a FACS Calibur flow cytometer using CellQuest Pro and analysed by FCAP array v3 software (BD Biosciences, Australia). Concentrations were normalised to the mass of tissue that was homogenised in PBS and are expressed as pg/ml ± SEM (per 1g of tissue). The experiment was repeated 3 times with representative results presented from one experiment.

8.2.3 Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 6.0b. Bacterial dissemination was compared between groups by two-way ANOVA, with Bonferroni post-hoc tests. Unpaired Student's T-tests were used for comparison of baseline and post-infection cytokine levels between diabetic and non-diabetic mice. Survival of diabetic and non-diabetic mice was compared using the Kaplan–Meier method and the Log-rank test. Statistical significance was determined at $P \leq 0.05$ and data are expressed as mean ± SEM.

8.3 Results

8.3.1 Early dissemination of *B. pseudomallei* in diabetic and non-diabetic mice

Following 15 weeks of diet intervention, diabetic and non-diabetic mice were infected subcutaneously with *B. pseudomallei*. At 1 hour post-infection, bacterial loads were comparable in the SAT of diabetic and non-diabetic mice (P = 0.730) and dissemination to other organs was evident (Figure 8.1). *B. pseudomallei* loads were comparable in the spleen (P = 1.00), liver (P = 0.524), LN (P = 1.00) and lung (P = 1.00) between diabetic and non-diabetic mice at 1 hour post-infection (Figure

8.1). Despite rapid dissemination of *B. pseudomallei*, presumably via the haematogenous route, levels of *B. pseudomallei* remained below the limit of detection in the blood at 1 hour post-infection.

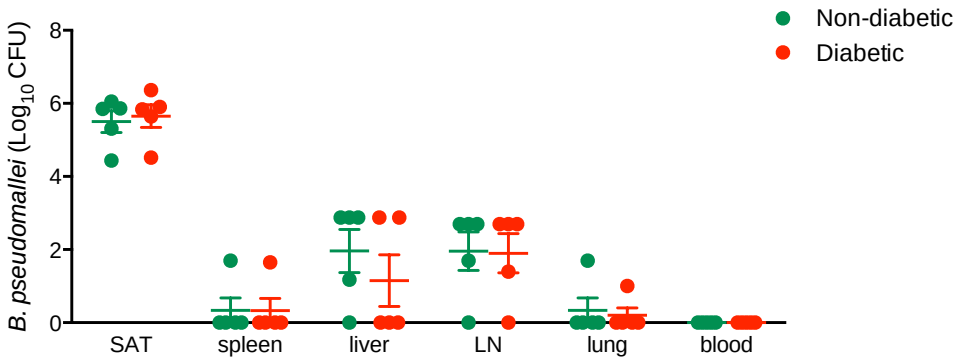

Figure 8.1 Dissemination of *B. pseudomallei* at 1 hour post-infection
At 1 hour post-infection, *B. pseudomallei* had spread from subcutaneous adipose tissue (SAT) at the site of infection to the spleen, liver, inguinal lymph nodes (LN) and lung of diabetic and non-diabetic mice. $n = 5$; mean Log_{10} CFU ± SEM.

At all time points examined, *B. pseudomallei* loads were comparable between diabetic and non-diabetic mice in SAT at the site of infection (Figure 8.2a). At 6 hours post-infection, levels of *B. pseudomallei* were comparable in the spleen but growth of *B. pseudomallei* tended to be greater in spleen of diabetic mice compared to non-diabetic mice by 12 hours post-infection ($P = 0.060$; Figure 8.2b). The burden of *B. pseudomallei* tended to be greater in spleen of diabetic mice at 24 hours post-infection ($P = 0.053$) and was significantly greater by 72 hours ($P < 0.05$). Growth of *B. pseudomallei* in liver followed a similar trend (Figure 8.2c). In contrast, the dissemination of *B. pseudomallei* in draining LN was similar between diabetic and non-diabetic mice (Figure 8.2d). Despite slightly higher *B. pseudomallei* loads in lungs of diabetic mice within the first 24 hours of infection, levels were also similar in lungs of diabetic and non-diabetic mice by 72 hours post-infection (Figure 8.2e). The growth of *B. pseudomallei* in blood was significantly greater in diabetic mice, with bacteraemia evident by 72 hours post-infection ($P < 0.05$; Figure 8.2f).

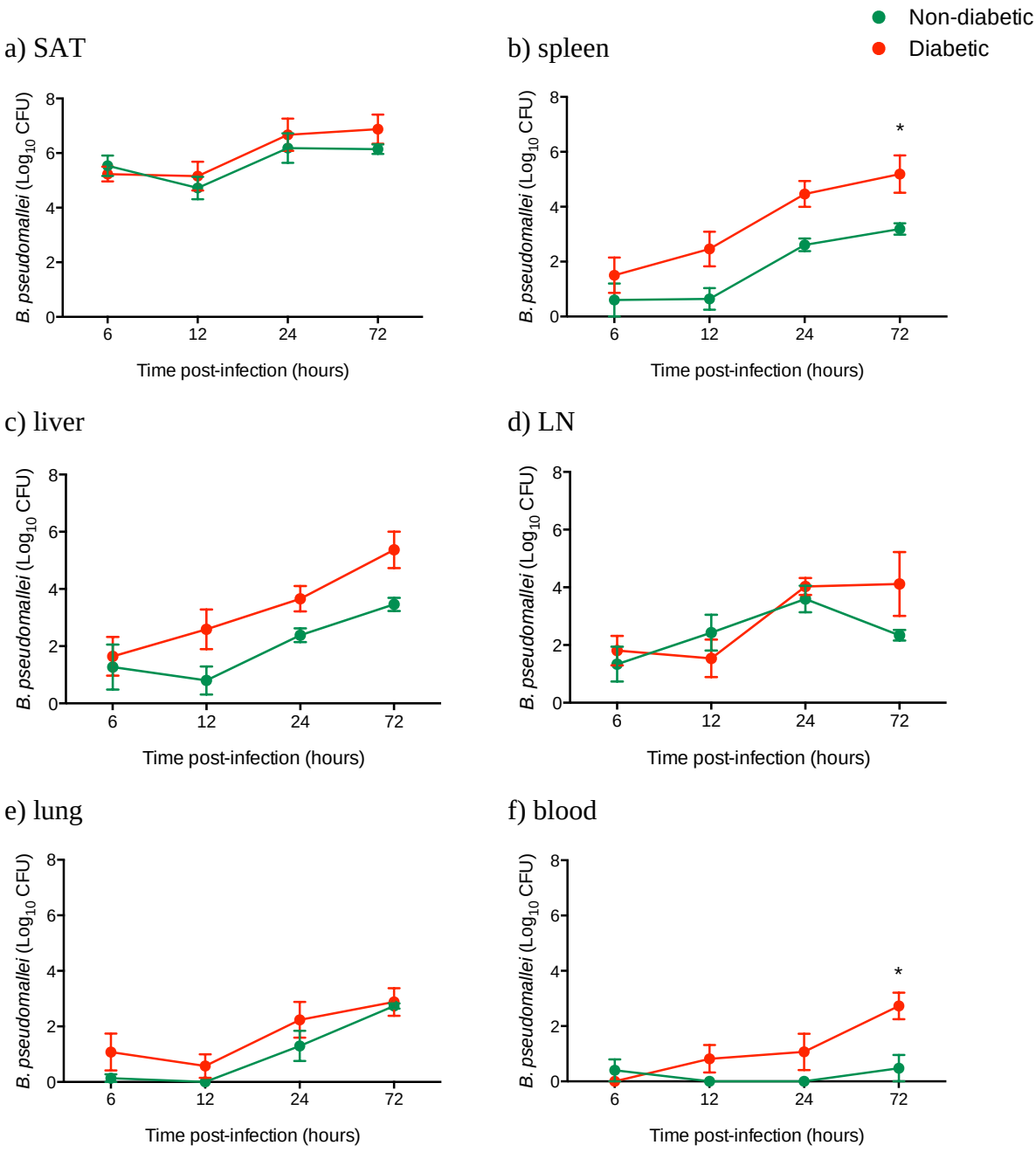

Figure 8.2 Bacterial dissemination following subcutaneous infection with *B. pseudomallei*

a) Levels of *B. pseudomallei* were comparable in the subcutaneous adipose tissue (SAT) of diabetic and non-diabetic mice in the first 72 hours post-infection. b) *B. pseudomallei* growth was increased in the spleen of diabetic mice and was significantly higher compared to non-diabetic mice by 72 hours post-infection. A similar trend was observed in the c) liver of diabetic mice although it did not reach significance. d) *B. pseudomallei* levels in the inguinal lymph node (LN) were comparable until 72 hours post-infection, tending to be higher in LN of diabetic mice compared to non-diabetic mice. e) *B. pseudomallei* levels tended to be higher in the lung of diabetic mice in the first 6 hours post-infection, but were comparable by 72 hours post-infection. f) Growth of *B. pseudomallei* was greater in the blood of diabetic mice, which were bacteraemic at 72 hours post-infection. n = 5 per group; * $P < 0.05$; Log_{10} CFU ± SEM.

8.3.2 Cytokine responses to *B. pseudomallei* infection in spleen of diabetic and non-diabetic mice

Concentrations of IL-10 were comparable at baseline in the spleen of diabetic and non-diabetic mice (Appendix 2.7), while levels of MCP-1, TNF-α, IL-12, IFN-γ and IL-6 were below the limit of detection. Between 6 and 12 hours post-infection, when *B. pseudomallei* growth tended to be greater in diabetic mice, levels of MCP-1 in spleen were reduced in diabetic mice compared to non-diabetic mice ($P < 0.05$; Figure 8.3a). Between 12 and 24 hours post-infection, concentrations of TNF-α, IL-10, IL-12 and IFN-γ were significantly lower in spleen of diabetic mice compared to non-diabetic mice ($P < 0.05$; Figure 8.3b-e).

8.3.3 Histological evidence of inflammation following subcutaneous infection with *B. pseudomallei*

While there was no evidence of inflammation in liver from non-diabetic mice at 1 hour post-infection (Figure 8.4a), small inflammatory foci were observed in the liver of diabetic mice (Figure 8.4b). At 1 hour post-infection, there was marked neutrophil infiltration in the spleen of non-diabetic and diabetic mice (Figure 8.4c and d). By 12 hours post-infection, inflammation was comparable in the liver of diabetic and non-diabetic mice (Figure 8.5a and b). Multiple inflammatory foci could be observed, of which neutrophils were the predominant constituent. Furthermore, significant neutrophil infiltration was observed in spleen of both diabetic and non-diabetic mice at 12 hours post-infection (Figure 8.5c and d). There was no evidence of inflammation in lung of non-diabetic and diabetic mice at these early time points (data not shown).

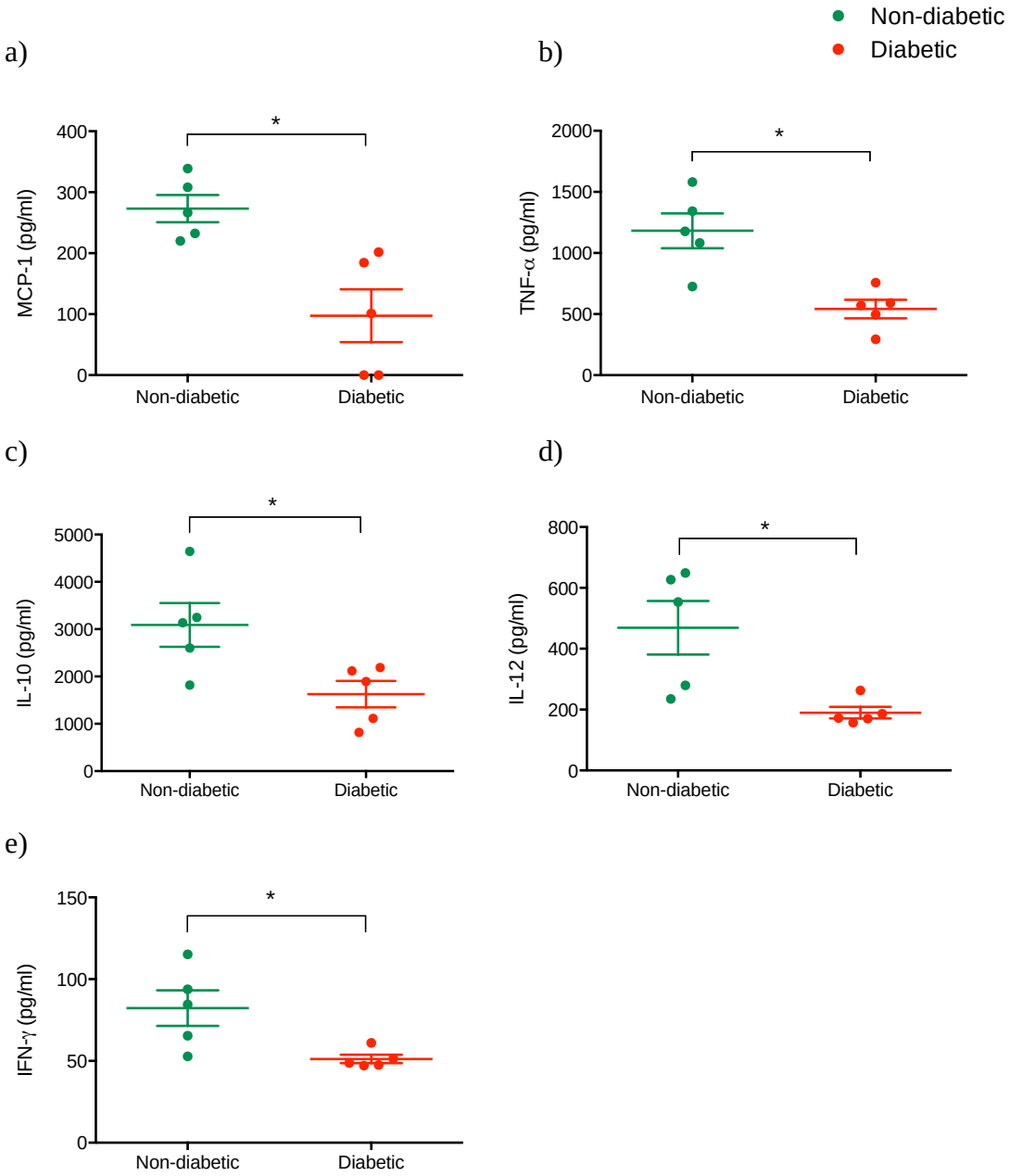

Figure 8.3 Concentration of MCP-1, TNF-α, IL-10, IL-12 and IFN-γ in spleen of diabetic and non-diabetic mice following infection with *B. pseudomallei*

Mice were subcutaneously infected with 9×10^5 CFU of *B. pseudomallei.* At 6 hours post-infection, secretion of MCP-1 was higher in spleen of non-diabetic mice compared to diabetic mice. At 12 hours post-infection, secretion of b) TNF-α, c) IL-10, d) IL-12 and e) IFN-γ was higher in spleen of non-diabetic mice compared to diabetic mice. $n = 5$ per group; * $P < 0.05$; mean ± SEM.

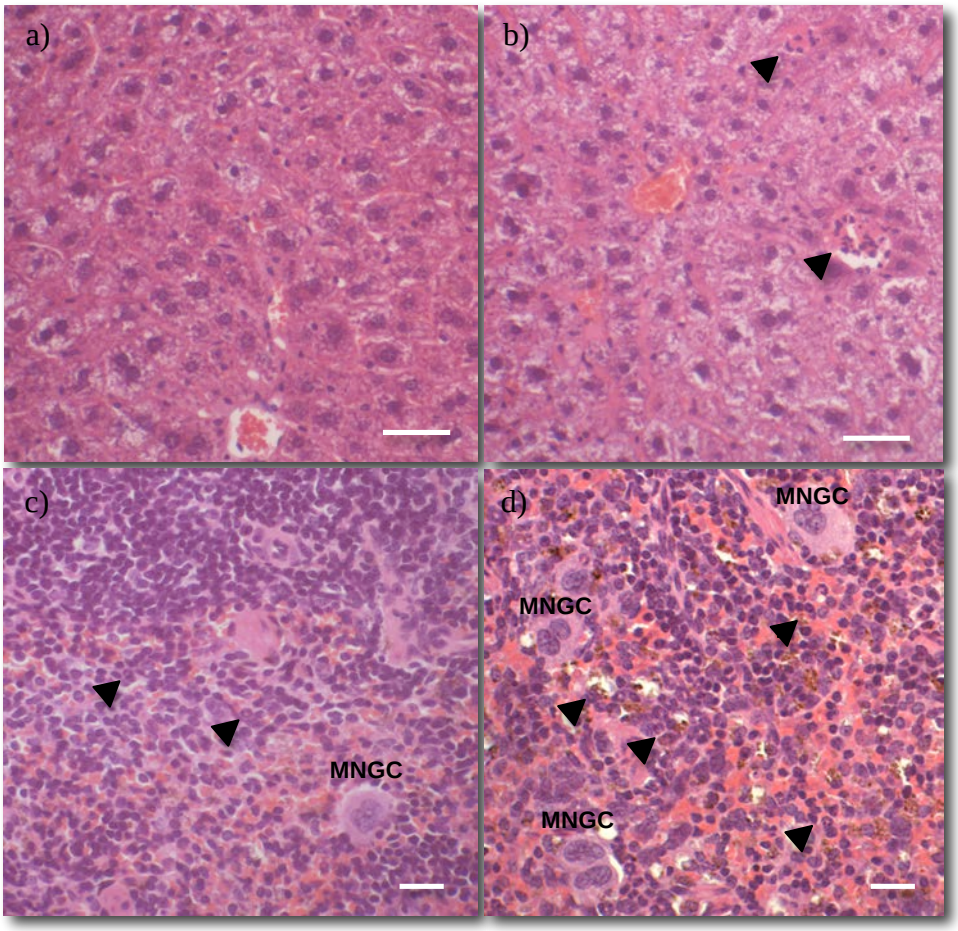

Figure 8.4 Inflammation in liver and spleen at 1 hour post-infection with *B. pseudomallei*
a) No inflammatory foci were observed in livers from non-diabetic mice at 1 hour post-infection. b) In contrast, multiple inflammatory foci of neutrophils (arrow heads) were observed in liver of diabetic mice (scale = 50µm; magnification = ×200). Neutrophil infiltration (arrow heads) was observed in spleen of c) non-diabetic and d) diabetic mice at 1 hour post-infection, in addition to the presence of multinucleated giant cells (MNGC; scale = 20µm; magnification = ×400). Images are representative of n = 3 mice per group.

8.4 Discussion

Observations from this study indicate that *B. pseudomallei* dissemination from the primary site to distant organs in the body occurs rapidly following subcutaneous infection. Infiltration of neutrophils in the spleen and liver of diabetic mice by 1 hour post-infection is consistent with the rapid dissemination of *B. pseudomallei*. However, the ability of diabetic mice to control *B. pseudomallei* infection is reduced compared to non-diabetic mice. Increased growth of *B. pseudomallei* in the first 24 hours following subcutaneous infection was observed in the spleen of diabetic mice

compared to non-diabetic mice resulting in a higher bacterial burden in this tissue by 72 hours post-infection. This coincided with bacteraemia in diabetic mice and was followed by 90% mortality within 5 days post-infection (Chapter 7). Intracellular survival of *B. pseudomallei* in phagocytic cells, including macrophages and DC, is an important virulence mechanism contributing to disease pathogenesis (Charoensap *et al.* 2009). Rapid bacterial dissemination from the site of infection most likely occurs via haematogenous and lymphatic spread of intracellular and extracellular *B. pseudomallei*, and is consistent with the widespread visceral involvement observed in clinical melioidosis, attributed to secondary bacteraemic spread (Cheng & Currie 2005).

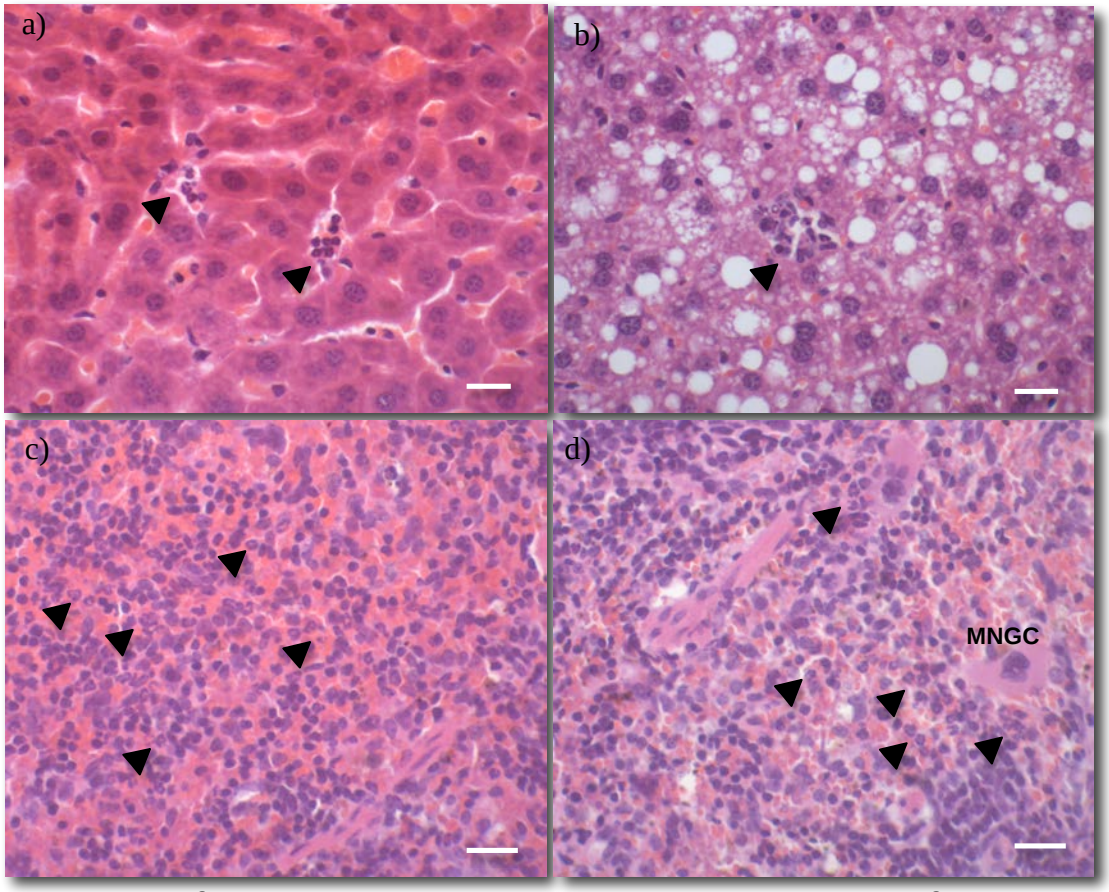

Figure 8.5 Inflammation in liver and spleen at 12 hours post-infection with *B. pseudomallei*

Comparable inflammatory infiltrate, consisting predominantly of neutrophils (arrow heads), was observed in liver from a) non-diabetic and b) diabetic mice at 12 hours post-infection. Extensive neutrophil infiltration was also evident in spleen of c) non-diabetic and d) diabetic mice at 12 hours post-infection (arrow heads; scale = 20μm; magnification = ×400). Images are representative of n = 3 mice per group.
MNGC – multinucleated giant cells.

The kinetics and concentration of various cytokines are critical in determining the balance between resistance to *B. pseudomallei* infection or development of immunopathology (Brown *et al.* 1991; Hoppe *et al.* 1999; Koo & Gan 2006; Lauw *et al.* 1999; Liu *et al.* 2002; Simpson *et al.* 2000; Suputtamongkol *et al.* 1999; Ulett, Ketheesan & Hirst 2000a, 2000b). Whilst inflammation is critical for early bacterial containment, uncontrolled amplification of the inflammatory response can result in excessive damage to tissues. The hyperinflammatory cytokine response during melioidosis sepsis has been extensively characterised in mice and humans (Ulett, Ketheesan & Hirst 2000a, 2000b; Wiersinga *et al.* 2007a). Previous studies using BALB/c mice have found that dissemination of *B. pseudomallei* to the spleen and liver precedes highly elevated levels of IL-1β, IL-6, TNF-α and IFN-γ at 48 to 72 hours post-infection, coinciding with overwhelming sepsis and mortality (Ulett, Ketheesan & Hirst 2000a, 2000b). This is supported by clinical evidence correlating high serum concentrations of IL-1β, IL-6, TNF-α, IFN-γ and IL-10 with poor outcome in patients with septic melioidosis (Wiersinga *et al.* 2007a). However, the mechanisms responsible for the initial failure to control *B. pseudomallei* growth in the first 12 hours post-infection, prior to the clear divergence in disease progression and development of sepsis, are still not clear.

In this study, reduced levels of MCP-1 were detected in spleen of diabetic mice, corresponding to higher bacterial loads in this tissue at 6 hours post-infection. MCP-1 is an important chemoattractant factor for the recruitment of monocytes, T cells and DC to sites of inflammation (Figure 8.6) (Carr *et al.* 1994; Xu *et al.* 1996). As such, secretion of MCP-1 is critical for host resistance to *B. pseudomallei* infection (Barnes *et al.* 2001). By 12 hours post-infection, there were also significantly lower levels of TNF-α, IL-12 and IFN-γ in spleen of diabetic compared to non-diabetic mice. This was not due to increased production of IL-10, an anti-inflammatory cytokine that inhibits synthesis of inflammatory cytokines, which was also reduced at this time. Importantly, these findings indicate that diabetic mice have a broadly reduced innate immune response to *B. pseudomallei* infection. This may be secondary to delayed immune cell activation and recruitment to sites of infection or a direct consequence of an innate defect in signalling pathways. Future research to understand the underlying mechanisms is warranted.

A significant source of IFN-γ in the early stages of *B. pseudomallei* infection is NK cells and $CD8^+$ T cells via antigen-independent activation (Figure 8.6) (Koo & Gan 2006; Lertmemongkolchai *et al.* 2001). This process is mediated by macrophage-derived IL-12 (Haque *et al.* 2006b; Lertmemongkolchai *et al.* 2001). Therefore, decreased secretion of IL-12 in the current study may influence the subsequent impairment in the early IFN-γ response, thereby negatively impacting on the microbicidal efficiency of *B. pseudomallei*-containing macrophages (Figure 8.6). This delay in fundamental cytokine responses in diabetic mice occurs at a critical time post-infection that corresponds to increased bacterial growth and possibly plays a central role in the reduced host resistance and accelerated disease progression.

The impaired cytokine production observed in diabetic mice in the current study may be a result of altered activation or functional responses of phagocytes. Delayed production of MCP-1 and TNF-α in diabetic mice may further contribute to impaired recruitment and activation of phagocytes and reduced clearance of *B. pseudomallei* infection (Figure 8.6). The immunomodulatory role of phagocytes through secretion of IL-12 and TNF-α is critical in early control of bacterial infections (Pedrosa *et al.* 2000; Tateda *et al.* 2001). IL-12 and TNF-α are key cytokines in the early innate immune response to *B. pseudomallei* infection (Barnes, Williams & Ketheesan 2008; Haque *et al.* 2006b; Wiersinga *et al.* 2006). Production of TNF-α by macrophages and neutrophils at sites of infection enhances immune activation and accelerates the inflammatory response, triggering the recruitment of DC required to initiate adaptive immunity. The synergistic effect of TNF-α and IFN-γ also enhances macrophage activation to enable killing of intracellular *B. pseudomallei*. These initial impairments in IL-12 secretion by phagocytes may be responsible for the subsequent impaired IFN-γ secretion by NK and $CD8^+$ T cells in response to *B. pseudomallei*. Macrophage activation by IFN-γ is absolutely critical for efficient killing of intracellular *B. pseudomallei*. Secretion of IL-12 and IFN-γ also drives the development of T_H1-type adaptive immune responses, important for effective clearance of *B. pseudomallei* infection (Haque *et al.* 2006a; Haque *et al.* 2006b; Romagnani 1997). Therefore, the delayed cytokine responses observed in diabetic mice in the current study may also impact on the development of adaptive immunity (Figure 8.6), although this is yet to be investigated.

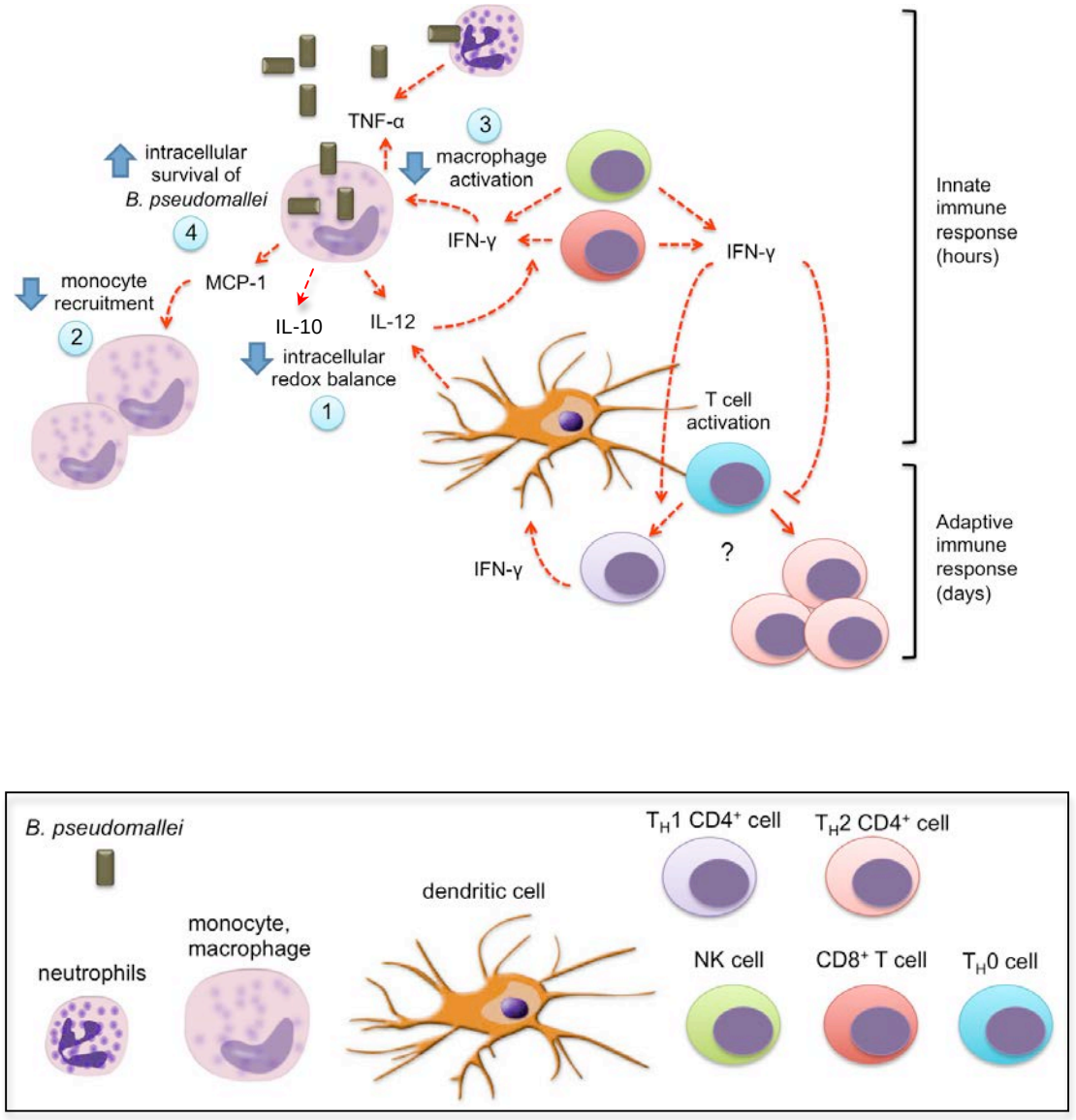

Figure 8.6 Putative dysregulation of immune response to *B. pseudomallei* infection in diabetic mice

The intracellular redox imbalance of macrophages may modulate cytokine responses to *B. pseudomallei* (1). Impaired secretion of MCP-1 may result in reduced recruitment of phagocytes to sites of infection (2), which may contribute to further decreases in levels of TNF-α and IL-12. The downstream consequences of reduced TNF-α and IL-12 include reduced IFN-γ production by natural killer (NK) and $CD8^+$ T cells and impaired macrophage activation (3). IFN-γ is critical for effective macrophage activation to enhance killing of *B. pseudomallei*, which can otherwise persist intracellularly (4). Reduced levels of IFN-γ may have consequences on subsequent naïve T (T_H0) cell differentiation, favouring development of T helper type 2 (T_H2) $CD4^+$ cells as opposed to T helper type 1 (T_H1) $CD4^+$ cells, which are critical in protection against intracellular pathogens. However, the dysregulation of adaptive immunity in diabetic mice is yet to be confirmed. Broken arrows indicate reduced activity of these pathways.

There is evidence to suggest that defects in neutrophil function may play a role in the comorbidity of T2D and melioidosis (Marhoffer *et al.* 1992; Muniyappa *et al.* 1998; Tseng *et al.* 1997). Neutrophils are rapidly recruited to sites of *B. pseudomallei* infection and are thought to be important for the initial containment of bacteria (Easton *et al.* 2007; Riyapa *et al.* 2012; Wiersinga *et al.* 2008a; Wiersinga *et al.* 2008b). It was shown in an *in vitro* study that neutrophils from individuals with T2D exhibited impaired phagocytosis of *B. pseudomallei* and reduced migration in response to IL-8 when compared to neutrophils from non-diabetic individuals (Chanchamroen *et al.* 2009). Although, in the current study, rapid infiltration of neutrophils was histologically observed in the liver and spleen of diabetic mice within 1 hour post-infection, it is possible that migration of neutrophils may be impaired in diabetic hosts after exposure to *B. pseudomallei*. Histological analysis has quantitative limitations and impaired migration of neutrophils in diabetic hosts cannot be confirmed without additional quantitative analysis such as flow cytometry. It is also possible that impaired function of neutrophils may contribute to increased bacterial survival in diabetic mice. Impaired NET release by neutrophils from diabetic individuals in response to stimulation with *B. pseudomallei in vitro* has been reported (Riyapa *et al.* 2012). This was directly associated with reduced killing of *B. pseudomallei* by neutrophils from diabetic individuals compared to non-diabetic, healthy controls (Riyapa *et al.* 2012). While this may contribute to increased bacterial growth in diabetic hosts, the importance of such neutrophil defects *in vivo* still needs to be confirmed. Control of *B. pseudomallei* infection is greatly assisted by the recruitment and appropriate activation of additional phagocytes, including macrophages and DC (Barnes, Williams & Ketheesan 2008). The complex interplay between neutrophils, macrophages and DC is crucial to host resistance against *B. pseudomallei* infection, and further research investigating these cellular interactions in diabetic hosts are warranted.

Macrophages are an essential component of the protective immune response to intracellular bacteria such as *B. pseudomallei* (Barnes, Williams & Ketheesan 2008). Compared to the neutrophil-dominant infiltrate in susceptible BALB/c mice, resistance of C57BL/6 mice to *B. pseudomallei* is associated with increased recruitment of mononuclear cells to sites of infection (Barnes *et al.* 2001; Barnes, Williams & Ketheesan 2008; Tan *et al.* 2008). Increased killing of *B. pseudomallei*

by IFN-γ-activated macrophages from C57BL/6 mice also contributes to their greater resistance to *B. pseudomallei* infection compared to BALB/c mice (Liu *et al.* 2002). Activation of macrophages by IFN-γ results in enhanced killing of intracellular *B. pseudomallei* and increased production of proinflammatory cytokines in experimental melioidosis, the absence of which leads to the development of septicaemia and host death in the first 24 hours of infection (Santanirand *et al.* 1999). Without activation by IFN-γ, internalised *B. pseudomallei* are able to escape from phagocytic vesicles and multiply in the cytoplasm, leading to the formation of MNGC, which facilitates intracellular spread of *B. pseudomallei* (Boddey *et al.* 2007; French *et al.* 2011; Kespichayawattana *et al.* 2000; Wong, Puthucheary & Vadivelu 1995). Depletion of both macrophages and TNF-α significantly increases the susceptibility of C57BL/6 mice to *B. pseudomallei* infection (Barnes, Williams & Ketheesan 2008; Breitbach *et al.* 2006). Therefore, the reduced levels of TNF-α, IL-12 and IFN-γ in the current study potentially contribute to impaired macrophage responses to *B. pseudomallei* and decreased clearance of infection in diabetic mice.

As an intracellular bacterium, many of the host protective responses to *M. tuberculosis* are believed to be analogous to those mounted against *B. pseudomallei*. Similar to melioidosis, T2D is also an important risk factor for tuberculosis. Significantly more knowledge regarding the immune responses in the comorbidity of tuberculosis and T2D is available, which may shed light on the mechanisms contributing to comorbid melioidosis. As observed for *B. pseudomallei* infection in diabetic mice in the current study, delayed IFN-γ and IL-12 production both contribute to disease severity in experimental models of *M. tuberculosis* (Cooper *et al.* 1993; Flynn *et al.* 1993; Kinjo *et al.* 2002). Secretion of IL-12 facilitates the development of T_H1 cells, contributing to heightened macrophage responses and the ability to kill intracellular pathogens (Trinchieri 1998). IL-12 is also important in mediating IFN-γ production and cytotoxicity of $CD8^+$ T cells in response to *M. tuberculosis* (Samten *et al.* 2003). Decreased production of IL-12 by macrophages both clinically and in experimental models of T2D in response to stimulation with *Mycobacterium bovis* bacille Calmette-Guérin (BCG) has been reported (Tsukaguchi *et al.* 1997; Tsukaguchi *et al.* 2002; Yamashiro *et al.* 2005). Attenuated production of IFN-γ and IL-12 in PBMC from diabetic individuals has

been described in response to *M. tuberculosis* stimulation *in vitro* (Tsukaguchi *et al.* 1997; Tsukaguchi *et al.* 2002) and in experimental mouse models of streptozotocin-induced diabetes (Yamashiro *et al.* 2005). This is consistent with our observations, suggesting that delayed production of IFN-γ and IL-12 may be a universal defect in T2D contributing to the impaired host resistance to intracellular bacteria such as *B. pseudomallei* and *M. tuberculosis.*

The mechanisms responsible for the delayed production of inflammatory cytokines in response to infections in diabetic hosts are not known. Differences in the intracellular redox state of macrophages and DC have been shown to modulate cytokine responses (Murata, Shimamura & Hamuro 2002). Disturbances in the redox balance of macrophages and DC leading to an oxidative state is associated with decreased production of IL-12 in response to LPS (Morris *et al.* 2013a; Murata *et al.* 2002b). This may be one putative mechanism contributing to depleted levels of IL-12 observed in diabetic mice following *B. pseudomallei* stimulation, caused by disturbances in redox-sensitive signalling pathways, particularly involving activation of NF-κB, as discussed in Chapter 9. Reduction in levels of IFN-γ in diabetic mice may subsequently occur due to the downstream decrease in IL-12-dependent production of IFN-γ by NK and $CD8^+$ T cells.

The appropriate timing and regulation of inflammatory cytokines in the early phase of *B. pseudomallei* infection are crucial in determining host resistance (Barnes, Williams & Ketheesan 2008; Morris *et al.* 2012b). Findings of the current study indicate novel differences in early cytokine responses between diabetic and non-diabetic mice following infection with *B. pseudomallei*. The delayed release of MCP-1, TNF-α, IL-12 and IFN-γ in diabetic mice occurring in the first 12 hours post-infection corresponds to increased growth of *B. pseudomallei*, ultimately leading to overwhelming bacteraemia and early death compared to non-diabetic mice. Further investigations are necessary to understand the mechanisms underlying the delayed cytokine response to *B. pseudomallei* in diabetic mice.

CHAPTER 9
THE POSSIBLE ROLE OF GLUTATHIONE IN SUSCEPTIBILITY OF MICE WITH TYPE 2 DIABETES TO *B. pseudomallei* INFECTION

9.1 Introduction

An important finding from Chapter 8 was the delayed cytokine response following *B. pseudomallei* infection in diabetic mice. This was suggestive of defective early activation and function of phagocytes. The intracellular redox state of phagocytes, specifically macrophages, has been shown to modulate cytokine production (Murata, Shimamura & Hamuro 2002). Glutathione (GSH) is the major regulator of the intracellular redox balance in eukaryotic cells (Seres *et al.* 2000). This provided the impetus to compare the levels of GSH and subsequent macrophage functional responses to *B. pseudomallei* between diabetic and non-diabetic mice.

The primary role of GSH is regulating the cellular redox balance by cycling between reduced (GSH) and oxidised (GSSG) states (Griffith 1999; Hayes & McLellan 1999; Tran *et al.* 2004). Under physiological conditions, high levels of intracellular GSH (>90%) are maintained compared to GSSG (<10%). The intracellular redox balance is indicated by the ratio of GSH/GSSG and is crucial for normal cell metabolism. Regulation of *de novo* synthesis of GSH and its metabolism involves a complex network of enzymes and pathways, illustrated in Figure 9.1. While GSH synthesis occurs in all cells of the body (Chen *et al.* 2007), hepatic tissue has the highest levels of GSH and hepatocyte GSH synthesis is intimately linked to host survival (Chen *et al.* 2007). Net loss of GSH occurs during the reduction of ROS by GSH peroxidase, which oxidises GSH to GSSG; GSH reductase can regenerate GSH from GSSG with reducing equivalents from NADPH. GSH is also depleted through the activity of GSH transferase, which is involved in detoxification through attachment of toxic compounds to GSH (Malmezat *et al.* 2000). During oxidative stress, GSH is consumed and GSSG is produced, shifting the intracellular redox balance to a lower GSH/GSSG ratio. Ultimately, the GSH concentration and overall intracellular redox balance are dependent on the combined activity of these processes (Malmezat *et al.* 2000).

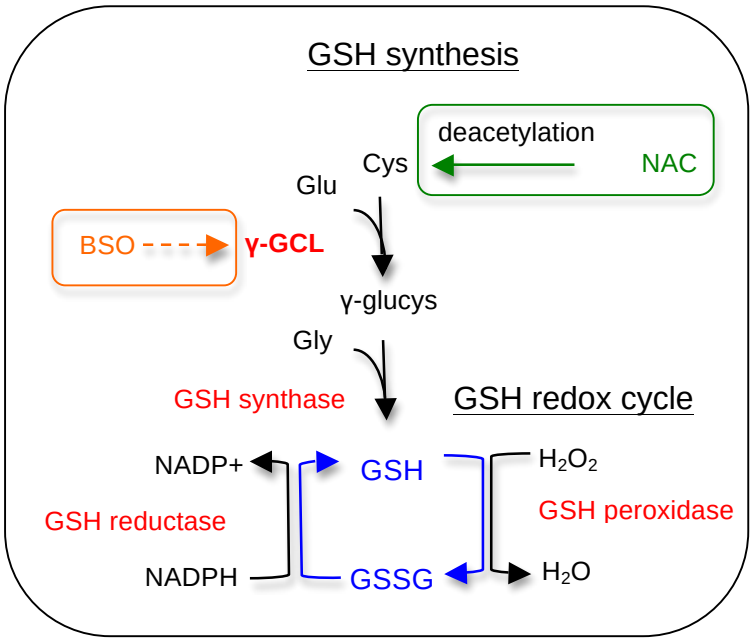

Figure 9.1 Pathways involved in the biosynthesis and metabolism of glutathione Cysteine is the rate-limiting factor in the biosynthesis of glutathione (GSH) and the generation of γ-glutamylcysteine by γ-glutamylcysteine synthetase (γ-GCS) is the rate-limiting step. In the process of eliminating oxidants, GSH is oxidised to GSSG and requires NADPH to be converted back to GSH, leading to the highly dynamic cycle between reduced (GSH) and oxidised (GSSG) states. *N*-acetyl-*L*-cysteine (NAC) is a thiol that is rapidly deacetylated to augment intracellular cysteine levels, thereby promoting augmenting GSH levels. *L*-buthionine-sulfoximine (BSO) inhibits *de novo* GSH synthesis by preventing γ-GCL activity. γ-GCL – γ-glutamylcysteine ligase, γ-glucys – γ-glutamylcysteine, Cys – cysteine, Glu – glutamine, Gly – glycine, GSH – glutathione (reduced), GSSG – glutathione disulfide (oxidised).

The function of GSH extends beyond protection from oxidative stress. In addition to antioxidant activities, GSH has many important cellular functions, including regulating synthesis of DNA precursors, sulfhydryl-dependent enzymes and gene expression (Barycki 2008; Cappel & Gilbert 1988; Gerard-Monnier & Chaudiere 1996; Kosower & Kosower 1969; Malmezat *et al.* 2000; Morris *et al.* 2012a; Staal *et al.* 1990; Zhang, Forman & Choi 2005). GSH deficiency has been described in many human diseases including cancer, neurodegenerative diseases, hepatitis, cystic fibrosis, chronic obstructive pulmonary disease (COPD), sepsis and T2D (Atkuri *et al.* 2007; Townsend, Tew & Tapiero 2003). Understanding of the regulation of GSH in these pathological conditions is incomplete but it is hypothesised that the consumption of GSH is induced by chronic inflammation, a condition that is thought to play a key role in the pathogenesis of T2D (Malmezat *et al.* 2000). Decreased

levels of GSH in T2D may be a consequence of increased oxidative stress from immune activation by AGE and additional glucolipotoxic mechanisms contributing to the pathogenesis of T2D (Tran *et al.* 2004). Depleted levels of NADPH due to increased flux of excess glucose through the polyol pathway may also lead to reduced conversion of GSSG to GSH, contributing to the build-up of GSSG levels (Bravi *et al.* 1997).

GSH deficiency has been described in T2D and excessive alcohol intake, which are also associated with an increased risk of infection. Clinical studies have documented reduced GSH levels in erythrocytes and plasma of patients with T2D (De Mattia *et al.* 1998; Jain & McVie 1994; Samiec *et al.* 1998), although findings from animal models of T2D have been less consistent (Sheng, Huang & Xu 2005; Unlucerci, Bekpinar & Kocak 2000). The mechanisms contributing to the depletion of GSH and its pathophysiology in humans is an active area of research. However, it evident that through its antioxidant activities, GSH can directly protect against oxidative stress, which is central to the pathogenesis of many of the complications associated with T2D (Osawa & Kato 2005). Furthermore, since oxidative stress contributes to the primary complication of impaired glucose metabolism in T2D, increasing circulating GSH levels has been shown to improve insulin sensitivity in people with T2D (Paolisso *et al.* 1993a; Paolisso *et al.* 1993b; Paolisso *et al.* 1992).

Deficiency in GSH is associated with an array of complications, one of which may involve the increased susceptibility to infections due to the potential for both direct and indirect effects of GSH. *In vitro* studies have shown that GSH is directly toxic to *M. tuberculosis* (Green, Seth & Connell 2000; Venketaraman *et al.* 2003). The basis of this toxicity and the extent to which it affects other microbes is not known (Green, Seth & Connell 2000). Since GSH is present in high concentrations in eukaryotic cells (up to 5 mM), the antibacterial properties of GSH could potentially contribute to improved killing of intracellular bacteria by macrophages. The sensitivity of *B. pseudomallei* to GSH has not yet been investigated. If GSH plays a direct role in regulating antimicrobial activity in macrophages, GSH deficiency in T2D may contribute to the reduced control of *B. pseudomallei* infection.

Furthermore, indirect effects of GSH, dependent on the GSH/GSSG ratio of macrophages, have been described. The intracellular GSH/GSSG ratio of macrophages has been shown to dictate cytokine responses to infection, subsequently impacting on the development of either T_H1 or T_H2 cytokine profiles (Kato, Mikami & Natsuno 2008; Murata *et al.* 2002a; Murata, Shimamura & Hamuro 2002; Peterson *et al.* 1998). Reductive macrophages, which have a high concentration of GSH, are implicated in eliciting T_H1 type responses, involving production of IL-12 and IFN-γ, while oxidative macrophages, with comparatively low GSH levels, inhibit these responses (Murata *et al.* 2002a; Parmely, Wang & Wright 2001). It has been proposed that this mechanism is responsible for the increased killing efficiency of macrophages with a higher GSH/GSSG ratio against intracellular pathogens such as *Leishmania* and *M. tuberculosis* (Buchmuller-Rouiller *et al.* 1995; Venketaraman *et al.* 2003). Given similarities in clinical features and pathogenesis, it is plausible that an increased GSH/GSSG ratio may be protective for *B. pseudomallei* infection. The pathogenesis of T2D is intricately linked to chronic inflammation which may lead to depletion of intracellular GSH levels, as has been documented in the T2D population. Alterations in the intracellular GSH/GSSG ratio in this murine model of T2D need to be confirmed. We hypothesise that lower GSH levels in diabetic mice may contribute to impaired killing of *B. pseudomallei* by macrophages. Based on the possible direct and indirect effects of GSH on progression of *B. pseudomallei* infection, modulation of GSH may influence disease outcome.

A role for GSH in influencing the progression of *B. pseudomallei* infection has recently been described (Tan *et al.* 2012). Tan *et al.* (2012) demonstrated that depletion of GSH in BALB/c mice results in rapid mortality by 72 hours post-infection with *B. pseudomallei,* compared to untreated mice. Decreased levels of GSH in PBMC from diabetic individuals coincided with impaired IL-12 and IFN-γ secretion and bacterial killing in response to *B. pseudomallei* (Tan *et al.* 2012). Therefore, depleted GSH levels have been postulated as a mechanism linking T2D and increased susceptibility to melioidosis (Tan *et al.* 2012). However, effects of GSH modulation have not previously been documented in a model of T2D and comorbid melioidosis. To confirm the putative role of GSH in the link between T2D and melioidosis, we investigated the impact of *in vivo* treatments with *L*-buthionine-

sulfoximine (BSO) and *N*-acetyl-*L*-cysteine (NAC), to deplete and replenish GSH, respectively, in our murine model of T2D and *B. pseudomallei* infection. BSO has been widely used as a chemotherapeutic agent to inhibit *de novo* GSH synthesis by preventing γ-GCL activity, the rate-limiting step in GSH synthesis (Engel & Evens 2006; Estrela, Ortega & Obrador 2006; Sobhakumari *et al.* 2012). We used BSO treatment to deplete GSH in non-diabetic mice to determine if this would increase susceptibility to *B. pseudomallei* infection.

The widely used GSH modulator, NAC, was used to determine if supplementing GSH levels in diabetic mice influences the progression of *B. pseudomallei* infection. NAC is a thiol that is rapidly deacetylated to augment intracellular cysteine levels, the rate-limiting substrate in GSH synthesis, thereby elevating GSH levels (De Flora *et al.* 1995; Meister 1983; Meister & Anderson 1983). Supplementation with GSH directly is not possible due to limited absorption in the gastrointestinal tract and poor transportation across cell membranes (De Flora *et al.* 2001; Witschi *et al.* 1992). NAC has been used clinically since the mid 1950s as a mucolytic agent and has been prescribed in the management of acetaminophen (paracetamol) overdose since the 1970s (Prescott & Critchley 1983; Sheffner 1963). Restoration of GSH by NAC is widely described in the literature and NAC treatment is currently the subject of intensive investigations as a potential therapeutic approach for management of a variety of diseases involving GSH deficiency, including neurological conditions, alcoholic hepatitis, COPD and influenza (Arakawa & Ito 2007; Geiler *et al.* 2010; Wang *et al.* 2011; Zafarullah *et al.* 2003). A deficiency in GSH in diabetic mice may contribute to the increased growth of *B. pseudomallei* and overall more severe outcome of melioidosis compared to non-diabetic littermates, as described in Chapter 8. Therefore, replenishing GSH by NAC treatment may improve survival of diabetic mice. The clinical application of NAC as a modulator of GSH and a potential adjuvant therapeutic agent is enhanced by its wide availability and relative affordability.

Efficient antimicrobial action of macrophages is critical for effective control of intracellular bacteria such as *B. pseudomallei* and may be directly or indirectly influenced by altered GSH/GSSG levels. We hypothesise that an imbalance in the intracellular redox status of macrophages in diabetic mice contributes to increased

intracellular persistence of *B. pseudomallei*. The purpose of the studies described in this chapter was to investigate the direct role of GSH in killing *B. pseudomallei* and indirect role of GSH/GSSG in modulating macrophage responses to *B. pseudomallei* and determining disease outcome. Therefore, in this chapter, the research aims were to:

i) determine the direct toxicity of GSH to *B. pseudomallei in vitro*
ii) compare *in vitro* killing of *B. pseudomallei* by macrophages from diabetic and non-diabetic mice and corresponding GSH/GSSG levels
iii) compare GSH/GSSG levels in diabetic and non-diabetic mice
iv) compare survival of diabetic and non-diabetic mice following modulation of GSH/GSSG levels

9.2 Materials and Methods

9.2.1 Direct effect of glutathione on *B. pseudomallei* growth *in vitro*

Twelve clinical isolates of *B. pseudomallei* were obtained from frozen stock cultures of the Immunopathogenesis and Infectious Diseases Research Group *B. pseudomallei* Isolate Collection (James Cook University, Australia; Table 9.1). *B. pseudomallei* isolates were cultured in TSB overnight with shaking (100rpm) at 37°C. After dilution in fresh TSB to optical density at 600nm (OD_{600nm}) of 0.150, GSH was added to a final concentration of 5mM, 10mM and 20mM, while an equal volume of distilled H_2O was added to the control (0mM GSH) group. Aliquots of 200µl were dispensed into 96 well plates in replicates of 6 and OD_{600nm} was measured hourly, for 12 hours. *In vitro* growth rates of *B. pseudomallei* isolates were calculated by determining the gradient of the line of best fit for each growth curve during the logarithmic phase (between 2 and 6 hours). Data is presented as the gradient (OD_{600nm} / hour) ± SEM.

It was subsequently determined that addition of GSH significantly acidified the culture medium (pH 5.0). To determine if the inhibitory effect of GSH was secondary to the decrease in pH, NaOH in combination with GSH was added to the culture medium to achieve a neutral pH equivalent to TSB alone (pH 7.2). An acid

control was also included with addition of HCl (pH 5.0). *In vitro* growth rates were determined following the protocols described above. In addition, *B. pseudomallei* concentration was determined at 6 hours of culture by enumeration of CFU on Ashdown agar (Chapter 3).

Table 9.1 Origin and virulence of *B. pseudomallei* isolates

Isolate Number	Risk Factor	Clinical Presentation	ID_{50} in BALB/c mice (CFU)
CL2	Diabetes	sepsis, pneumonia	$1.26x10^{6}$
CL3	None	sepsis	$7.49x10^{5}$
CL7	Diabetes	leg ulcer	$1.52x10^{4}$
CL9	Diabetes*	sepsis, pneumonia, UTI	$1.26x10^{4}$
CL14	Diabetes	sepsis, pneumonia, leg ulcer	$2.32x10^{3}$
CL16	None	sepsis, pneumonia	$2.00x10^{3}$
CL19	None	pneumonia, lung lesion	$3.76x10^{2}$
CL23	Diabetes*	sepsis	$1.50x10^{1}$
CL24	None	brain stem encephalitis	$1.00x10^{1}$
CL26	None	pneumonia, mediastinal mass	$3.00x10^{0}$
NCTC13179	Diabetes	leg ulcer	$1.52x10^{4}$
NCTC13178	None	sepsis	$1.00x10^{1}$

Adapted from Ulett *et al.* (2001). * additional risk factors present; CFU – colony forming units.

9.2.2 *In vitro* macrophage co-culture with *B. pseudomallei*

Diabetic and non-diabetic mice (n = 5 per group) were injected with 3ml of 3% thioglycollate (Sigma-Aldrich, Australia) to elicit peritoneal macrophages. After 72 hours, peritoneal macrophages were harvested from the abdominal cavity in 10ml of lavage medium (Appendix 1.2.2). Cell suspensions were pooled and cultured overnight in 24 well plates with single-strength culture medium (Appendix 1.2.1) at 37°C in 5% CO_2 (5×10^{5} cells/ml). After removal of non-adherent cells by washing 3 times with PBS, peritoneal macrophages were infected in triplicate with

1×10^6 CFU/ml of *B. pseudomallei* (multiplicity of infection [MOI] of 1:2) or PBS only (uninfected) for 4 hours prior to collection of cells for measurement of GSH concentrations as detailed above. Culture supernatants were collected and stored at -80°C until analysis of cytokine protein levels (MCP-1, TNF-α, IL-12, IFN-γ, IL-6) by CBA (Mouse Inflammation Kit, BD Biosciences, Australia). Intracellular survival of *B. pseudomallei* was compared after 4 hours of co-culture. Cells were first incubated with Kanamycin (250μg/ml) to kill extracellular bacteria. After washing 3 times with PBS, cells were lysed in 0.1% triton-X (Sigma-Aldrich, Australia) for 5 minutes and lysates were serially diluted for enumeration of CFU on Ashdown agar (Chapter 3). Viability of macrophages prior to lysis was determined in duplicate by staining with trypan blue exclusion dye and counting on a haemocytometer. Experiments were conducted in triplicate with results expressed as mean ± SEM.

9.2.3 Tissue collection for measurements of glutathione concentrations

Following 15 weeks of diet intervention, mice (n = 10 per group) were euthanased for collection of liver, spleen, blood and SAT. These sites were selected due to the typically high levels of GSH expected in hepatic tissue, the spleen and blood where increased *B. pseudomallei* growth was observed and SAT at the site of infection. Briefly, cardiac blood was collected in heparin (Appendix 1.2.2) and immediately added to an equal volume of ice-cold 5% 5-sulfosalicylic acid (Sigma-Aldrich, Australia) to precipitate proteins. Other organs were macerated at a concentration of 40mg/ml in ice-cold 5% SSA. Blood and organ homogenates were processed according to the DetectX® Glutathione Fluorescent Detection Kit manufacturer's instructions (Arbor Assays, USA) and protein free supernatants were collected and stored at -80°C until assayed. The remaining protein precipitate was resuspended in 0.1M NaOH and protein determination performed using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Australia) as per the manufacturer's instructions. Tissue concentrations of GSH and GSSG were normalised to total protein levels in samples.

In parallel, aliquots of whole blood and spleen homogenate were processed further to enable measurement of intracellular GSH and GSSG concentrations. Cells were

washed in cold PBS and pelleted by centrifugation at 450*g* for 5 minutes at 4°C. Cells were resuspended in Erythrocyte Lysis Buffer (Qiagen, Australia), washed twice and resuspended in PBS. A 100µl aliquot of cell suspension was separated to determine cell counts in duplicate by flow cytometry (FacsCalibur, BD Biosciences, Australia) using CellQuest Pro Software (BD Biosciences, Australia) and CountBright™ Absolute Counting Beads (Life Technologies, Australia). Cell counts (cells/µl) were calculated using the following equation:

$$\frac{\text{number of cell events}}{\text{number of bead events}} \times \frac{\text{bead count (beads/25µl)}}{\text{volume of sample}}$$

by collecting 2000 bead events and gating on the leukocyte population according to forward and side scatter parameters and excluding cell debris (Figure 9.2). Remaining cell suspensions were incubated with an equal volume of ice-cold 10% SSA and protein free supernatants were collected and stored at -80°C until assayed. Intracellular concentrations of GSH and GSSG were normalised to cell number in samples.

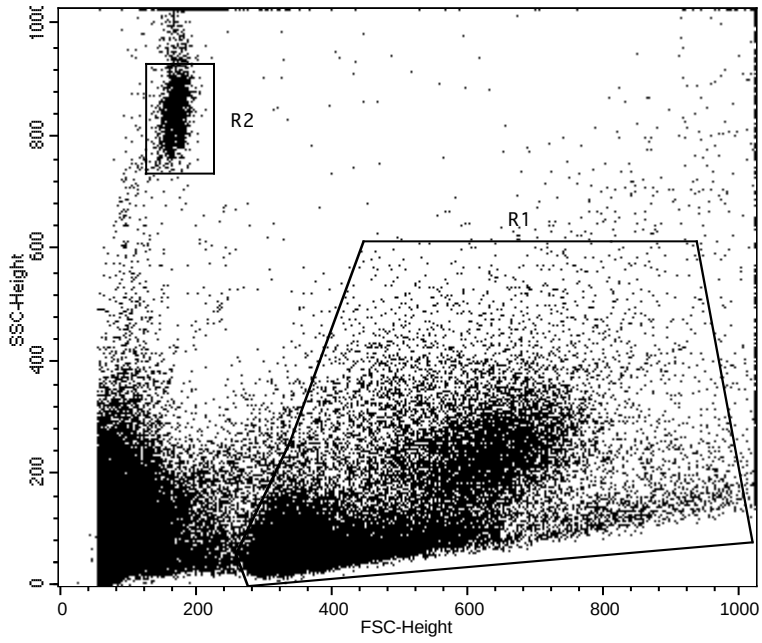

Figure 9.2 Flow cytometry gating parameters for cell counts
Cell counts were calculated by collecting 2000 bead events (R2) and gating on the leukocyte population (R1) according to forward and side scatter parameters. Image is representative of gating parameters used for all samples.

9.2.4 Measurement of glutathione concentrations

Glutathione concentration was determined using the DetectX® Glutathione Fluorescent Detection Kit (Arbor Assays, USA). Optimal dilutions for measurement of GSH were determined to be 100-fold for blood, spleen and liver samples and 20-fold for SAT and intracellular samples. To measure GSSG, an aliquot of undiluted sample was incubated with 2-vinylpyridine (2-VP; Appendix 1.3.3) at an optimal dilution of 10:1 (v/v) at room temperature for 1 hour. The assay was subsequently conducted according to manufacturer's instructions. Fluorescence was detected at an emission of 520nm with excitation at 390/10nm (FLUOstar Omega, BMG Labtech, Australia). All samples were run in duplicate and each assay included a GSSG positive control to confirm complete reduction of GSSG. After correction for background fluorescence, GSH concentrations were calculated by extrapolation from the standard curve. The limit of detection of the assay was 0.20 to 12.5µM GSH.

9.2.5 Indirect effect of glutathione modulation on survival of diabetic and non-diabetic mice following *B. pseudomallei* infection

Non-diabetic mice were treated with BSO (Appendix 1.3.1) to deplete cellular GSH levels, while diabetic mice received NAC treatments (Appendix 1.3.2) to restore cellular GSH levels. Preliminary experiments were performed to optimise the delivery of BSO and NAC and confirm modulation of tissue and intracellular GSH levels. Non-diabetic mice were treated with BSO (450mg/kg) or PBS (untreated) via intraperitoneal injections in a volume of 200µl according to the schedule illustrated in Figure 9.3. Mice were subcutaneously infected with *B. pseudomallei* (1.2×10^6 CFU) and survival was monitored for 10 days post-infection. At the end of the experimental period, surviving mice were euthanased and necropsied to confirm the presence of infection. Modulation of GSH levels in whole blood and leukocytes by BSO treatment was confirmed at 2 and 12 hours in uninfected mice (n = 5 per group). Untreated control mice received sham treatments with vehicle only.

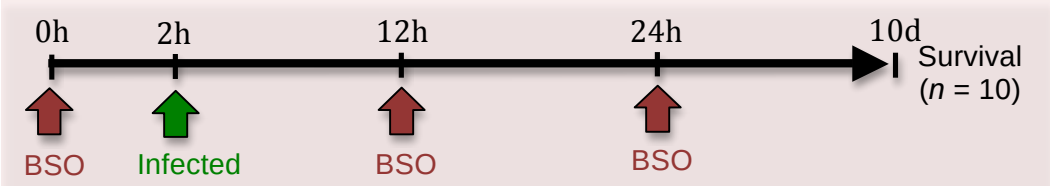

Figure 9.3 *L*-buthionine-sulfoximine treatment schedule
Non-diabetic mice were treated with *L*-buthionine-sulfoximine (BSO; 450mg/kg) at 0, 12 and 24 hours. Mice were infected with *B. pseudomallei* at 2 hours and survival was monitored for 10 days. d – days; h – hours.

Diabetic mice were treated with NAC (250mg/kg; Appendix 1.3.2) via intraperitoneal injection in a volume of 100µl every 12 hours for 5 days prior to infection and for the remaining duration of the experimental period (Figure 9.4a). Treated diabetic mice and untreated non-diabetic mice were subcutaneously infected with *B. pseudomallei* (1.2×10^6 CFU) and survival of mice was monitored for 10 days post-infection. Mice surviving to the end of the experimental period were euthanased and necropsied to confirm the presence of infection. An additional group of diabetic mice received an accelerated dosing schedule of NAC (250mg/kg) at 6 hourly intervals for 5 days prior to subcutaneous infection with *B. pseudomallei* (5.0×10^5 CFU; Figure 9.4b). Mice were euthanased at 12 and 24 hours post-infection to determine *B. pseudomallei* loads in spleen, liver, LN, SAT and blood by enumeration of CFU on Ashdown agar (Chapter 3). Levels of GSH in blood and spleen of uninfected mice was determined at 1, 6 and 12 hours following NAC treatment. Untreated control mice received sham treatments with vehicle only.

9.2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism Software Version 6.0b. Unpaired Student's T-tests were used for comparisons between diabetic and non-diabetic mice, with or without Welch's correction where appropriate. Comparisons between GSH and GSSG concentrations in macrophages of diabetic and non-diabetic mice before and after stimulation with *B. pseudomallei* were performed by two-way ANOVA with Bonferonni post-hoc tests. Two-way ANOVA was also performed for comparisons of *B. pseudomallei* organ loads between treatment groups

at 12 and 24 hours post-infection. Comparisons between *in vitro* bacterial growth rates and *in vivo* treatment groups were calculated by one-way ANOVA with Bonferonni post-hoc tests. Mortality rates were compared by Kaplan-Meier survival analysis. Statistical significance was determined at $P \leq 0.05$. All data is expressed as mean ± SEM except for *B. pseudomallei* loads, which are presented as mean Log_{10} CFU ± SEM.

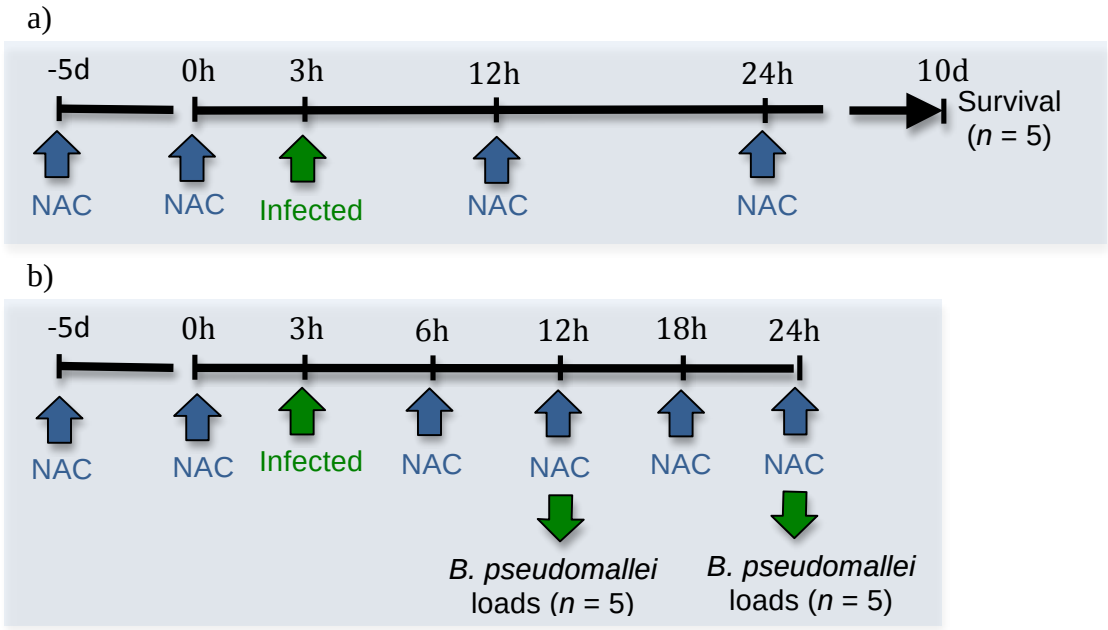

Figure 9.4 *N*-acetyl-*L*-cysteine treatment schedules
a) Diabetic mice were treated with *N*-acetyl-*L*-cysteine (NAC; 250mg/kg) every 12 hours for 5 days prior to infection and for the duration of the experiment. Mice were infected with *B. pseudomallei* 3 hours after NAC treatment and survival was monitored for 10 days. b) A parallel group of diabetic mice were treated with NAC (250mg/kg) every 6 hours beginning 5 days prior to infection with *B. pseudomallei*. Bacterial loads in the spleen were compared at 12 and 24 hours post-infection. d – days; h – hours.

9.3 Results

9.3.1 Direct effect of glutathione on *B. pseudomallei* growth *in vitro*

In vitro growth rates of *B. pseudomallei* isolates were calculated as the gradient of change in OD per hour during the logarithmic phase of bacterial growth (between 2 and 6 hours). Growth rates of all *B. pseudomallei* isolates tended to be inhibited by

20mM GSH as shown in Table 9.2. The inhibition of growth rate by increasing concentrations of GSH was calculated as a percentage relative to baseline growth of each *B. pseudomallei* isolate in TSB (Figure 9.5). There was a strong positive correlation between baseline growth rate and growth inhibition by 20mM GSH ($P < 0.05$; $r^2 = 0.51$), with the greatest inhibition in isolates with the highest growth rates as would typically be expected.

Table 9.2 Growth inhibition of *B. pseudomallei* isolates by glutathione

B. pseudomallei Isolate	Growth rate ($OD_{600nm}.hr^{-1}$)			
	0mM GSH	5mM GSH	10mM GSH	20mM GSH
CL2	0.0536 ± 0.0019	0.0627 * ± 0.0011	0.0578 ± 0.0013	0.0337 * ± 0.0025
CL3	0.0459 ± 0.0027	0.0524 ± 0.0030	0.0432 ± 0.0025	0.0348 * ± 0.0022
CL7	0.0372 ± 0.0031	0.0413 ± 0.0026	0.0428 ± 0.0045	0.0310 ± 0.0005
CL9	0.0308 ± 0.0019	0.0344 ± 0.0020	0.0363 ± 0.0012	0.0283 ± 0.0010
CL14	0.0260 ± 0.0012	0.0255 ± 0.0013	0.0275 ± 0.0017	0.0217 ± 0.0009
CL16	0.0300 ± 0.0015	0.0332 ± 0.0018	0.0304 ± 0.0026	0.0226 ± 0.0013
CL19	0.0108 ± 0.0010	0.0092 ± 0.0009	0.0094 ± 0.0016	0.0074 ± 0.0008
CL23	0.0505 ± 0.0011	0.0536 ± 0.0011	0.0504 ± 0.0015	0.0296 * ± 0.0015
CL24	0.0696 ± 0.0007	0.0685 ± 0.0022	0.0699 ± 0.0018	0.0294 * ± 0.0010
CL26	0.0280 ± 0.0017	0.0316 ± 0.0014	0.0317 ± 0.0029	0.0258 ± 0.0016
NCTC13179	0.0660 ± 0.0015	0.0641 ± 0.0012	0.0624 ± 0.0016	0.0305 * ± 0.0045
NCTC13178	0.0570 ± 0.0018	0.0591 ± 0.0023	0.0476 ± 0.0023	0.0424 * ± 0.0028

* $P < 0.05$ vs baseline (0mM GSH); mean ± SEM.

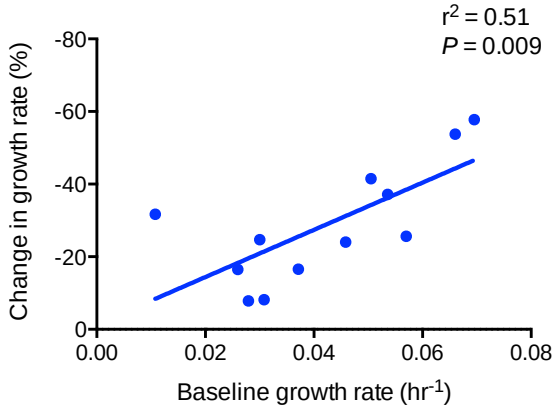

Figure 9.5 Correlation between growth inhibition of glutathione and baseline growth rate
There was a positive correlation between baseline growth rate and growth inhibition from 20mM GSH, having a greater effect on isolates with higher growth rates.

Whilst inhibition of *B. pseudomallei* growth was observed following the addition of 20mM GSH, it was subsequently noted that at this concentration of GSH the pH of the culture medium was significantly lowered. Therefore, it was difficult to determine the toxicity effect of GSH as opposed to acidic conditions *per se*. To address this, the growth rate of two well-characterised strains, NCTC13179 and NCTC13178, was compared in TSB (pH 7.2), acidified TSB (pH 5.0), TSB with 20mM GSH (pH 5.0) and TSB with 20mM GSH neutralised with NaOH (pH 7.2). Inhibition of growth rate of NCTC13178 (Figure 9.6a) and NCTC13179 (Figure 9.6b) was comparable between 20mM GSH (pH 5.0) and HCl (pH 5.0) and was significantly improved by neutralisation of GSH culture medium ($P < 0.05$). Although there was still a small but significant reduction in growth rate between 20mM GSH after neutralisation compared to baseline growth rate, the relevance of this *in vivo* is questionable given that overall *B. pseudomallei* numbers as determined by culture were comparable after 6 hours (Figure 9.7) and 20mM is well above the physiological levels of intracellular GSH.

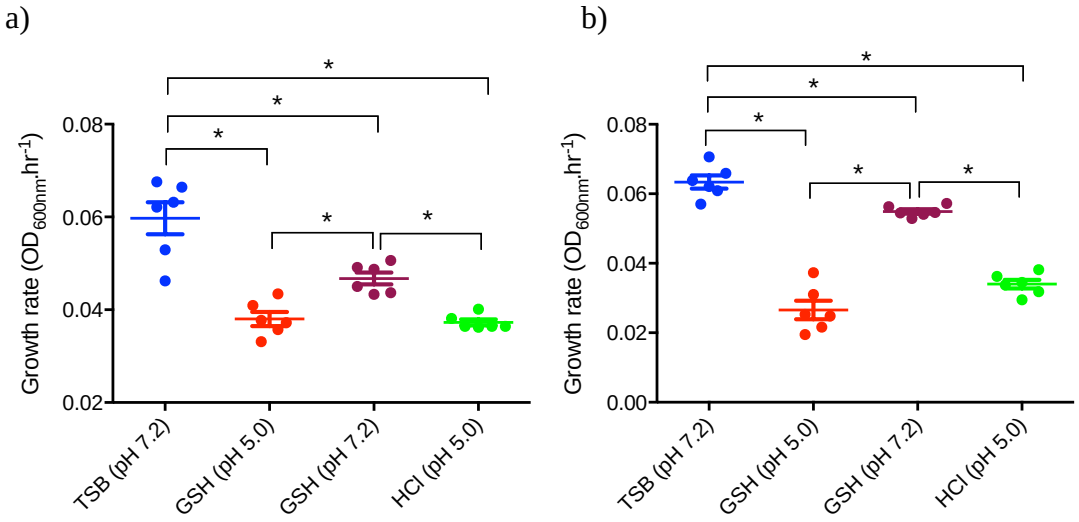

Figure 9.6 Growth rate of *B. pseudomallei in vitro* after adjustment of pH
Growth rate of *B. pseudomallei* isolates was compared in TSB (pH 7.2), acidified TSB (pH 5.0), 20mM GSH (5.0) and 20mM GSH (pH 7.2) neutralised with NaOH. a) Inhibition of growth rate of NCTC13178 was comparable between 20mM GSH (pH 5.0) and HCl (pH 5.0) and was significantly improved by neutralisation of GSH culture medium (pH 7.2). b) Similarly, inhibition of NCTC13179 was significantly improved by neutralisation of GSH culture medium (pH 7.2). * $P < 0.05$, mean ± SEM.

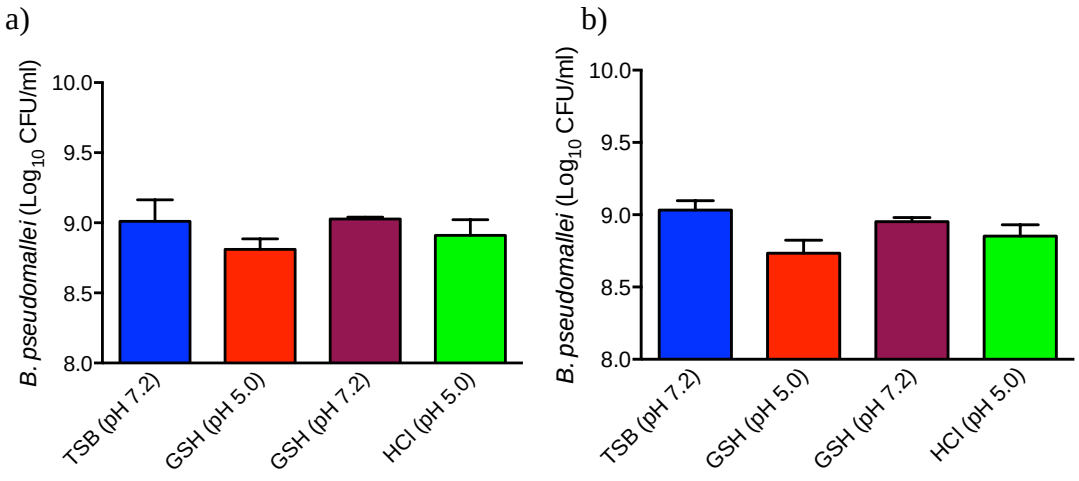

Figure 9.7 Bacterial counts of *B. pseudomallei* after *in vitro* culture with glutathione
Growth of *B. pseudomallei* isolates on Ashdown agar was compared in TSB (pH 7.2), acidified TSB (pH 5.0), 20mM GSH (pH 5.0) and 20mM GSH neutralised with NaOH (pH 7.2). There was no difference in bacterial loads of a) NCTC13178 or b) NCTC13179 after 6 hours of *in vitro* growth. mean Log_{10} CFU ± SEM.

9.3.2 Intracellular glutathione levels in macrophages from diabetic and non-diabetic mice and corresponding killing of *B. pseudomallei*

Intracellular GSH and GSSG levels were compared in macrophages from diabetic and non-diabetic mice. Prior to infection with *B. pseudomallei*, GSSG levels were significantly higher in macrophages from diabetic mice compared to macrophages from non-diabetic mice ($P < 0.05$; Figure 9.8b). This coincided with significantly higher baseline secretion of MCP-1 (Figure 9.8c) and TNF-α (Figure 9.8d) compared to macrophages from non-diabetic mice ($P < 0.05$). Co-culture with *B. pseudomallei* for 4 hours resulted in a significant elevation in intracellular GSH (Figure 9.8a) and GSSG levels (Figure 9.8b) in macrophages from non-diabetic mice. In contrast, levels of GSH and GSSG in macrophages from diabetic mice were not altered by co-culture with *B. pseudomallei*. Coinciding with this, production of MCP-1 (Figure 8.9c) was lower in macrophages from diabetic mice, although production of TNF-α was still significantly higher compared to macrophages from non-diabetic mice (Figure 8.9d). Intracellular survival of *B. pseudomallei* after 4 hours of co-culture was significantly increased in macrophages from diabetic mice ($P < 0.05$; Figure 9.8e). This also corresponded with reduced macrophage viability at 4 hours compared to macrophages from non-diabetic mice ($P < 0.05$; Figure 9.8f).

9.3.3 Baseline glutathione levels in diabetic and non-diabetic mice

Levels of GSH and GSSG were compared in blood, spleen, liver and SAT of diabetic and non-diabetic mice. In all organs examined, GSH levels were higher in non-diabetic mice compared to diabetic mice ($P < 0.05$; Figure 9.9). Circulating GSSG levels were higher in diabetic mice compared to non-diabetic mice ($P < 0.05$; Figure 9.10a). Due to reduced levels of GSH in diabetic mice, the overall GSH/GSSG ratio was significantly lower in whole blood ($P < 0.05$; Figure 9.11a) and SAT ($P < 0.05$; Figure 9.11d) and followed a similar trend in the spleen and liver (Figure 9.11b and c) compared to non-diabetic mice, although this was not statistically significant. Of note, there was a significant positive correlation observed between blood glucose levels and GSSG concentrations in whole blood ($P < 0.05$; $r^2 = 0.37$; Figure 9.12a) and leukocytes ($P < 0.05$; $r^2 = 0.42$; Figure 9.12b).

9.3.4 Modulation of glutathione levels and effect on disease progression after *B. pseudomallei* infection

Preliminary experiments indicated that a single intraperitoneal dose of BSO (450mg/kg) was sufficient to deplete GSH within 2 hours. Therefore, non-diabetic mice (n = 10 per group) were treated with BSO or vehicle (untreated), according to the treatment schedule (Figure 9.3). BSO treatment began 2 hours prior to infection with *B. pseudomallei* and mice were treated again at 12 and 24 hours post-infection. There was no significant difference in susceptibility between BSO treated and untreated groups, with a median survival of 12.5 to 13 days post-infection and a mean mortality rate of 90% by day 14 post-infection (P = 0.956; Figure 9.13).

Levels of GSH and GSSG in whole blood and within leukocytes were measured to confirm that GSH remained depleted between 12-hour doses of BSO. GSH was depleted by 60% in whole blood, 2 hours after BSO treatment ($P < 0.05$) and remained 50% lower at 12 hours post-infection compared to blood levels in untreated animals ($P < 0.05$; Figure 9.14a). Intracellular GSH concentrations in leukocytes followed a similar trend. Although the decrease at 2 hours post-treatment did not reach statistical significance (P = 0.147), by 12 hours, GSH concentration in leukocytes was reduced by 80% compared to leukocytes from untreated animals ($P < 0.05$; Figure 9.14b). There were no significant differences in levels of GSSG observed following BSO treatment (Figure 9.14c and d). Due to the depletion of GSH, the overall intracellular GSH/GSSG ratio tended to be lower in whole blood at 2 hours post-treatment (P = 0.074; Figure 9.14e) and was significantly lower in leukocytes after 2 and 12 hours ($P < 0.05$; Figure 9.14f).

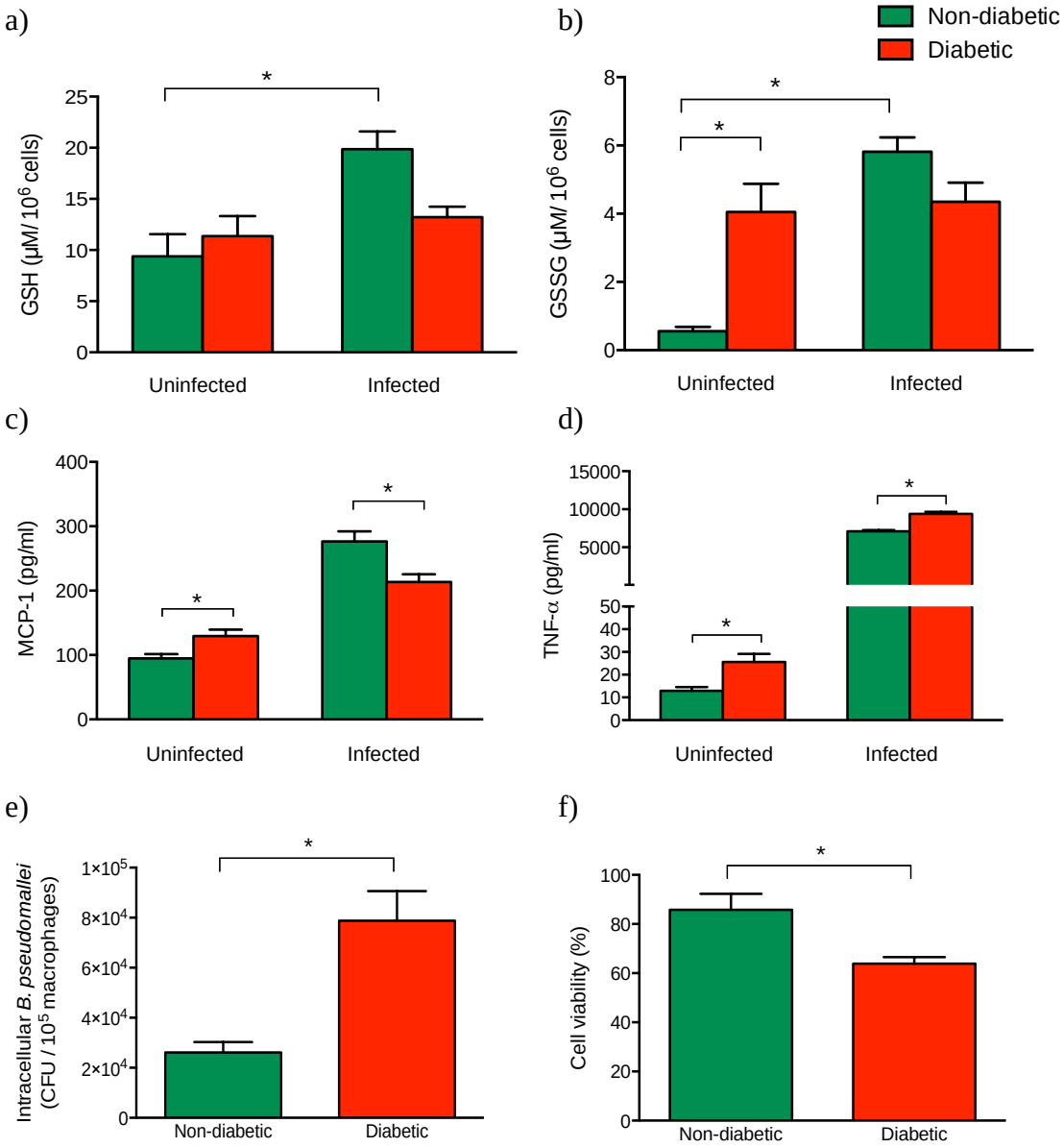

Figure 9.8 Comparison of glutathione levels, cytokine production, bacterial persistence and macrophage viability following co-culture with *B. pseudomallei*
Macrophages were cultured for 24 hours prior to infection with *B. pseudomallei* for 4 hours. a) Reduced (GSH) and b) oxidised (GSSG) glutathione levels significantly increased in macrophages from non-diabetic mice following infection with *B. pseudomallei*. Levels of b) GSSG and production of c) MCP-1 and d) TNF-α were significantly higher in macrophages from diabetic mice prior to infection ($P < 0.05$). After 4 hours of co-culture with *B. pseudomallei*, MCP-1 levels were higher in macrophages from non-diabetic mice ($P < 0.05$), while TNF-α levels were still higher in macrophages from diabetic mice ($P < 0.05$). e) Intracellular *B. pseudomallei* persistence was increased in macrophages from diabetic mice, which coincided with reduced f) macrophage viability compared to macrophages from non-diabetic mice. * $P < 0.05$; mean ± SEM.

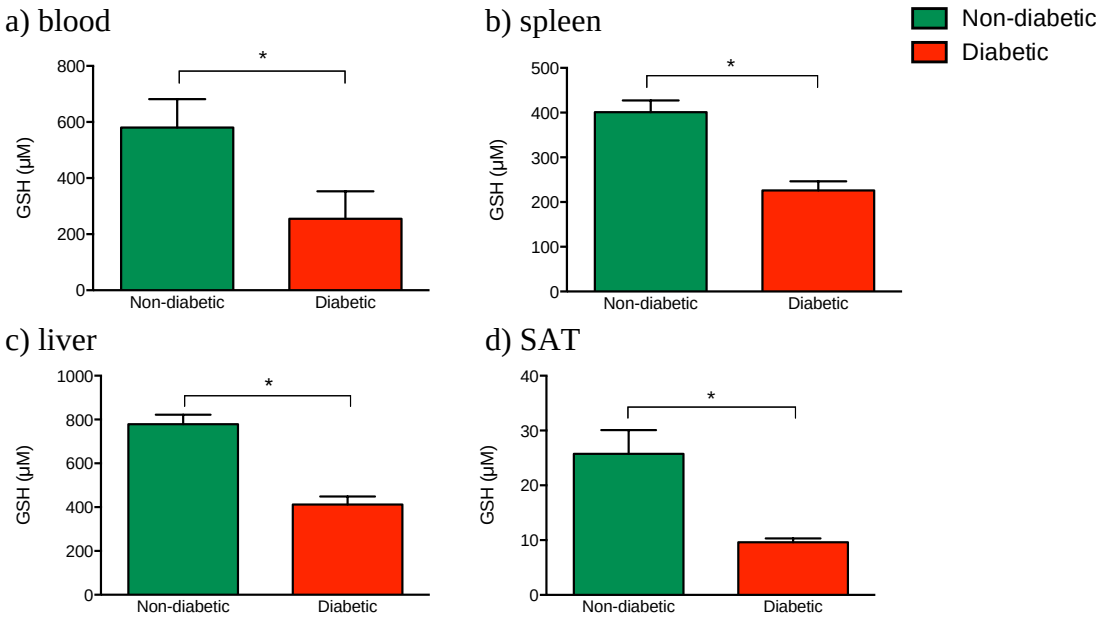

Figure 9.9 Baseline reduced glutathione levels in diabetic and non-diabetic mice
Levels of reduced glutathione (GSH) in a) whole blood were depleted in diabetic mice compared to non-diabetic littermates (n = 10 per group; $P < 0.05$). Significantly lower levels of GSH were also observed in b) spleen, c) liver and d) subcutaneous adipose tissue (SAT) of diabetic mice compared to non-diabetic mice (n = 5 per groups; $P < 0.05$). * $P < 0.05$; mean ± SEM.

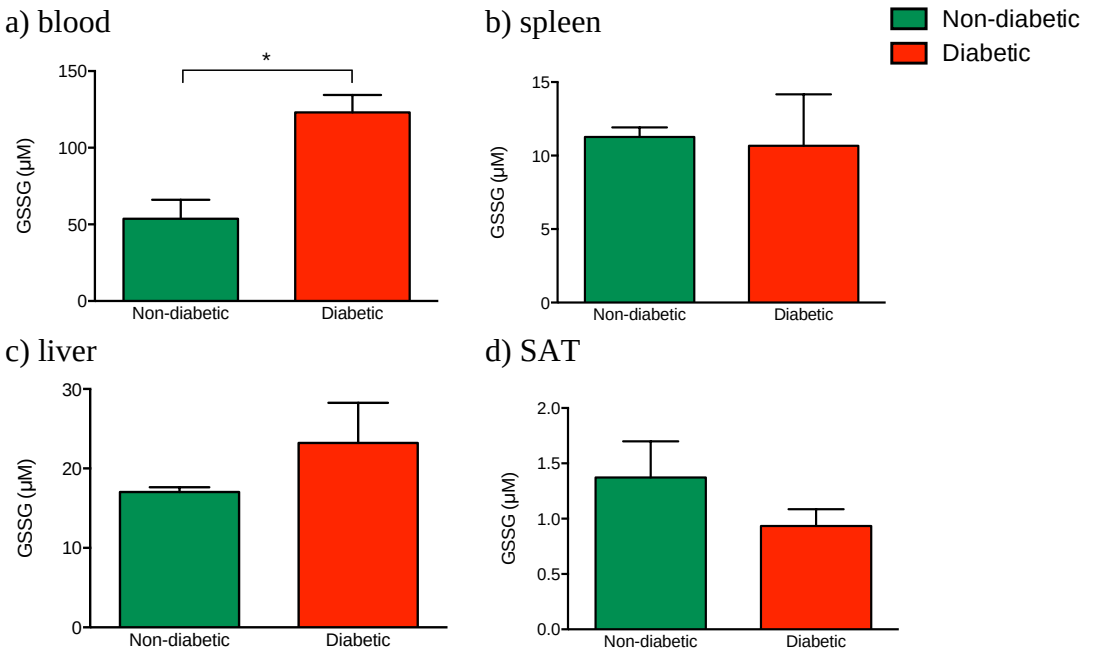

Figure 9.10 Baseline oxidised glutathione levels in diabetic and non-diabetic mice
Levels of oxidised glutathione (GSSG) were significantly higher in a) whole blood of diabetic mice compared to non-diabetic littermates (n = 10 per group; $P < 0.05$). GSSG levels were comparable in b) spleen, c) liver and d) subcutaneous adipose tissue (SAT) of diabetic and non-diabetic mice (n = 5 per group; $P < 0.05$). * $P < 0.05$; mean ± SEM.

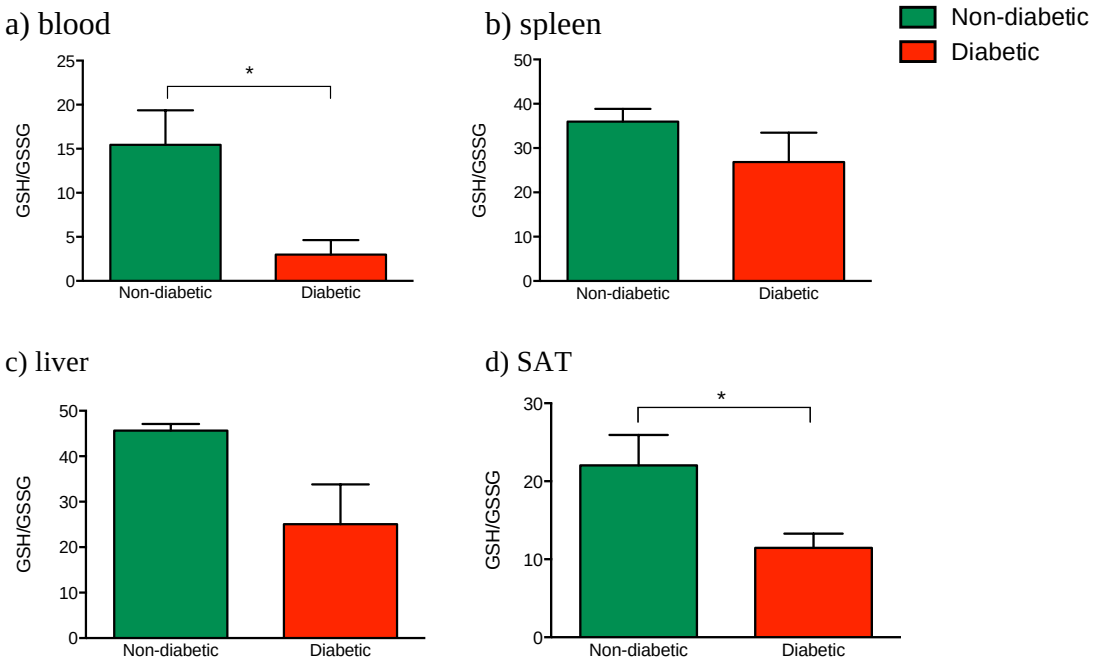

Figure 9.11 Baseline ratio of reduced and oxidised glutathione in diabetic and non-diabetic mice
The ratio of reduced to oxidised glutathione (GSH/GSSG) was significantly lower in a) whole blood of diabetic mice compared to non-diabetic mice (n = 10 per group, $P < 0.05$). A similar trend was observed in the b) spleen, c) liver and d) subcutaneous adipose tissue (SAT; n = 5 per group). * $P < 0.05$; mean ± SEM.

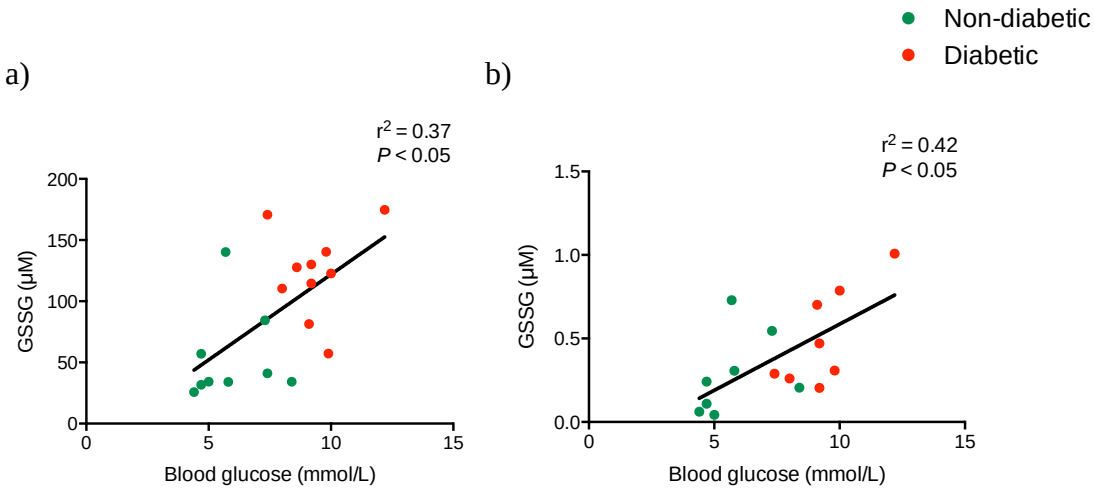

Figure 9.12 Positive correlation between oxidised glutathione and blood glucose
Blood glucose levels were positively correlated with oxidised glutathione (GSSG) levels in a) whole blood and b) leukocytes.

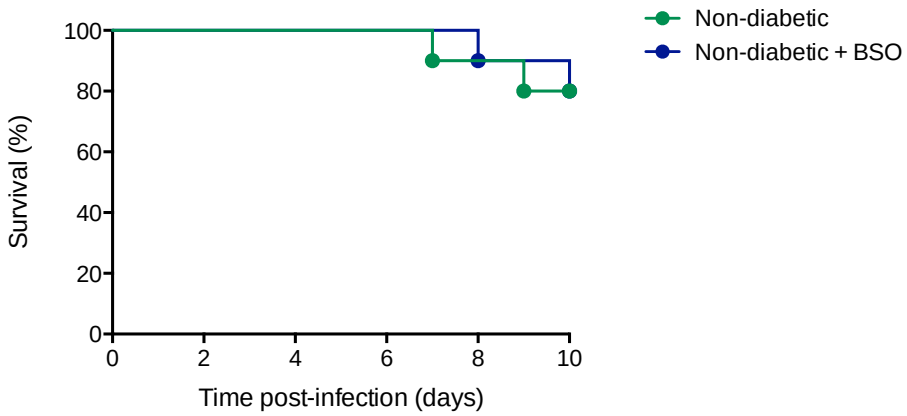

Figure 9.13 Susceptibility of non-diabetic mice to *B. pseudomallei* infection after treatment with *L*-buthionine-sulfoximine
Non-diabetic mice (n = 10 per group) were treated with *L*-buthionine-sulfoximine (BSO) or vehicle for 2 hours prior to subcutaneous infection with *B. pseudomallei* (1.5×10^6 CFU) and again at 12 and 24 hours post-infection. Mortality rate was comparable between groups with a median survival of 12.5 to 13 days.

Based on preliminary experiments, an intraperitoneal dose of NAC (250 mg/kg) was used to modulate GSH/GSSG levels in diabetic mice (n = 5 per group). Diabetic mice were treated 12-hourly for 5 days with NAC prior to subcutaneous infection with *B. pseudomallei*, according to the treatment schedule (Figure 9.4a). NAC treatments were continued every 12 hours following *B. pseudomallei* infection for the duration of the experimental period. Untreated, non-diabetic mice were also subcutaneously infected with *B. pseudomallei*. NAC treatment did not improve survival of diabetic mice, with animals succumbing to infection by day 4 after *B. pseudomallei* exposure with a median survival of 8 days (Figure 9.15). In comparison, the first fatality in non-diabetic mice occurred at 12 days post-infection with a median survival of 14 days for this group (Figure 9.15).

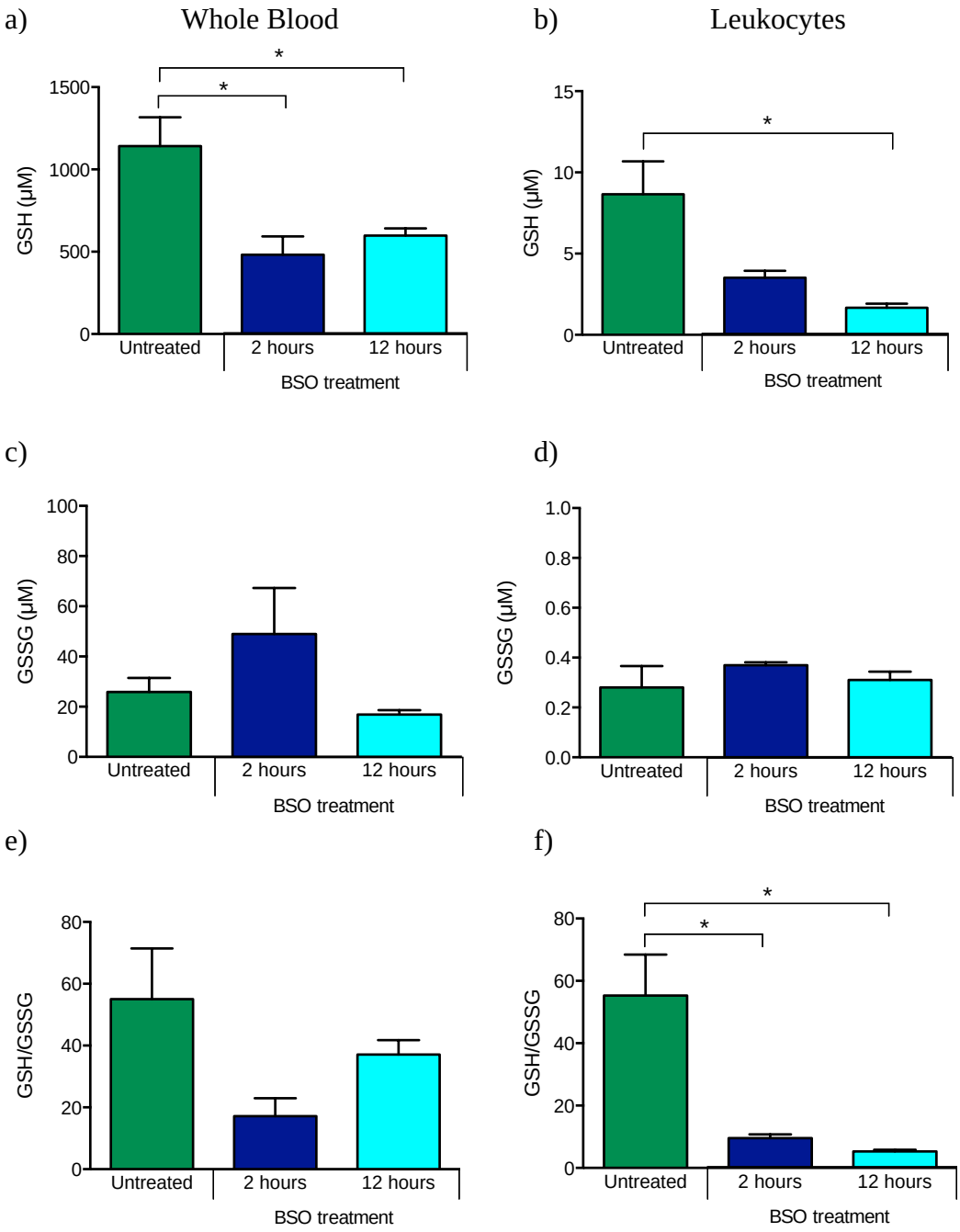

Figure 9.14 Kinetics of glutathione following treatment of non-diabetic mice with *L*-buthionine-sulfoximine

Non-diabetic mice received an intraperitoneal dose of *L*-buthionine-sulfoximine (BSO; 450 mg/kg) or vehicle only (untreated). Reduced glutathione (GSH) was measured in whole blood (a, c, e) and leukocytes (b, d, f). a) GSH was lower in blood of mice at 2 and 12 hours post-treatment ($P < 0.05$). b) Intracellular GSH levels in leukocytes tended to be lower at 2 hours post-treatment but did not reach significance until 12 hours post-treatment compared to untreated mice. No difference was observed in levels of oxidised glutathione (GSSG) in c) whole blood or d) leukocytes post-treatment. * $P < 0.05$; mean ± SEM.

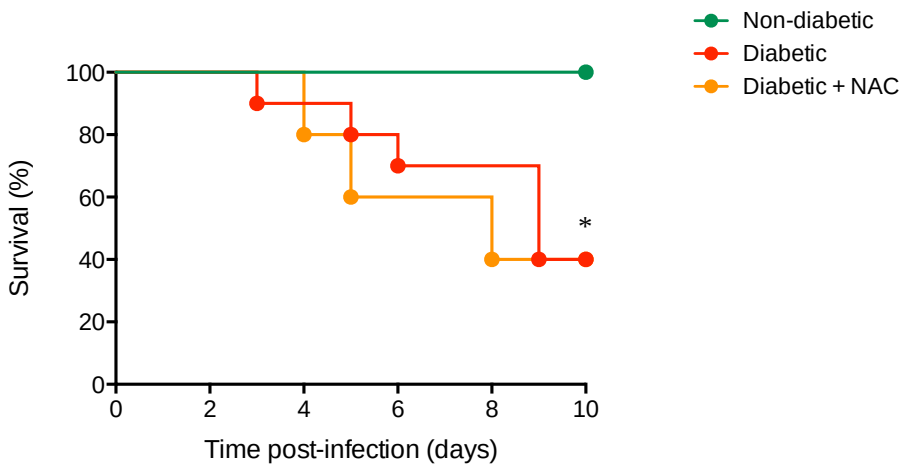

Figure 9.15 Susceptibility of diabetic mice to *B. pseudomallei* infection after treatment with *N*-acetyl-*L*-cysteine
Diabetic mice (n = 5 per group) were treated with an intraperitoneal dose of *N*-acetyl-*L*-cysteine (NAC; 250mg/kg) every 12 hours for the experimental period. After 5 days of treatment, diabetic mice were infected with *B. pseudomallei* in addition to untreated non-diabetic mice. NAC treatment did not improve survival of diabetic mice, which rapidly succumbed to infection compared to non-diabetic mice. * $P < 0.05$.

Given the similar disease progression observed for NAC-treated and untreated diabetic mice following *B. pseudomallei* infection, we hypothesised that the effect of NAC may have waned during the 12-hour interval between doses. Therefore, the NAC treatment schedule was increased from a 12-hourly to 6-hourly dosing regime and growth of *B. pseudomallei* in organs were determined within 24 hours of infection. There was no significant difference in *B. pseudomallei* loads in spleen between diabetic mice that were treated with NAC and untreated mice. Similar to previous experiments, growth of *B. pseudomallei* was significantly higher in the spleen of both groups of diabetic mice compared to non-diabetic mice ($P < 0.05$; Figure 9.16).

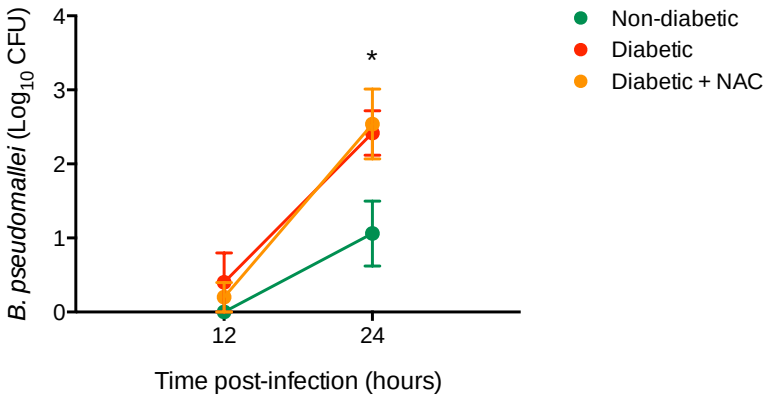

Figure 9.16 Growth of *B. pseudomallei* in spleen of diabetic mice following treatment with *N*-acetyl-*L*-cysteine
Diabetic mice were treated with *N*-acetyl-*L*-cysteine (NAC) every 6 hours for the experimental period. Three hours after the first NAC treatment, diabetic mice were infected with *B. pseudomallei* along with untreated non-diabetic and diabetic mice. *B. pseudomallei* growth was significantly greater in the spleen of both treated and untreated diabetic mice compared to non-diabetic littermates. * $P < 0.05$; mean Log_{10} CFU ± SEM.

No differences in overall mortality or bacterial loads in organs were observed following NAC treatment of diabetic mice using a dosing regime of 12 or 6 hours, respectively. To verify that GSH/GSSG levels remained elevated between 6 and 12-hourly dosing of NAC, an additional group of mice were treated and levels of GSH and GSSG determined at 1, 6 and 12 hours post-treatment. The levels of GSH in whole blood were only transiently elevated at 1 hour post-NAC treatment (Figure 9.17a). In contrast, GSSG levels in whole blood remained lower for at least 12 hours post-NAC treatment (Figure 9.17b). A similar trend for intracellular leukocyte levels of GSH (Figure 9.17c) and GSSG (Figure 9.17d) was observed.

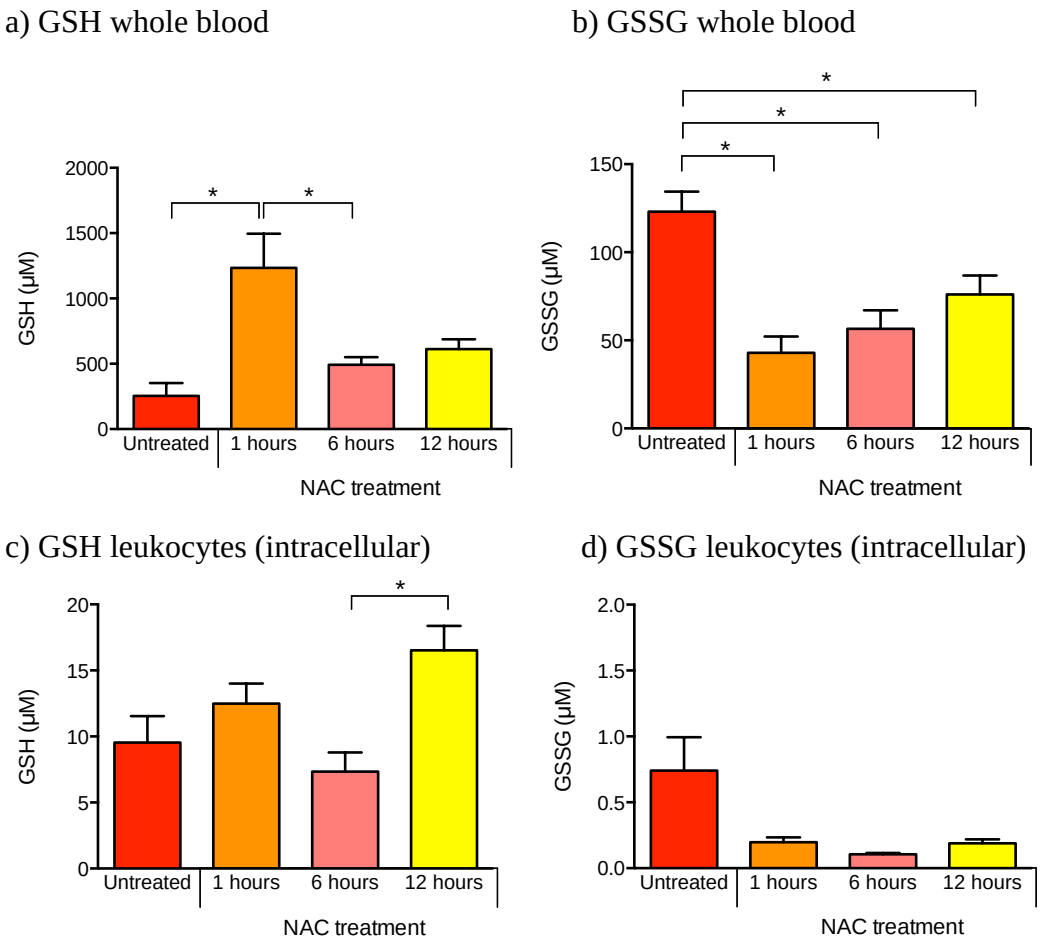

Figure 9.17 Levels of glutathione in whole blood and leukocytes of diabetic mice following treatment with *N*-acetyl-*L*-cysteine
Diabetic mice were treated with an intraperitoneal dose of *N*-acetyl-*L*-cysteine (NAC; 250mg/kg). a) Levels of GSH in whole blood of diabetic mice were significantly higher at 1 hour following NAC treatment but decreased by 6 hours. b) Levels of GSSG were significantly lower at all time points after NAC treatment compared to untreated diabetic mice. c) Intracellular levels of GSH in blood leukocytes tended to be higher by 12 hours after NAC treatment, intracellular levels of GSSG tended to be lower compared to untreated diabetic mice. * $P < 0.05$; mean ± SEM.

Modulation of tissue and intracellular GSH/GSSG levels by NAC was also measured in the spleen. The levels of GSH in spleen supernatants were not significantly altered by NAC treatment (Figure 9.18a), although GSSG levels tended to be lower (Figure 9.18b). Intracellular levels of GSH and GSSG in splenocytes followed a similar trend (Figure 9.18c and d). Overall, the GSH/GSSG ratio in whole blood of diabetic mice was significantly higher at 1 hour following NAC treatment compared

to untreated mice ($P < 0.05$). However, by 6 hours the GSH/GSSG ratio had declined to levels comparable to untreated diabetic mice (Figure 9.19a). In contrast, the intracellular GSH/GSSG ratio in leukocytes was higher after 1 hour ($P < 0.05$), and remained elevated after 6 ($P < 0.05$) and 12 hours ($P < 0.05$) post-NAC treatment (Figure 9.19b). Similar to whole blood, tissue levels of GSH/GSSG in the spleen were also transiently elevated at 1 hour after NAC treatment (Figure 9.19c). Intracellular GSH/GSSG levels in splenocytes tended to be higher at 1 and 6 hours following NAC treatment although it did not reach significance and had declined by 12 hours.

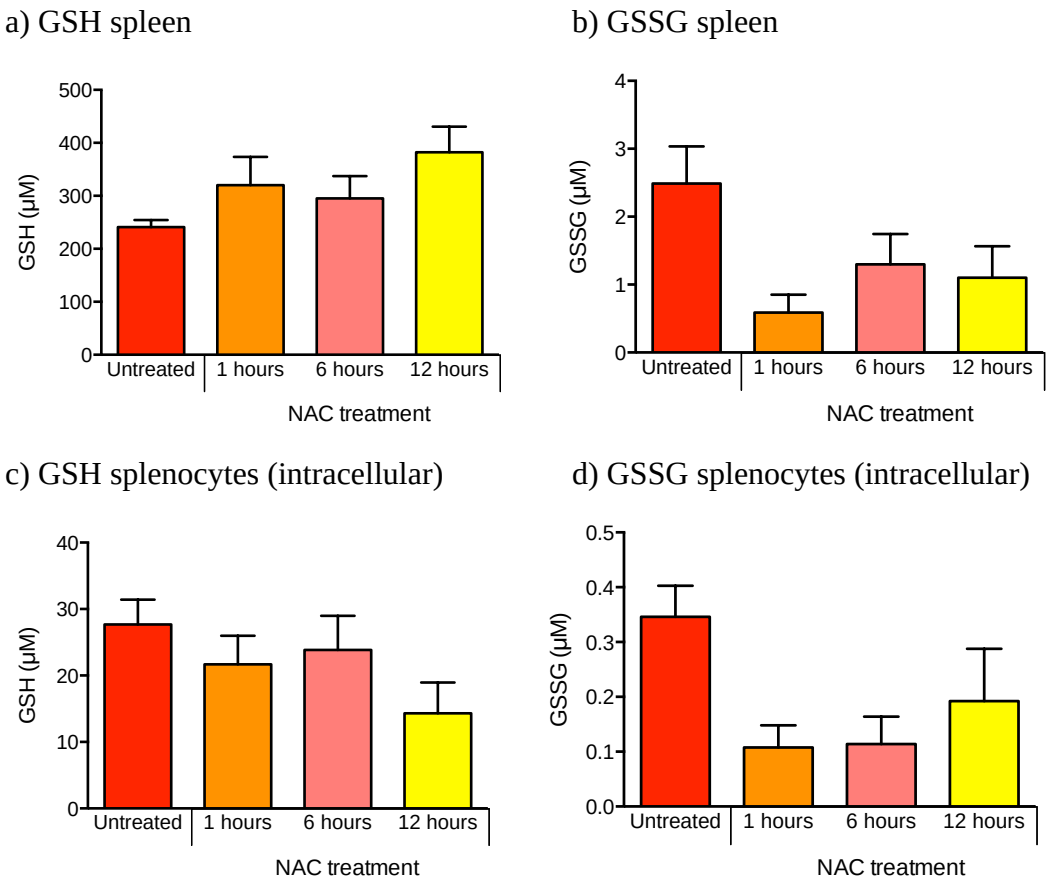

Figure 9.18 Tissue and intracellular levels of glutathione in spleen of diabetic mice following treatment with *N*-acetyl-*L*-cysteine
Diabetic mice were treated with an intraperitoneal dose of *N*-acetyl-*L*-cysteine (NAC; 250mg/kg). a) Levels of GSH in spleen of diabetic mice were comparable following NAC treatment, b) while splenic levels of GSSG tended to be lower after NAC treatment compared to untreated diabetic mice. c) NAC treatment did not alter intracellular levels of GSH in splenocytes. d) Similar to tissue levels, intracellular GSSG tended to be lower after NAC treatment. mean ± SEM.

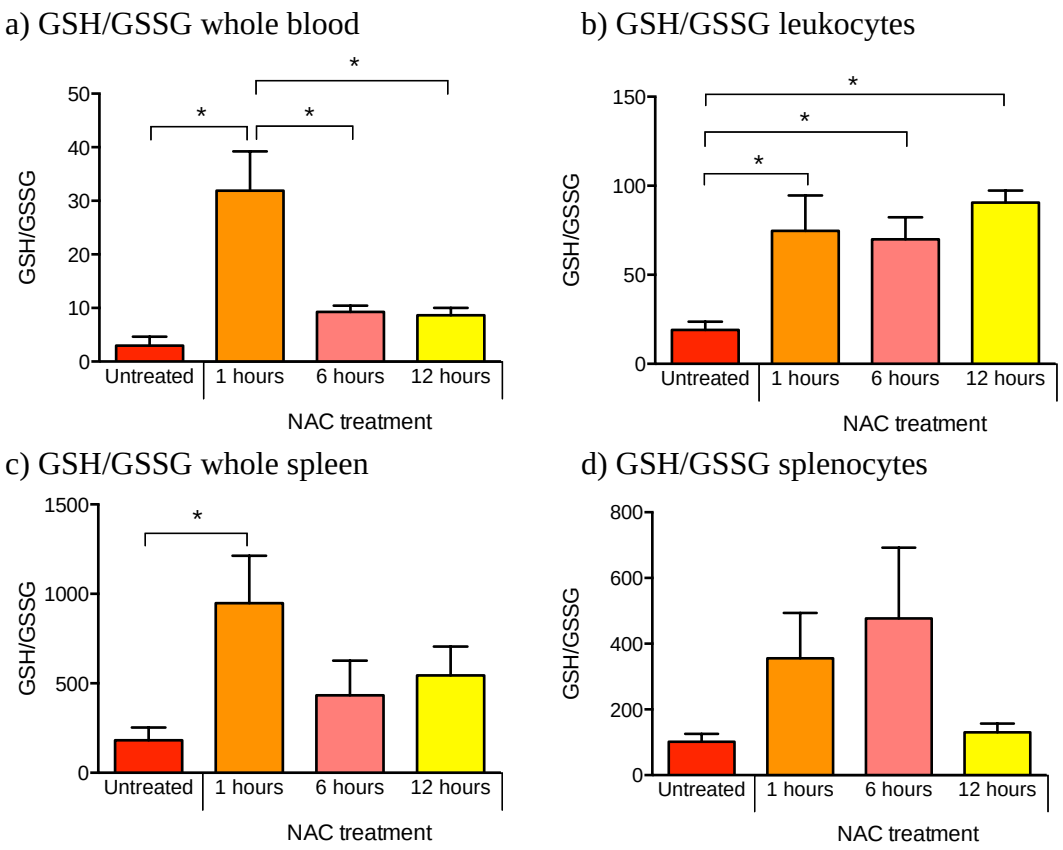

Figure 9.19 Ratio of reduced and oxidised glutathione in blood and spleen of diabetic mice following treatment with *N*-acetyl-*L*-cysteine
Diabetic mice were treated with an intraperitoneal dose of *N*-acetyl-*L*-cysteine (NAC; 250mg/kg). a) The GSH/GSSG ratio in whole blood was transiently increased at 1 hour following NAC treatment, falling again after 6 hours. b) However, the intracellular GSH/GSSG ratio in blood leukocytes remained elevated from 1 to 12 hours after NAC treatment. c) Similarly to whole blood, the tissue GSH/GSSG ratio of spleen was transiently increased at 1 hour after NAC treatment. d) While the intracellular GSH/GSSG ratio tended to be higher at 1 and 6 hours following NAC treatment, levels were comparable by 12 hours post-treatment. * $P < 0.05$; mean ± SEM.

Whilst the GSH/GSSG ratio observed in blood of diabetic mice only transiently increased after 1 hour of NAC treatment, the elevation in GSH/GSSG ratio in leukocytes was of longer term, persisting for at least 12 hours. Intracellular GSH/GSSG ratio is a more reliable indicator of true redox status since circulating GSH levels in whole blood are likely secondary from spillover of excess GSH from erythrocytes (De Mattia *et al.* 1998). Despite the increased GSH/GSSG ratio in diabetic mice following NAC treatment, there was no effect on survival after

infection with *B. pseudomallei* nor did NAC treatment reduce *B. pseudomallei* growth in tissues within the first 24 hours of infection.

9.4 Discussion

It has recently been proposed that the dysregulation of GSH, which has been described in clinical T2D, may be responsible for the increased susceptibility of people with T2D to *B. pseudomallei* infection (Tan *et al.* 2012). The direct and indirect effects of GSH on *B. pseudomallei* growth and the immune response to *B. pseudomallei* infection in a murine model of T2D have not previously been investigated. The direct effect of GSH on *in vitro B. pseudomallei* growth was of interest because direct inhibition of *M. tuberculosis* growth by GSH has previously been described, though the mechanisms of action behind these effects are not known (Venketaraman *et al.* 2005). It has been proposed that GSH may be an ancient penem-like precursor of antibiotics (Venketaraman *et al.* 2005), since the beta-lactam form of GSH has structural similarity to penicillin (Spallholz 1987). Therefore, the sensitivity of a variety of clinical isolates of *B. pseudomallei* to GSH was investigated in this study. Although growth inhibition of some isolates was observed at 20mM GSH, this was later found to be a result of the lower pH at this concentration of GSH rather than direct toxicity. Furthermore, this concentration of GSH is considerably more than would be expected intracellularly in most cells (ranging from 1-10mM) (Smith *et al.* 1996). Therefore, the findings of this study found no evidence for direct effects of GSH against *B. pseudomallei*. However, modulation of GSH/GSSG levels *in vivo* may have an indirect effect on disease progression of *B. pseudomallei* infection via important immunoregulatory mechanisms.

Given the importance of macrophages in the early host response against *B. pseudomallei* infection, we investigated the potential role of impaired macrophage function *ex vivo* and corresponding intracellular levels of GSH and GSSG in macrophages from diabetic and non-diabetic mice. The intracellular environment of macrophages from diabetic mice was more oxidative compared to macrophages from non-diabetic mice indicated by the higher GSSG content and consequently lower GSH/GSSG ratio at baseline. Following 4 hours of co-culture with *B. pseudomallei*,

GSSG content was increased in macrophages from non-diabetic mice. Activation of NADPH oxidase after phagocytosis of bacteria is important for production of ROS, such as H_2O_2, which is required for killing of engulfed bacteria (Yan *et al.* 2013). As previously described, GSH reacts with H_2O_2 to produce GSSG, potentially contributing to the increased GSSG levels observed after stimulation with *B. pseudomallei*. In contrast, there was no change in GSSG levels in macrophages from diabetic mice following stimulation with *B. pseudomallei*. As an indirect measure of ROS production, this may account for the increased intracellular survival of *B. pseudomallei* observed in macrophages from diabetic mice.

Coinciding with increases in GSSG levels, GSH content was also significantly increased in macrophages from non-diabetic mice following stimulation with *B. pseudomallei*. The increased GSH content after stimulation in macrophages from non-diabetic mice may be due to increased GSH synthesis to compensate for a potentially higher rate of utilisation during infection-induced oxidative stress, however this remains to be shown (Malmezat *et al.* 2000). GSH reductase, which catalyses the cycle of GSSG back to GSH by utilising NADPH, is also critical for preventing excessive oxidative stress (Yan *et al.* 2013). Following phagocytosis, the respiratory burst, which involves production of ROS such as H_2O_2 and O_2^-, is a critical bactericidal mechanism and is sustained by GSH recycling through the actions of GSH reductase (Yan *et al.* 2013). For this reason, impairment to the functional activity of GSH reductase has been directly correlated with reduced bacterial killing and increased susceptibility to bacterial infections (Yan *et al.* 2012; Yan *et al.* 2013). Compared to non-diabetic mice, there was no change in GSH levels in macrophages from diabetic mice after 4 hours of *B. pseudomallei* co-culture. This may be due to depletion of endogenous antioxidant activity in macrophages from diabetic mice prior to *B. pseudomallei* stimulation due to the already higher resting levels of GSSG or impairments in other parts of the biosynthetic pathway. Consumption of GSH without sufficient resynthesis may contribute to impaired cellular function and reduced viability (Yan *et al.* 2013).

The increase in GSH after an infectious insult is a protective mechanism to prevent host damage from increased oxidative stress and inflammatory mediators (Malmezat *et al.* 2000). The increased GSH observed in macrophages from non-diabetic at 4

hours post-stimulation with *B. pseudomallei* coincided with reduced intracellular survival of *B. pseudomallei* compared to macrophages from diabetic mice. Previous studies have also shown that GSH levels in macrophages are increased in the first 12 hours post-stimulation with other Gram negative bacteria such as *Porphyromonas gingivalis* (Kato, Mikami & Natsuno 2008). It has been demonstrated that activity of γ-GCL and GSH reductase is increased following infection, accounting for the increased GSH and conversion of GSSG to GSH to maintain a high GSH/GSSG ratio in the face of GSH depletion (Hunter & Grimble 1997; Malmezat *et al.* 2000). By protecting cells from the damaging effects of ROS, GSH promotes cell survival (Beaver & Waring 1995; Malorni *et al.* 1993). Since there was no increase in GSH in macrophages from diabetic mice following *B. pseudomallei*, we hypothesise that this may contribute to the concomitant reduction in cell viability that was observed. This observation is of clinical relevance since reduced viability of immune cells may undermine the effectiveness of the immune response during *B. pseudomallei* infection in diabetic mice. Given the functional importance of the intracellular ratio of GSH/GSSG in macrophages and the critical role macrophages play in controlling *B. pseudomallei* infection, further work is necessary to elucidate the effect of modulating GSH on cellular responses *in vitro*. It would also be worthwhile investigating the altered biosynthetic pathways of GSH/GSSG metabolism in more detail to understand what mechanisms are responsible for the dysregulation in GSH/GSSG levels observed in macrophages following stimulation with *B. pseudomallei*. It may be attributed to a combination of defects involving reduced activity of γ-GCL, GSH reductase or depletion of NADPH, but this is still unclear.

There is strong evidence to support the role of GSH in determining the cytokine propensities of antigen presenting cells, including macrophages and DC, thereby influencing the subsequent development of T_H1 or T_H2-type responses (Monick *et al.* 2003; Morris *et al.* 2013c). It has been demonstrated that depletion of GSH levels in macrophages and DC is associated with decreased production of IL-12, while supplementation of GSH has the opposite effect (Dobashi *et al.* 2001; Morris *et al.* 2013b; Murata, Shimamura & Hamuro 2002; Peterson *et al.* 1998). Therefore, the increased GSH/GSSG ratio of macrophages in non-diabetic mice may skew them towards production of IL-12 in response to *B. pseudomallei*. As previously described in Chapter 8, IL-12 is one of the decisive factors priming the development of IFN-γ

producing cells which drives the development of T_H1 responses that are protective against *B. pseudomallei* infection. In this study, macrophage production of IL-12 after 4 hours of co-culture with *B. pseudomallei* was below the limit of detection. It is likely that longer incubation is necessary to observe detectable levels of IL-12. Production of MCP-1 by macrophages from diabetic mice was significantly lower than those from non-diabetic mice, despite higher baseline levels prior to stimulation with *B. pseudomallei*. This is similar to *in vivo* observations as previously described in Chapter 7. Production of MCP-1 early during infection is critical for recruitment of inflammatory monocytes to sites of infection (Serbina & Pamer 2006; Tsou *et al.* 2007). Therefore, defects in chemokine production may contribute to reduced leukocyte mobilisation, impaired ability to control bacterial growth at the site of infection and increased bacterial dissemination.

Production of TNF-α was higher in macrophages from diabetic mice after 4 hours of co-culture with *B. pseudomallei* compared to macrophages from non-diabetic mice. This is despite reduced levels observed in diabetic mice following *B. pseudomallei* infection *in vivo* (Chapter 7). This may be a reflection of the different time points and relative dose of infection between *in vivo* and *ex vivo* studies or the distinct environment and cellular interactions that occur *in vivo* as opposed to cells treated in isolation *ex vivo*. It has previously been demonstrated that decreased cellular GSH/GSSG ratio is associated with elevated production of proinflammatory cytokines such as TNF-α (Morris *et al.* 2013a; Yan *et al.* 2013). At baseline TNF-α levels were higher in macrophages from diabetic mice consistent with the reduced GSH/GSSG ratio compared to macrophages from non-diabetic mice. TNF-α also stimulates generation of ROS, which indirectly increases GSSG levels and further enhances TNF-α production, representing a feedback loop that potentially contributes to the increased TNF-α levels observed *in vitro* in diabetic macrophages. It is also possible that increased TNF-α levels in macrophages from diabetic mice is a reflection of the greater bacterial burden at this time. While macrophages from diabetic mice may have adequate or enhanced capacity to produce TNF-α, reduced recruitment to sites of infection as a consequence of impaired chemotaxis and MCP-1 production may account for the reduced cytokine levels observed following *B. pseudomallei* infection *in vivo* (Chapter 8).

Alterations in the concentration of GSH and GSSG were observed in the blood, spleen, liver and SAT of diabetic mice in this study. Generally levels of GSH were lower while GSSG levels were higher in diabetic mice, resulting in the overall decreased GSH/GSSG ratio compared to non-diabetic mice. This is consistent with findings in clinical studies (Bravi *et al.* 2006; De Mattia *et al.* 1998; De Mattia *et al.* 1994; Murakami 1991; Murakami *et al.* 1989; Paolisso *et al.* 1993a; Paolisso *et al.* 1993b; Paolisso *et al.* 1992). We also observed a direct correlation between decreasing GSH/GSSG ratio and increasing blood glucose levels in this study. This finding is also reflective of clinical observations, highlighting the parallels of our model to clinical T2D (Jain & McVie 1994; Murakami *et al.* 1989; Sharma *et al.* 2000; Yoshida *et al.* 1995). Oxidative stress, indicated by decreasing GSH/GSSG ratio, is correlated to progression of T2D.

It is hypothesised that decreased levels of cellular GSH and GSH/GSSG ratio in T2D is due to the oxidative stress occurring as a result of immune activation of AGE and the glucolipotoxicity involved in the pathogenesis of T2D (Collier *et al.* 1990; Ghiselli *et al.* 1992; Jennings *et al.* 1987a; Jennings *et al.* 1987b; Tran *et al.* 2004). The metabolic pathways of GSH are highly complex, such that the GSH/GSSG ratio can be influenced by alterations in different sections of the cycle (Figure 9.20). It is hypothesised that depletion of GSH/GSSG ratio in T2D could be a reflection of the increased consumption of GSH due to oxidation to GSSG (Bravi *et al.* 2006). It has previously been shown that GSH is rapidly depleted in cells stimulated with TNF-α under conditions that activate NF-κB (Staal *et al.* 1990). This could also contribute to the depletion of GSH in diabetic mice, which have increased baseline levels of TNF-α, a marker of the chronic inflammation associated with T2D (Chapter 7). However, variations in GSH/GSSG ratio may also be caused by modifications in the synthesis of GSH and/or reconversion of GSSG back to GSH (Chapter 2).

This study provides preliminary findings indirectly linking impaired GSH levels to the increased intracellular persistence of *B. pseudomallei* in macrophages from diabetic mice. We hypothesise that this is caused by reduced microbicidal capacity of macrophages. We have shown that GSH has no direct bactericidal properties against *B. pseudomallei in vitro*. There is evidence that without adequate resynthesis of cellular GSH during infection, phagocytic activity is compromised and

phagocytes cannot sustain the respiratory burst required to kill engulfed bacteria (Yan *et al.* 2013). It has been shown that GSH reductase-deficient monocytes have significant impairments in uptake of pHrodo-conjugated *E. coli* particles, and exhibited impaired recruitment of inflammatory leukocytes and greater susceptibility to bacterial infection compared to wild-type mice (Yan *et al.* 2013).

We propose that alterations in the GSH/GSSG ratio of antigen presenting cells in diabetic mice hampers the development of T_H1-type CMI responses via immunomodulatory mechanisms involving the reduced production of IL-12. Additional research is necessary to confirm this by comparing the effect of GSH modulation on cellular cytokine responses and *B. pseudomallei* persistence. Other studies have shown that increasing GSH in macrophages and DC, through supplementation from addition of *L*-GSH or NAC, augments synthesis of IL-12 and reduces production of IL-10 (Alam *et al.* 2010; Morris *et al.* 2013b). This directly and indirectly favours T_H1 $CD4^+$ T cell responses. In contrast, depletion of GSH in macrophages and DC with BSO enhances the production of IL-10, promoting development of T_H2 T cell responses, and increasing intracellular growth of *M. tuberculosis* (Morris *et al.* 2013b). Decreased GSH levels in PBMC and erythrocytes from patients with active tuberculosis have been correlated with increased production of proinflammatory cytokines and *M. tuberculosis* growth (Venketaraman *et al.* 2008).

In vivo treatments with BSO and NAC were used to assess the effect of GSH modulation on *B. pseudomallei* dissemination and disease severity. BSO treatment to deplete GSH in non-diabetic mice was used to determine if this would increase susceptibility to melioidosis to a similar extent to that observed for diabetic mice (Chapter 8). BSO treated non-diabetic mice were infected 2 hours after BSO treatment when reduced levels of GSH were observed. BSO treatment was then continued for 24 hours following infection with *B. pseudomallei*. However, despite the depletion of GSH in non-diabetic mice after BSO treatment, susceptibility to *B. pseudomallei* was not increased.

A recent study has demonstrated that combined treatment with BSO and diethyl maleate (DEM) of BALB/c mice 2 hours prior to infection with *B. pseudomallei*

dramatically increases mortality compared to untreated mice (Tan *et al.* 2012). BSO treatment blocks *de novo* synthesis of GSH but has no effect on GSH already present in the cell, while DEM acts to deplete GSH already present (Griffith 1999). Although the majority of GSH is in the cytosol, 15-20% is located in subcellular compartments, particularly the mitochondria (Armstrong *et al.* 2002). The mitochondrial pool of GSH is of paramount importance for cell viability, and is relatively resistant to depletion by BSO treatment alone compared to treatment coupled with DEM (Armstrong *et al.* 2002). Furthermore, unlike BSO, DEM also depletes protein-bound GSH, which has important residual activity and can act as a source of soluble GSH (Buchmuller-Rouiller *et al.* 1995). This may explain why DEM combined with BSO is more potent than BSO treatment alone. It is plausible that the combination of depletion of GSH by DEM together with the inhibition of synthesis by BSO would have a greater effect on the response to infection by inhibiting repletion of GSH.

In this study, intraperitoneal administration of NAC resulted in a significant decrease in the GSSG content in diabetic mice, leading to a substantially higher GSH/GSSG ratio. Limited effect of NAC was observed when delivered *per os* (data not shown), potentially related to the low bioavailability that has been demonstrated from oral delivery (< 5%) (Arakawa & Ito 2007). While studies have shown increases in GSH after *per os* delivery of NAC, discrepancies between studies may be due to large differences in NAC concentrations used, ranging from 10mg/kg to 1,000mg/kg, and duration of treatment regimens (Garozzo *et al.* 2007; Guevara *et al.* 2000). We did not extend treatment duration of NAC for greater than 5 days since the metabolic pathway of GSH synthesis is self-regulated due to feedback inhibition by GSH. Therefore, increased duration of NAC treatment is not likely to further increase GSH levels.

It is plausible that alterations in γ-GCL activity, which have been described in T2D, may hamper the ability of NAC to increase GSH content in diabetic mice. It has been shown in *in vitro* studies that treatment with glutathione ethyl ester (GSH-Et) is more efficacious than NAC at much lower concentrations (Morris *et al.* 2013c). This is probably because GSH-Et is a bioavailable form of GSH, whilst NAC must be enzymatically broken down to produce cysteine before it can be used for *de novo*

synthesis of GSH. Therefore, treatment with GSH-Et may be able to circumvent this and may be more successful in modulating GSH and infection outcome in diabetic mice. This may be a potential avenue to pursue in future experiments. Beyond its ability to increase GSH content, NAC also has its own antioxidant properties and can thereby sequester ROS directly to diminish oxidative stress (Arakawa & Ito 2007; De Flora *et al.* 1995; Gamaley *et al.* 2006). This may account for the significant decrease in GSSG levels observed in diabetic mice and the overall increase in GSH/GSSG ratio.

There is evidence that NAC enhances intracellular killing of bacteria by neutrophils, macrophages and DC and is protective against intracellular bacterial infections such as *M. tuberculosis* (Guerra *et al.* 2011; Morris *et al.* 2013c; Venketaraman *et al.* 2006; Yan *et al.* 2013). This has been predominantly attributed to its ability to increase CMI responses by augmenting IL-12 and IFN-γ secretion. In this study, we report for the first time novel differences in GSH and GSSG levels in tissues and reduced GSH/GSSG ratio in macrophages of diet-induced diabetic mice compared to non-diabetic mice. Whilst NAC treatment of diabetic mice afforded no protection against *B. pseudomallei* infection using the regime described, we believe the use of other GSH modulators and antioxidants warrants further investigation. More research is needed to better understand the dysregulation of GSH redox pathways in T2D to identify appropriate targets for modulation and perhaps utilise these for the development of novel therapeutics of melioidosis in at-risk populations.

CHAPTER 10
GENERAL DISCUSSION

In 2011, the global prevalence of T2D increased to 371 million people, making it one of the most common non-communicable diseases worldwide (IDF 2011). Recent projections indicate that this figure will rise to 552 million by 2030 (IDF 2011). For this reason, T2D is regarded as one of the most challenging problems facing public health in the 21st century (IDF 2011). Although originally considered an affluent disease, epidemiological studies for the last two decades have confirmed that the greatest burden of T2D is in low and middle-income countries (IDF 2011).

Infectious diseases remain a leading cause of morbidity and mortality, particularly in low- and middle-income regions, which concurrently face the highest burden of T2D. This convergence presents new challenges for managing and preventing infectious diseases (Remais *et al.* 2013). The global epidemic of T2D has significant implications for the tropics with regards to the emergence and re-emergence of infectious diseases; melioidosis is one such disease. While the past decade has seen steady advancements in the detection of and response to melioidosis, the convergence of non-communicable diseases such as T2D will undoubtedly complicate disease management and accelerate the incidence of this bacterial infection. This is of particular importance since people with T2D have a relative risk for acquiring melioidosis that is 13-fold higher than the general population (Currie, 2004). Given the rapid population growth and increase of metabolic diseases in the tropics, an interdisciplinary approach to address the link between T2D and infectious diseases like melioidosis is imperative.

Melioidosis is inherently resistant to standard empiric antibiotic treatment and despite prolonged courses of appropriate antibiotic therapy, relapse often occurs due to the intracellular niche of *B. pseudomallei* (Leelarasamee 1998; Wiersinga *et al.* 2006; Wuthiekanun & Peacock 2006). Furthermore, acquired antibiotic resistance is a concern that could threaten the progress made in reducing mortality from melioidosis, as has been the case with the advent of multiple drug-resistant tuberculosis (Gillespie 2002). Due to the ease of transmission and infectivity, potential disease severity, environmental stability and lack of vaccine,

B. pseudomallei is classified as a Class B biological warfare agent (Kortepeter & Parker 1999). This has generated significant interest in melioidosis research over the past two decades.

Despite vast improvements in our understanding of the pathogenesis of *B. pseudomallei* infection over the past century, the specific immunological and molecular mechanisms underlying host susceptibility or resistance to *B. pseudomallei* infection remain poorly understood. The most significant risk factor for melioidosis is T2D, and as the epidemic of T2D continues and so to the number of people susceptible to this infection, it becomes even more imperative to unravel the link between these two important diseases. This is accentuated by the increased severity of *B. pseudomallei* infection in individuals with pre-existing risk factors such as T2D. The lack of a suitable animal model of T2D and comorbid melioidosis has hampered research efforts so far. Furthermore, we believe that the development of novel diagnostic and therapeutic agents should be targeted to the population most at risk, the efficacy of which cannot be evaluated without a reliable animal model. To address this need, we have established the first monogenic and polygenic murine models of T2D-melioidosis comorbidity, which will be invaluable for investigating the mechanisms underlying susceptibility of T2D and facilitating therapeutic advancements in treating melioidosis.

In this thesis, several murine models of T2D were compared to identify the most reflective model of clinical T2D. One of the most widely used models of T2D in the literature is the leptin signalling deficient $Lept^{db}$ mouse (Panchal & Brown 2011). $Lept^{db}$ mice are available on either a C57BL/6 or BKS genetic background, believed to result in distinct metabolic phenotypes. Therefore, metabolic parameters including body mass, blood glucose, lipid profiles and glucose tolerance were compared (Chapter 4). Homozygous *db/db* mice developed obesity and glucose intolerance with uncontrolled hyperglycaemia dependant on the genetic background. Although the *db/db* mutation resulted in comparable body mass and glucose intolerance between background strains, BKS *db/db* mice developed severe hyperglycaemia. In contrast, blood glucose levels were more variable and modest in C57BL/6 *db/db* mice. Both strains of $Lept^{db}$ mice were more susceptible to subcutaneous infection with *B. pseudomallei* than control mice and the increased severity of infection was

associated with decreased blood glucose, a phenomenon frequently described in sepsis.

Using this monogenic model of T2D, we described for the first time the early dissemination and pathological responses to *B. pseudomallei* infection in comorbid T2D (Chapter 4). Although these mice phenotypically resembled a T2D state, there were significant aetiological differences to clinical disease. The confounding complications of leptin signalling deficiency conferred a morbidly obese phenotype, not representative of the vast majority of patients with melioidosis and coexisting T2D. This was a useful proof of concept study, using a model that was readily available at the time, that indicated that dissemination of *B. pseudomallei* and mortality from the infection was greater in diabetic (*db/db*) compared to non-diabetic (*db/+*) mice. However, we were interested in developing a model that more closely reflected the comorbidity of melioidosis and T2D in endemic regions.

A polygenic diet-induced murine model of T2D is more reflective of the aetiopathology of T2D in humans. Rapid changes in technology and human behaviour, associated with increased consumption of energy dense diets are largely responsible for the global epidemic of metabolic diseases (Kong *et al.* 2013). Arguably, the development and characterisation of a polygenic diet-induced model of T2D provides a more clinically relevant tool for comprehensive investigation of the immunological basis of susceptibility to *B. pseudomallei* infection. The process of selecting and developing a murine model of diet-induced T2D was complicated by the vast inconsistencies in the literature, owing to the variability and incomplete description of diets, genetic backgrounds, genders, age and duration of feeding regimes. Therefore, considerable effort was spent in this project to optimise many of these parameters to enable development of a model that most closely reflects clinical disease.

In this study, male B6D2F1 mice were found to be more susceptible to metabolic complications induced by HFD feeding compared to female mice (Chapter 5). This parallels the gender susceptibility that has been documented in the development of clinical T2D, believed to be due to the involvement of sex hormones in insulin sensitivity although the mechanisms are incompletely understood. Although

B6D2F1 mice fed a HFD developed severe obesity and glucose intolerance, blood glucose levels were only moderately elevated.

Hyperglycaemia is the pathognomonic indicator of T2D in humans and is thus a critical endpoint of clinical T2D. Therefore, metabolic parameters were compared between B6D2F1 and C57BL/6 mice after HFD feeding to determine if the latter strain was more susceptible to HFD-induced hyperglycaemia (Chapter 6). Despite greater body weight gain in B6D2F1 mice fed a HFD, glucose intolerance was exacerbated in C57BL/6 mice following 10 weeks of HFD feeding. The genetic predisposition for development of insulin resistance in C57BL/6 mice at a lower degree of adiposity is clinically significant since obesity is not well described in patients with melioidosis and comorbid T2D (Simpson *et al.* 2003). This may be due to the genetic susceptibility of indigenous ethnicities in the Asia-Pacific region for developing impaired pancreatic beta cell function and insulin resistance at lower BMI compared to Caucasian populations (King *et al.* 2012; Nishi *et al.* 2005; Razak *et al.* 2007). Similar findings have been documented in Australian Aboriginals, with a 6-fold higher prevalence of T2D than in the general population (Gracey 1995; Leonard *et al.* 2002; McDermott, Li & Campbell 2010). It is also noteworthy that there is a significant secondary interaction between Aboriginal ethnicity and T2D, with a relative risk more than 20-fold for developing melioidosis (Currie *et al.* 2004).

Having identified C57BL/6 mice as a more ideal strain, we next sought to optimise the diet composition to intensify hyperglycaemic levels in these mice (Chapter 7). Consumption of a HF-HGD further increased blood glucose levels in C57BL/6 mice, which was also associated with microalbuminuria and baseline elevation of circulating TNF-α levels consistent with clinical T2D. Microalbuminuria is an important prognostic marker for kidney abnormalities in clinical T2D (Andersen *et al.* 2000). TNF-α has been implicated as a causative factor in insulin resistance and a biomarker of chronic inflammation in experimental and clinical T2D (Moller 2000). In this study, C57BL/6 mice consuming a moderately HF diet became overweight and insulin resistant but only developed hyperglycaemia when fed a HF-HGD, highlighting the involvement of refined carbohydrates in the aetiology of T2D. This has significant clinical implications for nutritional guidelines, which presently advocate low fat, high carbohydrate diets that may actually promote insulin

resistance and development of T2D (Acheson 2004). Given the current findings, further research on the role of dietary carbohydrates in the pathophysiology of T2D and associated complications is warranted. HF-HGD feeding of C57BL/6 mice for 15 weeks was found to induce a metabolic profile reflective of clinical T2D (Table 10.1) and was selected for subsequent experiments focussed on comparing disease progression and immune responses to *B. pseudomallei* infection.

Table 10.1 Comparison of murine models of type 2 diabetes with respect to clinical disease

Type 2 Diabetes Phenotype	Humans	C57BL/6 (Lept *db/db*)	BKS (Lept *db/db*)	B6D2F1 (HFD)	C57BL/6 (HFD)	C57BL/6 (HF/HGD)
Development	Diet-induced*	Spontaneous	Spontaneous	Diet-induced	Diet-induced	Diet-induced
Genetics	Polygenic	Monogenic	Monogenic	Polygenic	Polygenic	Polygenic
Onset	Mature*	Young	Young	Mature	Mature	Mature
Hyperphagia	No	Yes	Yes	No	No	No
Obesity	Variable	Severe	Severe	Severe	Variable	Variable
Hyperinsulinaemia	Moderate	Severe	Transient	Severe	Severe	Moderate
Glucose intolerance	Yes	Yes	Yes	Yes	Yes	Yes
Hyperglycaemia	Yes	Variable	Yes	Variable	Variable	Yes
Dyslipidaemia	Yes	Moderate	Moderate	Yes	Yes	Yes
Nephropathy	Yes	No	Mild	No	No	Yes
Liver steatosis	Yes	Yes	Yes	Yes	Yes	Yes

Adapted from The Jackson Laboratory (*Diabetes Research Resources* 2013). * majority of T2D in humans is related to dietary intake and occurs in people >20 years of age, however it is recognised that not all cases of T2D are diet-induced and development of T2D in childhood is increasing; HFD – high fat diet; HF/HGD – high fat, high glycaemic index diet.

An interesting finding from this study is that mice able to maintain moderate control of blood glucose, yet had metabolic complications such as glucose intolerance, increased body mass and dyslipidaemia, were still more susceptible to *B. pseudomallei* infection. This so-called ‘pre-diabetic state’ is well described clinically in individuals with impaired glucose tolerance but blood glucose levels

below the diagnostic criteria for T2D. The clinical significance of this is paramount since the prevalence of pre-diabetes is as high as 25% in some regions and most people are unaware they have the condition. Therefore, a larger number of people are potentially at increased risk of melioidosis than first thought. This may explain why of the non-diabetic patients with melioidosis, almost half have risk factors of pre-diabetes, including hypertension, dyslipidaemia and increased BMI (*personal communication*, Dr R. Norton, TTH Melioidosis Database). This is especially significant since T2D is an inherently insidious disease that can take years to become established prior to overt clinical symptoms. Further investigation into the prevalence of pre-diabetes risk factors in patients with melioidosis would be interesting to confirm this potential association.

This was the first study comparing *B. pseudomallei* dissemination and cytokine responses in the first 24 hours post-infection with *B. pseudomallei* in a diet-induced model of T2D (Chapter 8). Diabetic mice were significantly more susceptible to *B. pseudomallei* infection compared to non-diabetic littermates. Increased and rapid mortality in diabetic mice was associated with increased bacterial dissemination and growth in the spleen and liver within the first 24 hours post-infection and bacteraemia by 72 hours post-infection preceding death. Observations from this study emphasised the importance of innate immune responses within the first 24 hours post-infection contributing to divergent disease progression. Increased mortality of diabetic mice was preceded by delayed inflammatory cytokine responses in the first 12 hours post-infection.

Interestingly, the increased expression of TNF-α observed in diabetic mice prior to infection did not lead to a cumulative increase in inflammation at least in the first 24 hours following *B. pseudomallei* infection. Levels of MCP-1 were lower at 6 hours post-infection in the spleen of diabetic compared to non-diabetic mice. By 12 hours post-infection, levels of TNF-α, IL-12 and IFN-γ were also lower in the spleen of diabetic mice. This coincided with increased bacterial growth in the spleen of diabetic mice, with significantly higher bacterial burden in the spleen and bacteraemia by 24 hours post-infection. By this time, levels of inflammatory cytokines in the spleen were comparable between diabetic and non-diabetic mice.

We believe that delayed cytokine responses during the early phase of infection allows *B. pseudomallei* to establish a niche in the diabetic host and contributes to accelerated bacterial growth. This delayed response to *B. pseudomallei* infection in diabetic mice may be attributed to the compromised activation, recruitment or functional capacity of phagocytic cells in response to *B. pseudomallei*. Importantly, macrophages have an essential role in immune defence against *B. pseudomallei* infection and impaired function could contribute to increased *B. pseudomallei* growth and poor disease outcome in diabetic mice. Findings from our *in vitro* studies indicate that macrophages from diabetic mice have a reduced capacity to kill *B. pseudomallei* compared to macrophages from non-diabetic mice, which may provide an intracellular niche for *B. pseudomallei* and may contribute to greater bacterial dissemination (Chapter 9). We also observed reduced protein levels of MCP-1 in macrophages from diabetic mice, consistent with observations following *in vivo* infection. Delayed levels of MCP-1 suggest that chemotaxis of monocytes and macrophages to sites of infection may be hampered in diabetic mice. Additional cellular interactions and potential involvement of impaired neutrophil and DC function is yet to be investigated. Delayed recruitment and activation of macrophages may contribute to the lower levels of TNF-α and IL-12 leading to delayed production of IFN-γ and activation of protective inflammatory pathways. By the time the inflammatory response is initiated in diabetic mice at 24 hours post-infection, the bacterial burden is already high and it is too late to rescue the host from overt bacteraemia and probable death within 72 hours post-infection.

Our findings of reduced levels of IL-12 and IFN-γ in diabetic mice following infection *in vivo* is consistent with the recently described defects in IL-12 and IFN-γ production by PBMC from individuals with T2D after *in vitro* stimulation with *B. pseudomallei* (Tan *et al.* 2012). It has recently been proposed that alterations in the GSH/GSSG redox ratio may be involved in the dysregulated cytokine responses observed in T2D that contributes to susceptibility to *B. pseudomallei* infection (Tan *et al.* 2012). GSH is the most abundant antioxidant in mammalian cells. The primary function of GSH involves regulation of the cellular redox balance by cycling between reduced and oxidised states to protect against oxidative injury. Chronic oxidative stress is central to the pathogenesis of T2D however the implications of altered GSH/GSSG redox balance in T2D complications is unclear. GSH

concentrations in diabetic mice tended to be lower compared to non-diabetic mice, while GSSG content was significantly greater indicative of oxidative stress.

To ascertain the role of the GSH/GSSG redox balance in determining susceptibility to melioidosis, GSH modulators were used to deplete or restore GSH in non-diabetic and diabetic mice, respectively (Chapter 9). Although, short-term GSH modulation did not alter susceptibility to melioidosis in non-diabetic or diabetic mice in this study, further research is warranted to investigate effects of GSH modulation *in vitro*. In particular, evidence is mounting that the GSH/GSSG ratio has an important role in immune cell functional responses to bacterial infections. Impairments to the GSH/GSSG redox pathways are associated with defective phagocytic and bactericidal activity of neutrophils (Yan *et al.* 2012; Yan *et al.* 2013). This was associated with attenuated oxidative burst and impaired formation of NET after stimulation with bacteria including *Escherichia coli* and *Group B Streptococci* (Yan *et al.* 2012; Yan *et al.* 2013).

An inordinate amount of evidence exists for the immunoregulatory role of the GSH/GSSG ratio in macrophage and DC responses to *M. tuberculosis*. It has been shown that increasing the intracellular levels of GSH in macrophages and DC can inhibit intracellular growth of *M. tuberculosis,* accompanied by increased production of IL-12 and development of protective $CD4^+$ T_H1 cell responses (Morris *et al.* 2013c; Venketaraman *et al.* 2006). This is critical for IFN-γ production and appropriate activation of macrophages to enable killing of intracellular bacteria. As an intracellular bacterium that also shares T2D as an important risk factor, *M. tuberculosis* exhibits many similarities to *B. pseudomallei,* and these important findings linking GSH/GSSG ratio to immune dysregulation may be directly translatable to melioidosis and T2D comorbidity.

Using our murine model, we have documented decreased tissue and macrophage levels of GSH/GSSG in diabetic mice for the first time, which we believe has important functional consequences on the ability of immune cells to respond appropriately to infection (Chapter 9). In particular the decreased GSH/GSSG ratio in macrophages from diabetic mice coincides with reduced bactericidal activity in response to *B. pseudomallei* exposure and reduced viability of macrophages. We

believe this is also associated with the delayed production of inflammatory cytokines such as IL-12 observed *in vivo*, which facilitates bacterial dissemination and leads to overwhelming bacteraemia and host death. Therefore, improving the GSH/GSSG ratio in diabetic mice through the use of GSH modulators may represent a therapeutic approach to restore the bactericidal activity and cytokine response of phagocytic cells. However, NAC may not be the most appropriate choice as was selected in this study due to the inherent abnormalities in the GSH synthesis pathway that have been described in clinical T2D. This may explain why we did not find significant improvements with NAC treatment in this study, although we also recognise that the treatment regime may require further optimisation. It would be interesting to explore other GSH modulators such as GSH-Et, a source of GSH without the requirement for *de novo* synthesis, or even the potential of antioxidant supplements to reduce GSSG. Furthermore, it would be valuable to investigate the combined outcome of treatment with antibiotics in combination with GSH modulation in diabetic mice, to investigate the potential of GSH modulators as adjuvant, immunotherapeutic agents. The immunoregulatory role of GSH/GSSG redox pathways in T2D and melioidosis comorbidity would be an exciting avenue to pursue in the future and may hold the key to unlocking a potential target for adjunctive therapy.

The murine model of *B. pseudomallei* infection with comorbid T2D described in this thesis will enable fundamental research exploring the role of GSH and additional pathogenic mechanisms that link these two significant diseases. The increased susceptibility of diabetic mice to *B. pseudomallei* is consistent with the clinical comorbidity of melioidosis and T2D and highlights the value of this animal model for use in future studies to unravel the mechanisms behind this comorbidity. The use of an *in vivo* animal model has distinct advantages over *in vitro* and *ex vivo* studies, by facilitating investigation of disease progression and local cellular responses within the host in the early stages of infection, prior to the development of overt clinical signs and symptoms. It is also necessary to understand the complexity of cellular interactions that occur and are essential for development of protection against intracellular pathogens.

Thailand and India are experiencing some of the highest increases in T2D prevalence (Aekplakorn *et al.* 2003; Chan *et al.* 2009; Ramachandran, Ma & Snehalatha 2010; Yoon *et al.* 2006). As this risk factor becomes more prevalent in melioidosis endemic regions, it is anticipated that the prevalence and severity of melioidosis will expand. As Asia undergoes rapid modernisation and urbanisation, the prevalence of non-communicable diseases such as obesity and T2D has significantly increased (Chan *et al.* 2009; Kong *et al.* 2013; Misra & Khurana 2008; Ramachandran, Ma & Snehalatha 2010; Yoon *et al.* 2006). Socio-economic changes in this region have been met with an unprecedented escalation in non-communicable diseases such as T2D. This has been attributed to the rapid economic advancements and nutrition transition towards high-energy diets, combined with a genetic predisposition to metabolic complications (Popkin & Gordon-Larsen 2004; Rodriguez-Hernandez *et al.* 2013).

The co-occurrence of T2D and tropical infectious diseases has substantial public health implications. Melioidosis is a neglected tropical disease and with the advent of non-communicable diseases like T2D, it becomes even more pertinent to address the double burden of this comorbidity (Inglis *et al.* 2005). Undoubtedly, the rising prevalence of T2D will contribute to more clinical complications and protracted disease course of melioidosis. Improvements in treatment will rely heavily on fundamental research to understand host-pathogen interactions with particular relevance to the increased susceptibility of individuals with T2D. In light of the current T2D epidemic, the link between metabolic and infectious diseases will be an important area of research to pursue in the future. The murine model described in this study will serve as a valuable platform for future investigations.

REFERENCES

Abbasi, F, Brown, BW, Jr., Lamendola, C, McLaughlin, T & Reaven, GM 2002, 'Relationship between obesity, insulin resistance, and coronary heart disease risk', *J Am Coll Cardiol*, vol. 40, no. 5, pp. 937-43.

Abbasi, F, Chu, JW, Lamendola, C, McLaughlin, T, Hayden, J, Reaven, GM & Reaven, PD 2004, 'Discrimination between obesity and insulin resistance in the relationship with adiponectin', *Diabetes*, vol. 53, no. 3, pp. 585-90.

Acheson, KJ 2004, 'Carbohydrate and weight control: where do we stand?', *Curr Opin Clin Nutr Metab Care*, vol. 7, no. 4, pp. 485-92.

ADA 2013, 'Diagnosis and classification of diabetes mellitus', *Diabetes Care*, vol. 36 Suppl 1, pp. S67-74.

Aekplakorn, W, Bunnag, P, Woodward, M, Sritara, P, Cheepudomwit, S, Yamwong, S, Yipintsoi, T & Rajatanavin, R 2006, 'A risk score for predicting incident diabetes in the Thai population', *Diabetes Care*, vol. 29, no. 8, pp. 1872-7.

Aekplakorn, W, Stolk, RP, Neal, B, Suriyawongpaisal, P, Chongsuvivatwong, V, Cheepudomwit, S & Woodward, M 2003, 'The prevalence and management of diabetes in Thai adults: the international collaborative study of cardiovascular disease in Asia', *Diabetes Care*, vol. 26, no. 10, pp. 2758-63.

Ahmed, K, Enciso, HD, Masaki, H, Tao, M, Omori, A, Tharavichikul, P & Nagatake, T 1999, 'Attachment of *Burkholderia pseudomallei* to pharyngeal epithelial cells: a highly pathogenic bacteria with low attachment ability', *Am J Trop Med Hyg*, vol. 60, no. 1, pp. 90-3.

Ahmed, N, Babaei-Jadidi, R, Howell, SK, Beisswenger, PJ & Thornalley, PJ 2005, 'Degradation products of proteins damaged by glycation, oxidation and nitration in clinical type 1 diabetes', *Diabetologia*, vol. 48, no. 8, pp. 1590-603.

AIHW 2008, 'Diabetes: Australian facts 2008', no. Diabetes series no. 8, viewed 28 July 2013, http://www.aihw.gov.au/publication-detail/?id=6442468075%3E.

AIHW 2009, 'Diabetes prevalence in Australia: an assessment of national data sources', no. Diabetes series no. 12, viewed 28 July 2013, http://www.aihw.gov.au/publication-detail/?id=6442468288%3E.

AIHW 2011, 'Diabetes prevalence in Australia: detailed estimates for 2007-08', no. Diabetes series no. 17. Cat. no. CVD 56, viewed 28 July 2013, http://www.aihw.gov.au/publication-detail/?id=10737419311%3E.%3E.

Al-Attiyah, RJ & Mustafa, AS 2009, 'Mycobacterial antigen-induced T helper type 1 (Th1) and Th2 reactivity of peripheral blood mononuclear cells from diabetic and non-diabetic tuberculosis patients and *Mycobacterium bovis* bacilli Calmette-Guerin (BCG)-vaccinated healthy subjects', *Clin Exp Immunol*, vol. 158, no. 1, pp. 64-73.

Alam, K, Ghousunnissa, S, Nair, S, Valluri, VL & Mukhopadhyay, S 2010, 'Glutathione-redox balance regulates c-rel-driven IL-12 production in macrophages: possible implications in antituberculosis immunotherapy', *J Immunol*, vol. 184, no. 6, pp. 2918-29.

Alamgir, S, Volkova, NB & Peterson, MW 2006, 'Prognostic value of low blood glucose at the presentation of *E. coli* bacteremia', *Am J Med*, vol. 119, no. 11, pp. 952-7.

Alexander, J, Chang, GQ, Dourmashkin, JT & Leibowitz, SF 2006, 'Distinct phenotypes of obesity-prone AKR/J, DBA2J and C57BL/6J mice compared to control strains', *Int J Obes (Lond)*, vol. 30, no. 1, pp. 50-9.

Allwood, EM, Devenish, RJ, Prescott, M, Adler, B & Boyce, JD 2011, 'Strategies for Intracellular Survival of *Burkholderia pseudomallei*', *Front Microbiol*, vol. 2, p. 170.

Andersen, S, Blouch, K, Bialek, J, Deckert, M, Parving, HH & Myers, BD 2000, 'Glomerular permselectivity in early stages of overt diabetic nephropathy', *Kidney Int*, vol. 58, no. 5, pp. 2129-37.

Andersson, U, Rosen, L, Wierup, N, Ostman, E, Bjorck, I & Holm, C 2010, 'A low glycaemic diet improves oral glucose tolerance but has no effect on beta-cell function in C57BL/6J mice', *Diabetes Obes Metab*, vol. 12, no. 11, pp. 976-82.

Andrikopoulos, S, Blair, AR, Deluca, N, Fam, BC & Proietto, J 2008, 'Evaluating the glucose tolerance test in mice', *Am J Physiol Endocrinol Metab*, vol. 295, no. 6, pp. E1323-32.

Anuntagool, N, Naigowit, P, Petkanchanapong, V, Aramsri, P, Panichakul, T & Sirisinha, S 2000, 'Monoclonal antibody-based rapid identification of *Burkholderia pseudomallei* in blood culture fluid from patients with community-acquired septicaemia', *J Med Microbiol*, vol. 49, no. 12, pp. 1075-8.

Arakawa, M & Ito, Y 2007, 'N-acetylcysteine and neurodegenerative diseases: basic and clinical pharmacology', *Cerebellum*, vol. 6, no. 4, pp. 308-14.

Arita, Y, Kihara, S, Ouchi, N, Takahashi, M, Maeda, K, Miyagawa, J, Hotta, K, Shimomura, I, Nakamura, T, Miyaoka, K, Kuriyama, H, Nishida, M, Yamashita, S, Okubo, K, Matsubara, K, Muraguchi, M, Ohmoto, Y, Funahashi, T & Matsuzawa, Y 1999, 'Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity', *Biochem Biophys Res Commun*, vol. 257, no. 1, pp. 79-83.

Armitage, JA, Khan, IY, Taylor, PD, Nathanielsz, PW & Poston, L 2004, 'Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals?', *J Physiol*, vol. 561, no. Pt 2, pp. 355-77.

Armstrong, JS, Steinauer, KK, Hornung, B, Irish, JM, Lecane, P, Birrell, GW, Peehl, DM & Knox, SJ 2002, 'Role of glutathione depletion and reactive oxygen species generation in apoptotic signaling in a human B lymphoma cell line', *Cell Death Differ*, vol. 9, no. 3, pp. 252-63.

Arner, P 1998, 'Not all fat is alike', *Lancet*, vol. 351, no. 9112, pp. 1301-2.

Askeland, EJ, Newton, MR, O'Donnell, MA & Luo, Y 2012, 'Bladder Cancer Immunotherapy: BCG and Beyond', *Adv Urol*, vol. 2012, p. 181987.

Astrup, A & Finer, N 2000, 'Redefining type 2 diabetes: 'diabesity' or 'obesity dependent diabetes mellitus'?', *Obes Rev*, vol. 1, no. 2, pp. 57-9.

Atkuri, KR, Mantovani, JJ, Herzenberg, LA & Herzenberg, LA 2007, 'N-Acetylcysteine--a safe antidote for cysteine/glutathione deficiency', *Curr Opin Pharmacol*, vol. 7, no. 4, pp. 355-9.

Baeuerle, PA & Baltimore, D 1988a, 'Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor', *Cell*, vol. 53, no. 2, pp. 211-7.

Baeuerle, PA & Baltimore, D 1988b, 'I kappa B: a specific inhibitor of the NF-kappa B transcription factor', *Science*, vol. 242, no. 4878, pp. 540-6.

Baik, I, Curhan, GC, Rimm, EB, Bendich, A, Willett, WC & Fawzi, WW 2000, 'A prospective study of age and lifestyle factors in relation to community-acquired pneumonia in US men and women', *Arch Intern Med*, vol. 160, no. 20, pp. 3082-8.

Baker, A, Tahani, D, Gardiner, C, Bristow, KL, Greenhill, AR & Warner, J 2011a, 'Groundwater seeps facilitate exposure to *Burkholderia pseudomallei*', *Appl Environ Microbiol*, vol. 77, no. 20, pp. 7243-6.

Baker, MA, Harries, AD, Jeon, CY, Hart, JE, Kapur, A, Lonnroth, K, Ottmani, SE, Goonesekera, SD & Murray, MB 2011b, 'The impact of diabetes on tuberculosis treatment outcomes: a systematic review', *BMC Med*, vol. 9, p. 81.

Barman, P, Kaur, R & Kumar, K 2013, 'Clinically lesser known entity in India: A Report of two cases of Melioidosis', *Indian J Crit Care Med*, vol. 17, no. 1, pp. 46-8.

Barnes, JL & Ketheesan, N 2005, 'Route of infection in melioidosis', *Emerg Infect Dis*, vol. 11, no. 4, pp. 638-9.

Barnes, JL & Ketheesan, N 2007, 'Development of protective immunity in a murine model of melioidosis is influenced by the source of *Burkholderia pseudomallei* antigens', *Immunol Cell Biol*, vol. 85, no. 7, pp. 551-7.

Barnes, JL, Ulett, GC, Ketheesan, N, Clair, T, Summers, PM & Hirst, RG 2001, 'Induction of multiple chemokine and colony-stimulating factor genes in experimental *Burkholderia pseudomallei* infection', *Immunol Cell Biol*, vol. 79, no. 5, pp. 490-501.

Barnes, JL, Warner, J, Melrose, W, Durrheim, D, Speare, R, Reeder, JC & Ketheesan, N 2004, 'Adaptive immunity in melioidosis: a possible role for T cells in determining outcome of infection with *Burkholderia pseudomallei*', *Clin Immunol*, vol. 113, no. 1, pp. 22-8.

Barnes, JL, Williams, NL & Ketheesan, N 2008, 'Susceptibility to *Burkholderia pseudomallei* is associated with host immune responses involving tumor necrosis factor receptor-1 (TNFR1) and TNF receptor-2 (TNFR2)', *FEMS Immunol Med Microbiol*, vol. 52, no. 3, pp. 379-88.

Barycki, J 2008, 'Glutathione', in R Banerjee (ed.), *Redox Biochemistry*, John Wiley & Sons, New Jersey.

Beaver, JP & Waring, P 1995, 'A decrease in intracellular glutathione concentration precedes the onset of apoptosis in murine thymocytes', *Eur J Cell Biol*, vol. 68, no. 1, pp. 47-54.

Behera, B, Prasad Babu, TL, Kamalesh, A & Reddy, G 2012, 'Ceftazidime resistance in *Burkholderia pseudomallei*: first report from India', *Asian Pac J Trop Med*, vol. 5, no. 4, pp. 329-30.

Bergman, M 2013, 'Pathophysiology of prediabetes and treatment implications for the prevention of type 2 diabetes mellitus', *Endocrine*, vol. 43, no. 3, pp. 504-13.

Bo, S, Menato, G, Lezo, A, Signorile, A, Bardelli, C, De Michieli, F, Massobrio, M & Pagano, G 2001, 'Dietary fat and gestational hyperglycaemia', *Diabetologia*, vol. 44, no. 8, pp. 972-8.

Boddey, JA, Day, CJ, Flegg, CP, Ulrich, RL, Stephens, SR, Beacham, IR, Morrison, NA & Peak, IR 2007, 'The bacterial gene lfpA influences the potent induction of calcitonin receptor and osteoclast-related genes in *Burkholderia pseudomallei*-induced TRAP-positive multinucleated giant cells', *Cell Microbiol*, vol. 9, no. 2, pp. 514-31.

Boddey, JA, Flegg, CP, Day, CJ, Beacham, IR & Peak, IR 2006, 'Temperature-regulated microcolony formation by *Burkholderia pseudomallei* requires pilA and enhances association with cultured human cells', *Infect Immun*, vol. 74, no. 9, pp. 5374-81.

Boden, G 2011, 'Obesity, insulin resistance and free fatty acids', *Curr Opin Endocrinol Diabetes Obes*, vol. 18, no. 2, pp. 139-43.

Bonora, E, Kiechl, S, Willeit, J, Oberhollenzer, F, Egger, G, Meigs, JB, Bonadonna, RC & Muggeo, M 2007, 'Insulin resistance as estimated by homeostasis model assessment predicts incident symptomatic cardiovascular disease in caucasian subjects from the general population: the Bruneck study', *Diabetes Care*, vol. 30, no. 2, pp. 318-24.

Borkman, M, Storlien, LH, Pan, DA, Jenkins, AB, Chisholm, DJ & Campbell, LV 1993, 'The relation between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids', *N Engl J Med*, vol. 328, no. 4, pp. 238-44.

Boyko, EJ, Fujimoto, WY, Leonetti, DL & Newell-Morris, L 2000, 'Visceral adiposity and risk of type 2 diabetes: a prospective study among Japanese Americans', *Diabetes Care*, vol. 23, no. 4, pp. 465-71.

Brabant, G, Muller, G, Horn, R, Anderwald, C, Roden, M & Nave, H 2005, 'Hepatic leptin signaling in obesity', *FASEB J*, vol. 19, no. 8, pp. 1048-50.

Brand-Miller, J, McMillan-Price, J, Steinbeck, K & Caterson, I 2009, 'Dietary glycemic index: health implications', *J Am Coll Nutr*, vol. 28 Suppl, pp. 446S-9S.

Bravi, MC, Armiento, A, Laurenti, O, Cassone-Faldetta, M, De Luca, O, Moretti, A & De Mattia, G 2006, 'Insulin decreases intracellular oxidative stress in patients with type 2 diabetes mellitus', *Metabolism*, vol. 55, no. 5, pp. 691-5.

Bravi, MC, Pietrangeli, P, Laurenti, O, Basili, S, Cassone-Faldetta, M, Ferri, C & De Mattia, G 1997, 'Polyol pathway activation and glutathione redox status in non-insulin-dependent diabetic patients', *Metabolism*, vol. 46, no. 10, pp. 1194-8.

Breitbach, K, Klocke, S, Tschernig, T, van Rooijen, N, Baumann, U & Steinmetz, I 2006, 'Role of inducible nitric oxide synthase and NADPH oxidase in early control of *Burkholderia pseudomallei* infection in mice', *Infect Immun*, vol. 74, no. 11, pp. 6300-9.

Breitbach, K, Sun, GW, Kohler, J, Eske, K, Wongprompitak, P, Tan, G, Liu, Y, Gan, YH & Steinmetz, I 2009, 'Caspase-1 mediates resistance in murine melioidosis', *Infect Immun*, vol. 77, no. 4, pp. 1589-95.

Brett, PJ & Woods, DE 2000, 'Pathogenesis of and immunity to melioidosis', *Acta Trop*, vol. 74, no. 2-3, pp. 201-10.

Brown, AE, Dance, DA, Suputtamongkol, Y, Chaowagul, W, Kongchareon, S, Webster, HK & White, NJ 1991, 'Immune cell activation in melioidosis: increased serum levels of interferon-gamma and soluble interleukin-2 receptors without change in soluble CD8 protein', *J Infect Dis*, vol. 163, no. 5, pp. 1145-8.

Brown, LM, Gent, L, Davis, K & Clegg, DJ 2010, 'Metabolic impact of sex hormones on obesity', *Brain Res*, vol. 1350, pp. 77-85.

Brown, NF, Boddey, JA, Flegg, CP & Beacham, IR 2002, 'Adherence of *Burkholderia pseudomallei* cells to cultured human epithelial cell lines is regulated by growth temperature', *Infect Immun*, vol. 70, no. 2, pp. 974-80.

Brownlee, M 2001, 'Biochemistry and molecular cell biology of diabetic complications', *Nature*, vol. 414, no. 6865, pp. 813-20.

Brownlee, M 2005, 'The pathobiology of diabetic complications: a unifying mechanism', *Diabetes*, vol. 54, no. 6, pp. 1615-25.

Brunzell, JD & Ayyobi, AF 2003, 'Dyslipidemia in the metabolic syndrome and type 2 diabetes mellitus', *Am J Med*, vol. 115 Suppl 8A, pp. 24S-8S.

Bryant, NJ, Govers, R & James, DE 2002, 'Regulated transport of the glucose transporter GLUT4', *Nat Rev Mol Cell Biol*, vol. 3, no. 4, pp. 267-77.

Buchmuller-Rouiller, Y, Corrandin, SB, Smith, J, Schneider, P, Ransijn, A, Jongeneel, CV & Mauel, J 1995, 'Role of glutathione in macrophage activation: effect of cellular glutathione depletion on nitrite production and leishmanicidal activity', *Cell Immunol*, vol. 164, no. 1, pp. 73-80.

Burivong, W, Wu, X, Saenkote, W & Stern, EJ 2012, 'Thoracic radiologic manifestations of melioidosis', *Curr Probl Diagn Radiol*, vol. 41, no. 6, pp. 199-209.

Burtnick, MN & Woods, DE 1999, 'Isolation of polymyxin B-susceptible mutants of *Burkholderia pseudomallei* and molecular characterization of genetic loci involved in polymyxin B resistance', *Antimicrob Agents Chemother*, vol. 43, no. 11, pp. 2648-56.

Butler, AE, Janson, J, Bonner-Weir, S, Ritzel, R, Rizza, RA & Butler, PC 2003, 'Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes', *Diabetes*, vol. 52, no. 1, pp. 102-10.

Cai, D, Yuan, M, Frantz, DF, Melendez, PA, Hansen, L, Lee, J & Shoelson, SE 2005, 'Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB', *Nat Med*, vol. 11, no. 2, pp. 183-90.

Caldefie-Chezet, F, Poulin, A, Tridon, A, Sion, B & Vasson, MP 2001, 'Leptin: a potential regulator of polymorphonuclear neutrophil bactericidal action?', *J Leukoc Biol*, vol. 69, no. 3, pp. 414-8.

Caldefie-Chezet, F, Poulin, A & Vasson, MP 2003, 'Leptin regulates functional capacities of polymorphonuclear neutrophils', *Free Radic Res*, vol. 37, no. 8, pp. 809-14.

Campbell, BW, Addison, CC, Charles, L & Thurston, DA 2003, 'Cardiovascular risk factors among women in Mississippi in the 1990s', *J Am Med Womens Assoc*, vol. 58, no. 2, pp. 105-11.

Cancello, R, Henegar, C, Viguerie, N, Taleb, S, Poitou, C, Rouault, C, Coupaye, M, Pelloux, V, Hugol, D, Bouillot, JL, Bouloumie, A, Barbatelli, G, Cinti, S, Svensson,

PA, Barsh, GS, Zucker, JD, Basdevant, A, Langin, D & Clement, K 2005, 'Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss', *Diabetes*, vol. 54, no. 8, pp. 2277-86.

Canturk, Z, Canturk, NZ, Cetinarslan, B, Utkan, NZ & Tarkun, I 2003, 'Nosocomial infections and obesity in surgical patients', *Obes Res*, vol. 11, no. 6, pp. 769-75.

Cappel, RE & Gilbert, HF 1988, 'Thiol/disulfide exchange between 3-hydroxy-3-methylglutaryl-CoA reductase and glutathione. A thermodynamically facile dithiol oxidation', *J Biol Chem*, vol. 263, no. 25, pp. 12204-12.

Carr, MW, Roth, SJ, Luther, E, Rose, SS & Springer, TA 1994, 'Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant', *Proc Natl Acad Sci U S A*, vol. 91, no. 9, pp. 3652-6.

Ceballos-Olvera, I, Sahoo, M, Miller, MA, Del Barrio, L & Re, F 2011, 'Inflammasome-dependent pyroptosis and IL-18 protect against *Burkholderia pseudomallei* lung infection while IL-1beta is deleterious', *PLoS Pathog*, vol. 7, no. 12, p. e1002452.

Chalkley, SM, Hettiarachchi, M, Chisholm, DJ & Kraegen, EW 2002, 'Long-term high-fat feeding leads to severe insulin resistance but not diabetes in Wistar rats', *Am J Physiol Endocrinol Metab*, vol. 282, no. 6, pp. E1231-8.

Chan, JC, Malik, V, Jia, W, Kadowaki, T, Yajnik, CS, Yoon, KH & Hu, FB 2009, 'Diabetes in Asia: epidemiology, risk factors, and pathophysiology', *JAMA*, vol. 301, no. 20, pp. 2129-40.

Chan, JM, Rimm, EB, Colditz, GA, Stampfer, MJ & Willett, WC 1994, 'Obesity, fat distribution, and weight gain as risk factors for clinical diabetes in men', *Diabetes Care*, vol. 17, no. 9, pp. 961-9.

Chan, KP, Low, JG, Raghuram, J, Fook-Chong, SM & Kurup, A 2005, 'Clinical characteristics and outcome of severe melioidosis requiring intensive care', *Chest*, vol. 128, no. 5, pp. 3674-8.

Chan, WB, Tong, PC, Chow, CC, So, WY, Ng, MC, Ma, RC, Osaki, R, Cockram, CS & Chan, JC 2004, 'The associations of body mass index, C-peptide and metabolic status in Chinese Type 2 diabetic patients', *Diabet Med*, vol. 21, no. 4, pp. 349-53.

Chan, YY, Ong, YM & Chua, KL 2007, 'Synergistic interaction between phenothiazines and antimicrobial agents against *Burkholderia pseudomallei*', *Antimicrob Agents Chemother*, vol. 51, no. 2, pp. 623-30.

Chanchamroen, S, Kewcharoenwong, C, Susaengrat, W, Ato, M & Lertmemongkolchai, G 2009, 'Human polymorphonuclear neutrophil responses to *Burkholderia pseudomallei* in healthy and diabetic subjects', *Infect Immun*, vol. 77, no. 1, pp. 456-63.

Chandalia, M & Abate, N 2007, 'Metabolic complications of obesity: inflated or inflamed?', *J Diabetes Complications*, vol. 21, no. 2, pp. 128-36.

Chantratita, N, Wuthiekanun, V, Boonbumrung, K, Tiyawisutsri, R, Vesaratchavest, M, Limmathurotsakul, D, Chierakul, W, Wongratanacheewin, S, Pukritiyakamee, S, White, NJ, Day, NP & Peacock, SJ 2007, 'Biological relevance of colony morphology and phenotypic switching by *Burkholderia pseudomallei*', *J Bacteriol*, vol. 189, no. 3, pp. 807-17.

Chaowagul, W, White, NJ, Dance, DA, Wattanagoon, Y, Naigowit, P, Davis, TM, Looareesuwan, S & Pitakwatchara, N 1989, 'Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand', *J Infect Dis*, vol. 159, no. 5, pp. 890-9.

Charoensap, J, Utaisincharoen, P, Engering, A & Sirisinha, S 2009, 'Differential intracellular fate of *Burkholderia pseudomallei* 844 and *Burkholderia thailandensis* UE5 in human monocyte-derived dendritic cells and macrophages', *BMC Immunol*, vol. 10, p. 20.

Chen, M, Bergman, RN, Pacini, G & Porte, D, Jr. 1985, 'Pathogenesis of age-related glucose intolerance in man: insulin resistance and decreased beta-cell function', *J Clin Endocrinol Metab*, vol. 60, no. 1, pp. 13-20.

Chen, Y, Yang, Y, Miller, ML, Shen, D, Shertzer, HG, Stringer, KF, Wang, B, Schneider, SN, Nebert, DW & Dalton, TP 2007, 'Hepatocyte-specific Gclc deletion leads to rapid onset of steatosis with mitochondrial injury and liver failure', *Hepatology*, vol. 45, no. 5, pp. 1118-28.

Cheng, AC & Currie, BJ 2005, 'Melioidosis: epidemiology, pathophysiology, and management', *Clin Microbiol Rev*, vol. 18, no. 2, pp. 383-416.

Cheng, AC, Currie, BJ, Dance, DA, Funnell, SG, Limmathurotsakul, D, Simpson, AJ & Peacock, SJ 2013, 'Clinical definitions of melioidosis', *Am J Trop Med Hyg*, vol. 88, no. 3, pp. 411-3.

Cheng, AC, Dasari, P & Currie, BJ 2004, 'Granulocyte colony-stimulating factor and an *in vitro* whole blood model of melioidosis', *Eur J Clin Microbiol Infect Dis*, vol. 23, no. 3, pp. 205-7.

Cheng, AC, Limmathurotsakul, D, Chierakul, W, Getchalarat, N, Wuthiekanun, V, Stephens, DP, Day, NP, White, NJ, Chaowagul, W, Currie, BJ & Peacock, SJ 2007, 'A randomized controlled trial of granulocyte colony-stimulating factor for the treatment of severe sepsis due to melioidosis in Thailand', *Clin Infect Dis*, vol. 45, no. 3, pp. 308-14.

Cheng, AC, Mayo, MJ, Gal, D & Currie, BJ 2003, 'Chlorination and pH of drinking water do not correlate with rates of melioidosis in the Northern Territory, Australia', *Trans R Soc Trop Med Hyg*, vol. 97, no. 5, pp. 511-2.

Cheng, AC, West, TE, Limmathurotsakul, D & Peacock, SJ 2008, 'Strategies to reduce mortality from bacterial sepsis in adults in developing countries', *PLoS Med*, vol. 5, no. 8, p. e175.

Chiang, SH, Bazuine, M, Lumeng, CN, Geletka, LM, Mowers, J, White, NM, Ma, JT, Zhou, J, Qi, N, Westcott, D, Delproposto, JB, Blackwell, TS, Yull, FE & Saltiel, AR 2009, 'The protein kinase IKKepsilon regulates energy balance in obese mice', *Cell*, vol. 138, no. 5, pp. 961-75.

Chin, CY, Monack, DM & Nathan, S 2012, 'Delayed activation of host innate immune pathways in streptozotocin-induced diabetic hosts leads to more severe disease during infection with *Burkholderia pseudomallei*', *Immunology*, vol. 135, no. 4, pp. 312-32.

Choo, HJ, Kim, JH, Kwon, OB, Lee, CS, Mun, JY, Han, SS, Yoon, YS, Yoon, G, Choi, KM & Ko, YG 2006, 'Mitochondria are impaired in the adipocytes of type 2 diabetic mice', *Diabetologia*, vol. 49, no. 4, pp. 784-91.

Chou, DW, Chung, KM, Chen, CH & Cheung, BM 2007, 'Bacteremic melioidosis in southern Taiwan: clinical characteristics and outcome', *J Formos Med Assoc*, vol. 106, no. 12, pp. 1013-22.

Clee, SM, Nadler, ST & Attie, AD 2005, 'Genetic and genomic studies of the BTBR *ob/ob* mouse model of type 2 diabetes', *Am J Ther*, vol. 12, no. 6, pp. 491-8.

Cnop, M, Havel, PJ, Utzschneider, KM, Carr, DB, Sinha, MK, Boyko, EJ, Retzlaff, BM, Knopp, RH, Brunzell, JD & Kahn, SE 2003, 'Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex', *Diabetologia*, vol. 46, no. 4, pp. 459-69.

Colditz, GA, Willett, WC, Rotnitzky, A & Manson, JE 1995, 'Weight gain as a risk factor for clinical diabetes mellitus in women', *Ann Intern Med*, vol. 122, no. 7, pp. 481-6.

Colditz, GA, Willett, WC, Stampfer, MJ, Manson, JE, Hennekens, CH, Arky, RA & Speizer, FE 1990, 'Weight as a risk factor for clinical diabetes in women', *Am J Epidemiol*, vol. 132, no. 3, pp. 501-13.

Coleman, DL 1978, 'Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice', *Diabetologia*, vol. 14, no. 3, pp. 141-8.

Coleman, DL 2010, 'A historical perspective on leptin', *Nat Med*, vol. 16, no. 10, pp. 1097-9.

Coleman, DL & Hummel, KP 1973, 'The influence of genetic background on the expression of the obese (Ob) gene in the mouse', *Diabetologia*, vol. 9, no. 4, pp. 287-93.

Collier, A, Wilson, R, Bradley, H, Thomson, JA & Small, M 1990, 'Free radical activity in type 2 diabetes', *Diabet Med*, vol. 7, no. 1, pp. 27-30.

Colman, E, Toth, MJ, Katzel, LI, Fonong, T, Gardner, AW & Poehlman, ET 1995, 'Body fatness and waist circumference are independent predictors of the age-associated increase in fasting insulin levels in healthy men and women', *Int J Obes Relat Metab Disord*, vol. 19, no. 11, pp. 798-803.

Cooper, A, Williams, NL, Morris, JL, Norton, RE, Ketheesan, N & Schaeffer, PM 2013, 'ELISA and immuno-polymerase chain reaction assays for the sensitive detection of melioidosis', *Diagn Microbiol Infect Dis*, vol. 75, no. 2, pp. 135-8.

Cooper, AM, Dalton, DK, Stewart, TA, Griffin, JP, Russell, DG & Orme, IM 1993, 'Disseminated tuberculosis in interferon gamma gene-disrupted mice', *J Exp Med*, vol. 178, no. 6, pp. 2243-7.

Cottew, GS, Sutherland, AK & Meehan, JF 1952, 'Melioidosis in sheep in Queensland', *The Australian Veterinary Journal*, no. May, pp. 113-23.

Crepaldi, G & Tiengo, A 1976, '[Hyperlipoproteinemia and diabetes]', *Minerva Med*, vol. 67, no. 4, pp. 290-300.

Crotty, JM, Bromwich, AF, Quinn, JV & Brotherton, J 1963, 'Meliodosis in the Northern Territory: a report of two cases.', *Med J Aust*, no. 1, pp. 274-5.

Currie, BJ 2003, 'Melioidosis: an important cause of pneumonia in residents of and travellers returned from endemic regions', *Eur Respir J*, vol. 22, no. 3, pp. 542-50.

Currie, BJ, Chaowagul, W & Cheng, AC 2012, 'Section IV.1 Clinical features of acute melioidosis', in N Ketheesan (ed.), *Melioidosis - A Century of Observation and Research*, First edn, Elsevier B. V., The Netherlands.

Currie, BJ, Dance, DA & Cheng, AC 2008, 'The global distribution of *Burkholderia pseudomallei* and melioidosis: an update', *Trans R Soc Trop Med Hyg*, vol. 102 Suppl 1, pp. S1-4.

Currie, BJ, Fisher, DA, Howard, DM, Burrow, JN, Lo, D, Selva-Nayagam, S, Anstey, NM, Huffam, SE, Snelling, PL, Marks, PJ, Stephens, DP, Lum, GD, Jacups, SP & Krause, VL 2000a, 'Endemic melioidosis in tropical northern Australia: a 10-year prospective study and review of the literature', *Clin Infect Dis*, vol. 31, no. 4, pp. 981-6.

Currie, BJ, Fisher, DA, Howard, DM, Burrow, JNC, Selvanayagam, S, Snelling, PL, Anstey, NM & Mayo, MJ 2000b, 'The epidemiology of melioidosis in Australia and Papua New Guinea', *Acta Trop*, vol. 74, pp. 121-7.

Currie, BJ & Jacups, SP 2003, 'Intensity of rainfall and severity of melioidosis, Australia', *Emerg Infect Dis*, vol. 9, no. 12, pp. 1538-42.

Currie, BJ, Jacups, SP, Cheng, AC, Fisher, DA, Anstey, NM, Huffam, SE & Krause, VL 2004, 'Melioidosis epidemiology and risk factors from a prospective whole-population study in northern Australia', *Trop Med Int Health*, vol. 9, no. 11, pp. 1167-74.

Currie, BJ, Ward, L & Cheng, AC 2010, 'The epidemiology and clinical spectrum of melioidosis: 540 cases from the 20 year darwin prospective study', *PLoS Negl Trop Dis*, vol. 4, no. 11, p. e900.

D'Eon, TM, Souza, SC, Aronovitz, M, Obin, MS, Fried, SK & Greenberg, AS 2005, 'Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways', *J Biol Chem*, vol. 280, no. 43, pp. 35983-91.

Dance, DA 1991, 'Melioidosis: the tip of the iceberg?', *Clin Microbiol Rev*, vol. 4, no. 1, pp. 52-60.

Dance, DA 2012, 'Section II.1 Melioidosis as an emerging disease', in N Ketheesan (ed.), *Melioidosis - A Century of Observation and Research*, First edn, Elsevier B. V., The Netherlands.

Danforth, E, Jr. 2000, 'Failure of adipocyte differentiation causes type II diabetes mellitus?', *Nat Genet*, vol. 26, no. 1, p. 13.

Dannenberg, AM, Jr. & Scott, EM 1958, 'Melioidosis: pathogenesis and immunity in mice and hamsters. I. Studies with virulent strains of Malleomyces pseudomallei', *J Exp Med*, vol. 107, no. 1, pp. 153-66.

Day, C & Bailey, CJ 2011, 'Obesity in the Pathogenesis of Type 2 Diabetes', *British Journal of Diabetes and Vascular Disease*, vol. 11, no. 2, pp. 55-61.

De Flora, S, Cesarone, CF, Balansky, RM, Albini, A, D'Agostini, F, Bennicelli, C, Bagnasco, M, Camoirano, A, Scatolini, L, Rovida, A & et al. 1995, 'Chemopreventive properties and mechanisms of N-Acetylcysteine. The experimental background', *J Cell Biochem Suppl*, vol. 22, pp. 33-41.

De Flora, S, Izzotti, A, D'Agostini, F & Balansky, RM 2001, 'Mechanisms of N-acetylcysteine in the prevention of DNA damage and cancer, with special reference to smoking-related end-points', *Carcinogenesis*, vol. 22, no. 7, pp. 999-1013.

De Mattia, G, Bravi, MC, Laurenti, O, Cassone-Faldetta, M, Armiento, A, Ferri, C & Balsano, F 1998, 'Influence of reduced glutathione infusion on glucose metabolism in patients with non-insulin-dependent diabetes mellitus', *Metabolism*, vol. 47, no. 8, pp. 993-7.

De Mattia, G, Laurenti, O, Bravi, C, Ghiselli, A, Iuliano, L & Balsano, F 1994, 'Effect of aldose reductase inhibition on glutathione redox status in erythrocytes of diabetic patients', *Metabolism*, vol. 43, no. 8, pp. 965-8.

Deeds, MC, Anderson, JM, Armstrong, AS, Gastineau, DA, Hiddinga, HJ, Jahangir, A, Eberhardt, NL & Kudva, YC 2011, 'Single dose streptozotocin-induced diabetes: considerations for study design in islet transplantation models', *Lab Anim*, vol. 45, no. 3, pp. 131-40.

Defronzo, RA 1979, 'Glucose intolerance and aging: evidence for tissue insensitivity to insulin', *Diabetes*, vol. 28, no. 12, pp. 1095-101.

Dejsirilert, S, Kondo, E, Chiewsilp, D & Kanai, K 1991, 'Growth and survival of *Pseudomonas pseudomallei* in acidic environments', *Jpn J Med Sci Biol*, vol. 44, no. 2, pp. 63-74.

Del Prato, S 2009, 'Role of glucotoxicity and lipotoxicity in the pathophysiology of Type 2 diabetes mellitus and emerging treatment strategies', *Diabet Med*, vol. 26, no. 12, pp. 1185-92.

Delamaire, M, Maugendre, D, Moreno, M, Le Goff, MC, Allannic, H & Genetet, B 1997, 'Impaired leucocyte functions in diabetic patients', *Diabet Med*, vol. 14, no. 1, pp. 29-34.

Desfaits, AC, Serri, O & Renier, G 1998, 'Normalization of plasma lipid peroxides, monocyte adhesion, and tumor necrosis factor-alpha production in NIDDM patients after gliclazide treatment', *Diabetes Care*, vol. 21, no. 4, pp. 487-93.

DeShazer, D, Brett, PJ, Carlyon, R & Woods, DE 1997, 'Mutagenesis of *Burkholderia pseudomallei* with Tn5-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene', *J Bacteriol*, vol. 179, no. 7, pp. 2116-25.

DeShazer, D, Brett, PJ & Woods, DE 1998, 'The type II O-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence', *Mol Microbiol*, vol. 30, no. 5, pp. 1081-100.

Despres, JP 2001, 'Health consequences of visceral obesity', *Ann Med*, vol. 33, no. 8, pp. 534-41.

Despres, JP & Lemieux, I 2006, 'Abdominal obesity and metabolic syndrome', *Nature*, vol. 444, no. 7121, pp. 881-7.

Diabetes Research Resources 2013, The Jackson Laboratory, viewed 10 October 2013, http://jaxmice.jax.org/diabetes/comparison.html%3E.

Diamond, MP, Thornton, K, Connolly-Diamond, M, Sherwin, RS & DeFronzo, RA 1995, 'Reciprocal variations in insulin-stimulated glucose uptake and pancreatic insulin secretion in women with normal glucose tolerance', *J Soc Gynecol Investig*, vol. 2, no. 5, pp. 708-15.

Dixit, VD, Mielenz, M, Taub, DD & Parvizi, N 2003, 'Leptin induces growth hormone secretion from peripheral blood mononuclear cells via a protein kinase C- and nitric oxide-dependent mechanism', *Endocrinology*, vol. 144, no. 12, pp. 5595-603.

Dobashi, K, Aihara, M, Araki, T, Shimizu, Y, Utsugi, M, Iizuka, K, Murata, Y, Hamuro, J, Nakazawa, T & Mori, M 2001, 'Regulation of LPS induced IL-12 production by IFN-gamma and IL-4 through intracellular glutathione status in human alveolar macrophages', *Clin Exp Immunol*, vol. 124, no. 2, pp. 290-6.

Dooley, KE & Chaisson, RE 2009, 'Tuberculosis and diabetes mellitus: convergence of two epidemics', *Lancet Infect Dis*, vol. 9, no. 12, pp. 737-46.

Douglas, MW, Lum, G, Roy, J, Fisher, DA, Anstey, NM & Currie, BJ 2004, 'Epidemiology of community-acquired and nosocomial bloodstream infections in tropical Australia: a 12-month prospective study', *Trop Med Int Health*, vol. 9, no. 7, pp. 795-804.

Drivsholm, T, Ibsen, H, Schroll, M, Davidsen, M & Borch-Johnsen, K 2001, 'Increasing prevalence of diabetes mellitus and impaired glucose tolerance among 60-year-old Danes', *Diabet Med*, vol. 18, no. 2, pp. 126-32.

Du, XL, Edelstein, D, Rossetti, L, Fantus, IG, Goldberg, H, Ziyadeh, F, Wu, J & Brownlee, M 2000, 'Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation', *Proc Natl Acad Sci U S A*, vol. 97, no. 22, pp. 12222-6.

Dyck, DJ, Heigenhauser, GJ & Bruce, CR 2006, 'The role of adipokines as regulators of skeletal muscle fatty acid metabolism and insulin sensitivity', *Acta Physiol (Oxf)*, vol. 186, no. 1, pp. 5-16.

Easton, A, Haque, A, Chu, K, Lukaszewski, R & Bancroft, GJ 2007, 'A critical role for neutrophils in resistance to experimental infection with *Burkholderia pseudomallei*', *J Infect Dis*, vol. 195, no. 1, pp. 99-107.

Eckardt, K, Sell, H & Eckel, J 2008, 'Novel aspects of adipocyte-induced skeletal muscle insulin resistance', *Arch Physiol Biochem*, vol. 114, no. 4, pp. 287-98.

Eckel, LA 2011, 'The ovarian hormone estradiol plays a crucial role in the control of food intake in females', *Physiol Behav*, vol. 104, no. 4, pp. 517-24.

Ekchariyawat, P, Pudla, S, Limposuwan, K, Arjcharoen, S, Sirisinha, S & Utaisincharoen, P 2005, '*Burkholderia pseudomallei*-induced expression of suppressor of cytokine signaling 3 and cytokine-inducible src homology 2-containing protein in mouse macrophages: a possible mechanism for suppression of the response to gamma interferon stimulation', *Infect Immun*, vol. 73, no. 11, pp. 7332-9.

Eknoyan, G & Nagy, J 2005, 'A history of diabetes mellitus or how a disease of the kidneys evolved into a kidney disease', *Adv Chronic Kidney Dis*, vol. 12, no. 2, pp. 223-9.

Engel, RH & Evens, AM 2006, 'Oxidative stress and apoptosis: a new treatment paradigm in cancer', *Front Biosci*, vol. 11, pp. 300-12.

Esper, AM, Moss, M & Martin, GS 2009, 'The effect of diabetes mellitus on organ dysfunction with sepsis: an epidemiological study', *Crit Care*, vol. 13, no. 1, p. R18.

Essex-Lopresti, AE, Boddey, JA, Thomas, R, Smith, MP, Hartley, MG, Atkins, T, Brown, NF, Tsang, CH, Peak, IR, Hill, J, Beacham, IR & Titball, RW 2005, 'A type IV pilin, PilA, Contributes To Adherence of *Burkholderia pseudomallei* and virulence *in vivo*', *Infect Immun*, vol. 73, no. 2, pp. 1260-4.

Estrela, JM, Ortega, A & Obrador, E 2006, 'Glutathione in cancer biology and therapy', *Crit Rev Clin Lab Sci*, vol. 43, no. 2, pp. 143-81.

Faa, AG & Holt, PJ 2002, 'Melioidosis in the Torres Strait islands of far North Queensland', *Commun Dis Intell*, vol. 26, no. 2, pp. 279-83.

FAO 2002, *World agriculture: towards 2015/2030. Summary report*, Food and Agriculture Organization of the United Nations, Rome.

Feinstein, R, Kanety, H, Papa, MZ, Lunenfeld, B & Karasik, A 1993, 'Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates', *J Biol Chem*, vol. 268, no. 35, pp. 26055-8.

Fernandez-Fernandez, R, Martini, AC, Navarro, VM, Castellano, JM, Dieguez, C, Aguilar, E, Pinilla, L & Tena-Sempere, M 2006, 'Novel signals for the integration of energy balance and reproduction', *Mol Cell Endocrinol*, vol. 254-255, pp. 127-32.

Feskens, EJ, Virtanen, SM, Rasanen, L, Tuomilehto, J, Stengard, J, Pekkanen, J, Nissinen, A & Kromhout, D 1995, 'Dietary factors determining diabetes and impaired glucose tolerance. A 20-year follow-up of the Finnish and Dutch cohorts of the Seven Countries Study', *Diabetes Care*, vol. 18, no. 8, pp. 1104-12.

Fickova, M, Hubert, P, Klimes, I, Staedel, C, Cremel, G, Bohov, P & Macho, L 1994, 'Dietary fish oil and olive oil improve the liver insulin receptor tyrosine kinase activity in high sucrose fed rats', *Endocr Regul*, vol. 28, no. 4, pp. 187-97.

Field, AE, Coakley, EH, Must, A, Spadano, JL, Laird, N, Dietz, WH, Rimm, E & Colditz, GA 2001, 'Impact of overweight on the risk of developing common chronic diseases during a 10-year period', *Arch Intern Med*, vol. 161, no. 13, pp. 1581-6.

Filkins, JP & Cornell, RP 1974, 'Depression of hepatic gluconeogenesis and the hypoglycemia of endotoxin shock', *Am J Physiol*, vol. 227, no. 4, pp. 778-81.

Flynn, JL, Chan, J, Triebold, KJ, Dalton, DK, Stewart, TA & Bloom, BR 1993, 'An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection', *J Exp Med*, vol. 178, no. 6, pp. 2249-54.

Forbes, JM & Cooper, ME 2013, 'Mechanisms of diabetic complications', *Physiol Rev*, vol. 93, no. 1, pp. 137-88.

Ford, ES, Giles, WH & Mokdad, AH 2004, 'Increasing prevalence of the metabolic syndrome among U.S. Adults', *Diabetes Care*, vol. 27, no. 10, pp. 2444-9.

Ford, ES, Williamson, DF & Liu, S 1997, 'Weight change and diabetes incidence: findings from a national cohort of US adults', *Am J Epidemiol*, vol. 146, no. 3, pp. 214-22.

Franconi, F, Seghieri, G, Canu, S, Straface, E, Campesi, I & Malorni, W 2008, 'Are the available experimental models of type 2 diabetes appropriate for a gender perspective?', *Pharmacol Res*, vol. 57, no. 1, pp. 6-18.

French, CT, Toesca, IJ, Wu, TH, Teslaa, T, Beaty, SM, Wong, W, Liu, M, Schroder, I, Chiou, PY, Teitell, MA & Miller, JF 2011, 'Dissection of the *Burkholderia* intracellular life cycle using a photothermal nanoblade', *Proc Natl Acad Sci U S A*, vol. 108, no. 29, pp. 12095-100.

Fruebis, J, Tsao, TS, Javorschi, S, Ebbets-Reed, D, Erickson, MR, Yen, FT, Bihain, BE & Lodish, HF 2001, 'Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice', *Proc Natl Acad Sci U S A*, vol. 98, no. 4, pp. 2005-10.

Fukuhara, A, Matsuda, M, Nishizawa, M, Segawa, K, Tanaka, M, Kishimoto, K, Matsuki, Y, Murakami, M, Ichisaka, T, Murakami, H, Watanabe, E, Takagi, T, Akiyoshi, M, Ohtsubo, T, Kihara, S, Yamashita, S, Makishima, M, Funahashi, T, Yamanaka, S, Hiramatsu, R, Matsuzawa, Y & Shimomura, I 2005, 'Visfatin: a protein secreted by visceral fat that mimics the effects of insulin', *Science*, vol. 307, no. 5708, pp. 426-30.

Fukushima, M, Suzuki, H & Seino, Y 2004, 'Insulin secretion capacity in the development from normal glucose tolerance to type 2 diabetes', *Diabetes Res Clin Pract*, vol. 66 Suppl 1, pp. S37-43.

Furuhashi, M, Fucho, R, Gorgun, CZ, Tuncman, G, Cao, H & Hotamisligil, GS 2008, 'Adipocyte/macrophage fatty acid-binding proteins contribute to metabolic deterioration through actions in both macrophages and adipocytes in mice', *J Clin Invest*, vol. 118, no. 7, pp. 2640-50.

Gallou-Kabani, C, Vige, A, Gross, MS, Rabes, JP, Boileau, C, Larue-Achagiotis, C, Tome, D, Jais, JP & Junien, C 2007, 'C57BL/6J and A/J mice fed a high-fat diet delineate components of metabolic syndrome', *Obesity (Silver Spring)*, vol. 15, no. 8, pp. 1996-2005.

Gamaley, I, Efremova, T, Kirpichnikova, K, Kever, L, Komissarchik, Y, Polozov, Y & Khaitlina, S 2006, 'N-acetylcysteine-induced changes in susceptibility of transformed eukaryotic cells to bacterial invasion', *Cell Biol Int*, vol. 30, no. 4, pp. 319-25.

Gan, YH 2005, 'Interaction between *Burkholderia pseudomallei* and the host immune response: sleeping with the enemy?', *J Infect Dis*, vol. 192, no. 10, pp. 1845-50.

Gan, YH, Chua, KL, Chua, HH, Liu, B, Hii, CS, Chong, HL & Tan, P 2002, 'Characterization of *Burkholderia pseudomallei* infection and identification of novel

virulence factors using a *Caenorhabditis elegans* host system', *Mol Microbiol*, vol. 44, no. 5, pp. 1185-97.

Gao, H, Liang, M, Bergdahl, A, Hamren, A, Lindholm, MW, Dahlman-Wright, K & Nilsson, BO 2006, 'Estrogen attenuates vascular expression of inflammation associated genes and adhesion of monocytes to endothelial cells', *Inflamm Res*, vol. 55, no. 8, pp. 349-53.

Garozzo, A, Tempera, G, Ungheri, D, Timpanaro, R & Castro, A 2007, 'N-acetylcysteine synergizes with oseltamivir in protecting mice from lethal influenza infection', *Int J Immunopathol Pharmacol*, vol. 20, no. 2, pp. 349-54.

Garris, DR & Garris, BL 2004, 'Cytochemical analysis of pancreatic islet hypercytolipidemia following diabetes (*db/db*) and obese (*ob/ob*) mutation expression: influence of genomic background', *Pathobiology*, vol. 71, no. 5, pp. 231-40.

Gauthier, YP, Hagen, RM, Brochier, GS, Neubauer, H, Splettstoesser, WD, Finke, EJ & Vidal, DR 2001, 'Study on the pathophysiology of experimental *Burkholderia pseudomallei* infection in mice', *FEMS Immunol Med Microbiol*, vol. 30, no. 1, pp. 53-63.

Geerlings, SE & Hoepelman, AI 1999, 'Immune dysfunction in patients with diabetes mellitus (DM)', *FEMS Immunol Med Microbiol*, vol. 26, no. 3-4, pp. 259-65.

Gehrmann, W, Elsner, M & Lenzen, S 2010, 'Role of metabolically generated reactive oxygen species for lipotoxicity in pancreatic beta-cells', *Diabetes Obes Metab*, vol. 12 Suppl 2, pp. 149-58.

Geiler, J, Michaelis, M, Naczk, P, Leutz, A, Langer, K, Doerr, HW & Cinatl, J, Jr. 2010, 'N-acetyl-L-cysteine (NAC) inhibits virus replication and expression of pro-inflammatory molecules in A549 cells infected with highly pathogenic H5N1 influenza A virus', *Biochem Pharmacol*, vol. 79, no. 3, pp. 413-20.

Gerard-Monnier, D & Chaudiere, J 1996, '[Metabolism and antioxidant function of glutathione]', *Pathol Biol (Paris)*, vol. 44, no. 1, pp. 77-85.

Ghiselli, A, Laurenti, O, De Mattia, G, Maiani, G & Ferro-Luzzi, A 1992, 'Salicylate hydroxylation as an early marker of in vivo oxidative stress in diabetic patients', *Free Radic Biol Med*, vol. 13, no. 6, pp. 621-6.

Ghosh, S & Baltimore, D 1990, 'Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B', *Nature*, vol. 344, no. 6267, pp. 678-82.

Giacco, F & Brownlee, M 2010, 'Oxidative stress and diabetic complications', *Circ Res*, vol. 107, no. 9, pp. 1058-70.

Gibney, KB, Cheng, AC & Currie, BJ 2008, 'Cutaneous melioidosis in the tropical top end of Australia: a prospective study and review of the literature', *Clin Infect Dis*, vol. 47, no. 5, pp. 603-9.

Gilbert, ER, Fu, Z & Liu, D 2011, 'Development of a nongenetic mouse model of type 2 diabetes', *Exp Diabetes Res*, vol. 2011, p. 416254.

Gillespie, SH 2002, 'Evolution of drug resistance in *Mycobacterium tuberculosis*: clinical and molecular perspective', *Antimicrob Agents Chemother*, vol. 46, no. 2, pp. 267-74.

Gilmore, G, Barnes, J, Ketheesan, N & Norton, R 2007, 'Use of antigens derived from *Burkholderia pseudomallei*, *B. thailandensis*, and *B. cepacia* in the indirect hemagglutination assay for melioidosis', *Clin Vaccine Immunol*, vol. 14, no. 11, pp. 1529-31.

Ginsberg, H, Olefsky, JM & Reaven, GM 1974, 'Further evidence that insulin resistance exists in patients with chemical diabetes', *Diabetes*, vol. 23, no. 8, pp. 674-8.

Ginsberg, HN & Huang, LS 2000, 'The insulin resistance syndrome: impact on lipoprotein metabolism and atherothrombosis', *J Cardiovasc Risk*, vol. 7, no. 5, pp. 325-31.

Gnacinska, M, Malgorzewicz, S, Stojek, M, Lysiak-Szydlowska, W & Sworczak, K 2009, 'Role of adipokines in complications related to obesity: a review', *Adv Med Sci*, vol. 54, no. 2, pp. 150-7.

Godoy, D, Randle, G, Simpson, AJ, Aanensen, DM, Pitt, TL, Kinoshita, R & Spratt, BG 2003, 'Multilocus sequence typing and evolutionary relationships among the causative agents of melioidosis and glanders, *Burkholderia pseudomallei* and *Burkholderia mallei*', *J Clin Microbiol*, vol. 41, no. 5, pp. 2068-79.

Goldin, A, Beckman, JA, Schmidt, AM & Creager, MA 2006, 'Advanced glycation end products: sparking the development of diabetic vascular injury', *Circulation*, vol. 114, no. 6, pp. 597-605.

Goodyear, A, Kellihan, L, Bielefeldt-Ohmann, H, Troyer, R, Propst, K & Dow, S 2009, 'Protection from pneumonic infection with burkholderia species by inhalational immunotherapy', *Infect Immun*, vol. 77, no. 4, pp. 1579-88.

Gottschlich, MM, Mayes, T, Khoury, JC & Warden, GD 1993, 'Significance of obesity on nutritional, immunologic, hormonal, and clinical outcome parameters in burns', *J Am Diet Assoc*, vol. 93, no. 11, pp. 1261-8.

Gracey, M 1995, 'New World syndrome in Western Australian aborigines', *Clin Exp Pharmacol Physiol*, vol. 22, no. 3, pp. 220-5.

Green, RM, Seth, A & Connell, ND 2000, 'A peptide permease mutant of *Mycobacterium bovis* BCG resistant to the toxic peptides glutathione and S-nitrosoglutathione', *Infect Immun*, vol. 68, no. 2, pp. 429-36.

Greene, DA, Stevens, MJ & Feldman, EL 1999, 'Diabetic neuropathy: scope of the syndrome', *Am J Med*, vol. 107, no. 2B, pp. 2S-8S.

Griffin, ME, Marcucci, MJ, Cline, GW, Bell, K, Barucci, N, Lee, D, Goodyear, LJ, Kraegen, EW, White, MF & Shulman, GI 1999, 'Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade', *Diabetes*, vol. 48, no. 6, pp. 1270-4.

Griffith, OW 1999, 'Biologic and pharmacologic regulation of mammalian glutathione synthesis', *Free Radic Biol Med*, vol. 27, no. 9-10, pp. 922-35.

Grimble, RF 2002, 'Inflammatory status and insulin resistance', *Curr Opin Clin Nutr Metab Care*, vol. 5, no. 5, pp. 551-9.

Guard, RW, Khafagi, FA, Brigden, MC & Ashdown, LR 1984, 'Melioidosis in Far North Queensland. A clinical and epidemiological review of twenty cases', *Am J Trop Med Hyg*, vol. 33, no. 3, pp. 467-73.

Guerra, C, Morris, D, Sipin, A, Kung, S, Franklin, M, Gray, D, Tanzil, M, Guilford, F, Khasawneh, FT & Venketaraman, V 2011, 'Glutathione and adaptive immune responses against *Mycobacterium tuberculosis* infection in healthy and HIV infected individuals', *PLoS One*, vol. 6, no. 12, p. e28378.

Guevara, AG, Guilvard, E, Borges, MM, Cordeiro da Silva, A & Ouaissi, A 2000, 'N-Acetylcysteine and glutathione modulate the behaviour of *Trypanosoma cruzi* experimental infection', *Immunol Lett*, vol. 71, no. 2, pp. 79-83.

Gupta, S, Koirala, J, Khardori, R & Khardori, N 2007, 'Infections in diabetes mellitus and hyperglycemia', *Infect Dis Clin North Am*, vol. 21, no. 3, pp. 617-38, vii.

Hao, L, Wang, Y, Duan, Y & Bu, S 2010, 'Effects of treadmill exercise training on liver fat accumulation and estrogen receptor alpha expression in intact and ovariectomized rats with or without estrogen replacement treatment', *Eur J Appl Physiol*, vol. 109, no. 5, pp. 879-86.

Haque, A, Chu, K, Easton, A, Stevens, MP, Galyov, EE, Atkins, T, Titball, R & Bancroft, GJ 2006a, 'A live experimental vaccine against *Burkholderia pseudomallei* elicits CD4+ T cell-mediated immunity, priming T cells specific for 2 type III secretion system proteins', *J Infect Dis*, vol. 194, no. 9, pp. 1241-8.

Haque, A, Easton, A, Smith, D, O'Garra, A, Van Rooijen, N, Lertmemongkolchai, G, Titball, RW & Bancroft, GJ 2006b, 'Role of T cells in innate and adaptive immunity against murine *Burkholderia pseudomallei* infection', *J Infect Dis*, vol. 193, no. 3, pp. 370-9.

Harrington, G, Russo, P, Spelman, D, Borrell, S, Watson, K, Barr, W, Martin, R, Edmonds, D, Cocks, J, Greenbough, J, Lowe, J, Randle, L, Castell, J, Browne, E, Bellis, K & Aberline, M 2004, 'Surgical-site infection rates and risk factor analysis in coronary artery bypass graft surgery', *Infect Control Hosp Epidemiol*, vol. 25, no. 6, pp. 472-6.

Harris, P, Engler, C & Norton, R 2011, 'Comparative *in vitro* susceptibility of *Burkholderia pseudomallei* to doripenem, ertapenem, tigecycline and moxifloxacin', *Int J Antimicrob Agents*, vol. 37, no. 6, pp. 547-9.

Harris, PN, Ketheesan, N, Owens, L & Norton, RE 2009, 'Clinical features that affect indirect-hemagglutination-assay responses to *Burkholderia pseudomallei*', *Clin Vaccine Immunol*, vol. 16, no. 6, pp. 924-30.

Harris, PN, Williams, NL, Morris, JL, Ketheesan, N & Norton, RE 2011, 'Evidence of *Burkholderia pseudomallei*-specific immunity in patient sera persistently nonreactive by the indirect hemagglutination assay', *Clin Vaccine Immunol*, vol. 18, no. 8, pp. 1288-91.

Hassan, MR, Pani, SP, Peng, NP, Voralu, K, Vijayalakshmi, N, Mehanderkar, R, Aziz, NA & Michael, E 2010, 'Incidence, risk factors and clinical epidemiology of melioidosis: a complex socio-ecological emerging infectious disease in the Alor Setar region of Kedah, Malaysia', *BMC Infect Dis*, vol. 10, p. 302.

Hayes, JD & McLellan, LI 1999, 'Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress', *Free Radic Res*, vol. 31, no. 4, pp. 273-300.

He, Q, Horlick, M, Thornton, J, Wang, J, Pierson, RN, Jr., Heshka, S & Gallagher, D 2002, 'Sex and race differences in fat distribution among Asian, African-American, and Caucasian prepubertal children', *J Clin Endocrinol Metab*, vol. 87, no. 5, pp. 2164-70.

Healey, GD, Elvin, SJ, Morton, M & Williamson, ED 2005, 'Humoral and cell-mediated adaptive immune responses are required for protection against *Burkholderia pseudomallei* challenge and bacterial clearance postinfection', *Infect Immun*, vol. 73, no. 9, pp. 5945-51.

Heine, PA, Taylor, JA, Iwamoto, GA, Lubahn, DB & Cooke, PS 2000, 'Increased adipose tissue in male and female estrogen receptor-alpha knockout mice', *Proc Natl Acad Sci U S A*, vol. 97, no. 23, pp. 12729-34.

Hentze, MW, Rouault, TA, Harford, JB & Klausner, RD 1989, 'Oxidation-reduction and the molecular mechanism of a regulatory RNA-protein interaction', *Science*, vol. 244, no. 4902, pp. 357-9.

Heymsfield, SB, Segal, KR, Hauptman, J, Lucas, CP, Boldrin, MN, Rissanen, A, Wilding, JP & Sjostrom, L 2000, 'Effects of weight loss with orlistat on glucose tolerance and progression to type 2 diabetes in obese adults', *Arch Intern Med*, vol. 160, no. 9, pp. 1321-6.

Himsworth, HP 1936, 'Diabetes mellitus: its differentiation into insulin sensitive and insulin insensitive types', *Lancet*, no. 1, pp. 127-30.

Ho, M, Schollaardt, T, Smith, MD, Perry, MB, Brett, PJ, Chaowagul, W & Bryan, LE 1997, 'Specificity and functional activity of anti-*Burkholderia pseudomallei* polysaccharide antibodies', *Infect Immun*, vol. 65, no. 9, pp. 3648-53.

Hodgson, KA, Morris, JL, Feterl, ML, Govan, BL & Ketheesan, N 2011, 'Altered macrophage function is associated with severe *Burkholderia pseudomallei* infection in a murine model of type 2 diabetes', *Microbes Infect*, vol. 13, no. 14-15, pp. 1177-84.

Holden, MT, Titball, RW, Peacock, SJ, Cerdeno-Tarraga, AM, Atkins, T, Crossman, LC, Pitt, T, Churcher, C, Mungall, K, Bentley, SD, Sebaihia, M, Thomson, NR, Bason, N, Beacham, IR, Brooks, K, Brown, KA, Brown, NF, Challis, GL, Cherevach, I, Chillingworth, T, Cronin, A, Crossett, B, Davis, P, DeShazer, D, Feltwell, T, Fraser, A, Hance, Z, Hauser, H, Holroyd, S, Jagels, K, Keith, KE, Maddison, M, Moule, S, Price, C, Quail, MA, Rabbinowitsch, E, Rutherford, K, Sanders, M, Simmonds, M, Songsivilai, S, Stevens, K, Tumapa, S, Vesaratchavest, M, Whitehead, S, Yeats, C, Barrell, BG, Oyston, PC & Parkhill, J 2004, 'Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*', *Proc Natl Acad Sci U S A*, vol. 101, no. 39, pp. 14240-5.

Homma, H, Kurachi, H, Nishio, Y, Takeda, T, Yamamoto, T, Adachi, K, Morishige, K, Ohmichi, M, Matsuzawa, Y & Murata, Y 2000, 'Estrogen suppresses transcription of lipoprotein lipase gene. Existence of a unique estrogen response element on the lipoprotein lipase promoter', *J Biol Chem*, vol. 275, no. 15, pp. 11404-11.

Hoppe, I, Brenneke, B, Rohde, M, Kreft, A, Haussler, S, Reganzerowski, A & Steinmetz, I 1999, 'Characterization of a murine model of melioidosis: comparison of different strains of mice', *Infect Immun*, vol. 67, no. 6, pp. 2891-900.

Horton, RE, Grant, GD, Matthews, B, Batzloff, M, Owen, SJ, Kyan, S, Flegg, CP, Clark, AM, Ulett, GC, Morrison, N, Peak, IR & Beacham, IR 2013, 'Quorum sensing negatively regulates multinucleate cell formation during intracellular growth of *Burkholderia pseudomallei* in macrophage-like cells', *PLoS One*, vol. 8, no. 5, p. e63394.

Hosogai, N, Fukuhara, A, Oshima, K, Miyata, Y, Tanaka, S, Segawa, K, Furukawa, S, Tochino, Y, Komuro, R, Matsuda, M & Shimomura, I 2007, 'Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation', *Diabetes*, vol. 56, no. 4, pp. 901-11.

Hotamisligil, GS, Shargill, NS & Spiegelman, BM 1993, 'Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance', *Science*, vol. 259, no. 5091, pp. 87-91.

Hu, FB, Manson, JE, Stampfer, MJ, Colditz, G, Liu, S, Solomon, CG & Willett, WC 2001, 'Diet, lifestyle, and the risk of type 2 diabetes mellitus in women', *N Engl J Med*, vol. 345, no. 11, pp. 790-7.

Hu, FB, van Dam, RM & Liu, S 2001, 'Diet and risk of Type II diabetes: the role of types of fat and carbohydrate', *Diabetologia*, vol. 44, no. 7, pp. 805-17.

Hull, RL, Kodama, K, Utzschneider, KM, Carr, DB, Prigeon, RL & Kahn, SE 2005, 'Dietary-fat-induced obesity in mice results in beta cell hyperplasia but not increased insulin release: evidence for specificity of impaired beta cell adaptation', *Diabetologia*, vol. 48, no. 7, pp. 1350-8.

Hummel, KP, Dickie, MM & Coleman, DL 1966, 'Diabetes, a new mutation in the mouse', *Science*, vol. 153, no. 3740, pp. 1127-8.

Hunter, EA & Grimble, RF 1997, 'Dietary sulphur amino acid adequacy influences glutathione synthesis and glutathione-dependent enzymes during the inflammatory response to endotoxin and tumour necrosis factor-alpha in rats', *Clin Sci (Lond)*, vol. 92, no. 3, pp. 297-305.

Huxley, R, James, WP, Barzi, F, Patel, JV, Lear, SA, Suriyawongpaisal, P, Janus, E, Caterson, I, Zimmet, P, Prabhakaran, D, Reddy, S & Woodward, M 2008, 'Ethnic comparisons of the cross-sectional relationships between measures of body size with diabetes and hypertension', *Obes Rev*, vol. 9 Suppl 1, pp. 53-61.

Hwang, C, Sinskey, AJ & Lodish, HF 1992, 'Oxidized redox state of glutathione in the endoplasmic reticulum', *Science*, vol. 257, no. 5076, pp. 1496-502.

IDF 2011, *IDF Diabetes Atlas, 5th edn.*, International Diabetes Federation, Brussels, Belgium.

Imaeda, A, Kaneko, T, Aoki, T, Kondo, Y & Nagase, H 2002, 'DNA damage and the effect of antioxidants in streptozotocin-treated mice', *Food Chem Toxicol*, vol. 40, no. 7, pp. 979-87.

Inadera, H 2013, 'Developmental origins of obesity and type 2 diabetes: molecular aspects and role of chemicals', *Environ Health Prev Med*, vol. 18, no. 3, pp. 185-97.

Inglis, TJ, Merritt, A, Chidlow, G, Aravena-Roman, M & Harnett, G 2005, 'Comparison of diagnostic laboratory methods for identification of *Burkholderia pseudomallei*', *J Clin Microbiol*, vol. 43, no. 5, pp. 2201-6.

Inglis, TJ & Sousa, AQ 2009, 'The public health implications of melioidosis', *Braz J Infect Dis*, vol. 13, no. 1, pp. 59-66.

Itani, SI, Ruderman, NB, Schmieder, F & Boden, G 2002, 'Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha', *Diabetes*, vol. 51, no. 7, pp. 2005-11.

Jain, SK & McVie, R 1994, 'Effect of glycemic control, race (white versus black), and duration of diabetes on reduced glutathione content in erythrocytes of diabetic patients', *Metabolism*, vol. 43, no. 3, pp. 306-9.

Jeddeloh, JA, Fritz, DL, Waag, DM, Hartings, JM & Andrews, GP 2003, 'Biodefense-driven murine model of pneumonic melioidosis', *Infect Immun*, vol. 71, no. 1, pp. 584-7.

Jenkins, DJ, Wolever, TM, Taylor, RH, Barker, H, Fielden, H, Baldwin, JM, Bowling, AC, Newman, HC, Jenkins, AL & Goff, DV 1981, 'Glycemic index of foods: a physiological basis for carbohydrate exchange', *Am J Clin Nutr*, vol. 34, no. 3, pp. 362-6.

Jennings, PE, Chirico, S, Jones, AF, Lunec, J & Barnett, AH 1987a, 'Vitamin C metabolites and microangiopathy in diabetes mellitus', *Diabetes Res*, vol. 6, no. 3, pp. 151-4.

Jennings, PE, Jones, AF, Florkowski, CM, Lunec, J & Barnett, AH 1987b, 'Increased diene conjugates in diabetic subjects with microangiopathy', *Diabet Med*, vol. 4, no. 5, pp. 452-6.

Joshi, N, Caputo, GM, Weitekamp, MR & Karchmer, AW 1999, 'Infections in patients with diabetes mellitus', *N Engl J Med*, vol. 341, no. 25, pp. 1906-12.

Jubber, AS 2004, 'Respiratory complications of obesity', *Int J Clin Pract*, vol. 58, no. 6, pp. 573-80.

Kahn, SE 2001, 'Clinical review 135: The importance of beta-cell failure in the development and progression of type 2 diabetes', *J Clin Endocrinol Metab*, vol. 86, no. 9, pp. 4047-58.

Kanasaki, K & Koya, D 2011, 'Biology of obesity: lessons from animal models of obesity', *J Biomed Biotechnol*, vol. 2011, p. 197636.

Kanety, H, Feinstein, R, Papa, MZ, Hemi, R & Karasik, A 1995, 'Tumor necrosis factor alpha-induced phosphorylation of insulin receptor substrate-1 (IRS-1). Possible mechanism for suppression of insulin-stimulated tyrosine phosphorylation of IRS-1', *J Biol Chem*, vol. 270, no. 40, pp. 23780-4.

Karasawa, H, Nagata-Goto, S, Takaishi, K & Kumagae, Y 2009, 'A novel model of type 2 diabetes mellitus based on obesity induced by high-fat diet in BDF1 mice', *Metabolism*, vol. 58, no. 3, pp. 296-303.

Kato, C, Mikami, M & Natsuno, T 2008, 'Participation of glutathione in the elimination of *Porphyromonas gingivalis in vivo*', *Oral Microbiol Immunol*, vol. 23, no. 6, pp. 441-8.

Kaukonen, KM, Bailey, M, Suzuki, S, Pilcher, D & Bellomo, R 2014, 'Mortality related to severe sepsis and septic shock among critically ill patients in Australia and New Zealand, 2000-2012', *JAMA*, vol. 311, no. 13, pp. 1308-16.

Kazumi, T, Yoshino, G, Fujii, S & Baba, S 1978, 'Tumorigenic action of streptozotocin on the pancreas and kidney in male Wistar rats', *Cancer Res*, vol. 38, no. 7, pp. 2144-7.

Kellerer, M, Lammers, R, Fritsche, A, Strack, V, Machicao, F, Borboni, P, Ullrich, A & Haring, HU 2001, 'Insulin inhibits leptin receptor signalling in HEK293 cells at the level of janus kinase-2: a potential mechanism for hyperinsulinaemia-associated leptin resistance', *Diabetologia*, vol. 44, no. 9, pp. 1125-32.

Kespichayawattana, W, Rattanachetkul, S, Wanun, T, Utaisincharoen, P & Sirisinha, S 2000, '*Burkholderia pseudomallei* induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading', *Infect Immun*, vol. 68, no. 9, pp. 5377-84.

Ketheesan, N, Barnes, JL, Ulett, GC, VanGessel, HJ, Norton, RE, Hirst, RG & LaBrooy, JT 2002, 'Demonstration of a cell-mediated immune response in melioidosis', *J Infect Dis*, vol. 186, no. 2, pp. 286-9.

Khovidhunkit, W, Kim, MS, Memon, RA, Shigenaga, JK, Moser, AH, Feingold, KR & Grunfeld, C 2004, 'Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host', *J Lipid Res*, vol. 45, no. 7, pp. 1169-96.

Khwannimit, B & Bhurayanontachai, R 2009, 'The epidemiology of, and risk factors for, mortality from severe sepsis and septic shock in a tertiary-care university hospital setting', *Epidemiol Infect*, vol. 137, no. 9, pp. 1333-41.

King, GL, McNeely, MJ, Thorpe, LE, Mau, ML, Ko, J, Liu, LL, Sun, A, Hsu, WC & Chow, EA 2012, 'Understanding and addressing unique needs of diabetes in Asian Americans, native Hawaiians, and Pacific Islanders', *Diabetes Care*, vol. 35, no. 5, pp. 1181-8.

King, H, Aubert, RE & Herman, WH 1998, 'Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections', *Diabetes Care*, vol. 21, no. 9, pp. 1414-31.

Kinjo, Y, Kawakami, K, Uezu, K, Yara, S, Miyagi, K, Koguchi, Y, Hoshino, T, Okamoto, M, Kawase, Y, Yokota, K, Yoshino, K, Takeda, K, Akira, S & Saito, A 2002, 'Contribution of IL-18 to Th1 response and host defense against infection by *Mycobacterium tuberculosis*: a comparative study with IL-12p40', *J Immunol*, vol. 169, no. 1, pp. 323-9.

Kissebah, AH & Krakower, GR 1994, 'Regional adiposity and morbidity', *Physiol Rev*, vol. 74, no. 4, pp. 761-811.

Knowler, WC, Barrett-Connor, E, Fowler, SE, Hamman, RF, Lachin, JM, Walker, EA, Nathan, DM & Diabetes Prevention Program Research, G 2002, 'Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin', *N Engl J Med*, vol. 346, no. 6, pp. 393-403.

Koh, GC, Maude, RR, Schreiber, MF, Limmathurotsakul, D, Wiersinga, WJ, Wuthiekanun, V, Lee, SJ, Mahavanakul, W, Chaowagul, W, Chierakul, W, White, NJ, van der Poll, T, Day, NP, Dougan, G & Peacock, SJ 2011, 'Glyburide is anti-inflammatory and associated with reduced mortality in melioidosis', *Clin Infect Dis*, vol. 52, no. 6, pp. 717-25.

Kong, AP, Xu, G, Brown, N, So, WY, Ma, RC & Chan, JC 2013, 'Diabetes and its comorbidities-where East meets West', *Nat Rev Endocrinol*.

Koo, GC & Gan, YH 2006, 'The innate interferon gamma response of BALB/c and C57BL/6 mice to *in vitro Burkholderia pseudomallei* infection', *BMC Immunol*, vol. 7, p. 19.

Kooptiwut, S, Zraika, S, Thorburn, AW, Dunlop, ME, Darwiche, R, Kay, TW, Proietto, J & Andrikopoulos, S 2002, 'Comparison of insulin secretory function in two mouse models with different susceptibility to beta-cell failure', *Endocrinology*, vol. 143, no. 6, pp. 2085-92.

Kortepeter, MG & Parker, GW 1999, 'Potential biological weapons threats', *Emerg Infect Dis*, vol. 5, no. 4, pp. 523-7.

Kosower, EM & Kosower, NS 1969, 'Lest I forget thee, glutathione', *Nature*, vol. 224, no. 5215, pp. 117-20.

Kuroe, A, Fukushima, M, Usami, M, Ikeda, M, Nakai, Y, Taniguchi, A, Matsuura, T, Suzuki, H, Kurose, T, Yasuda, K, Yamada, Y & Seino, Y 2003, 'Impaired beta-cell function and insulin sensitivity in Japanese subjects with normal glucose tolerance', *Diabetes Res Clin Pract*, vol. 59, no. 1, pp. 71-7.

La Cava, A & Matarese, G 2004, 'The weight of leptin in immunity', *Nat Rev Immunol*, vol. 4, no. 5, pp. 371-9.

Lam, TK, Carpentier, A, Lewis, GF, van de Werve, G, Fantus, IG & Giacca, A 2003, 'Mechanisms of the free fatty acid-induced increase in hepatic glucose production', *Am J Physiol Endocrinol Metab*, vol. 284, no. 5, pp. E863-73.

Lamas, O, Marti, A & Martinez, JA 2002, 'Obesity and immunocompetence', *Eur J Clin Nutr*, vol. 56 Suppl 3, pp. S42-5.

Lamkanfi, M, Mueller, JL, Vitari, AC, Misaghi, S, Fedorova, A, Deshayes, K, Lee, WP, Hoffman, HM & Dixit, VM 2009, 'Glyburide inhibits the Cryopyrin/Nalp3 inflammasome', *J Cell Biol*, vol. 187, no. 1, pp. 61-70.

Lang, CH, Spolarics, Z, Ottlakan, A & Spitzer, JJ 1993, 'Effect of high-dose endotoxin on glucose production and utilization', *Metabolism*, vol. 42, no. 10, pp. 1351-8.

Lardinois, CK & Starich, GH 1991, 'Polyunsaturated fats enhance peripheral glucose utilization in rats', *J Am Coll Nutr*, vol. 10, no. 4, pp. 340-5.

Lauw, FN, Simpson, AJ, Prins, JM, Smith, MD, Kurimoto, M, van Deventer, SJ, Speelman, P, Chaowagul, W, White, NJ & van der Poll, T 1999, 'Elevated plasma concentrations of interferon (IFN)-gamma and the IFN-gamma-inducing cytokines interleukin (IL)-18, IL-12, and IL-15 in severe melioidosis', *J Infect Dis*, vol. 180, no. 6, pp. 1878-85.

Lazar Adler, NR, Govan, B, Cullinane, M, Harper, M, Adler, B & Boyce, JD 2009, 'The molecular and cellular basis of pathogenesis in melioidosis: how does *Burkholderia pseudomallei* cause disease?', *FEMS Microbiol Rev*, vol. 33, no. 6, pp. 1079-99.

Le Hello, S, Currie, BJ, Godoy, D, Spratt, BG, Mikulski, M, Lacassin, F & Garin, B 2005, 'Melioidosis in New Caledonia', *Emerg Infect Dis*, vol. 11, no. 10, pp. 1607-9.

Leakey, AK, Ulett, GC & Hirst, RG 1998, 'BALB/c and C57Bl/6 mice infected with virulent *Burkholderia pseudomallei* provide contrasting animal models for the acute and chronic forms of human melioidosis', *Microb Pathog*, vol. 24, no. 5, pp. 269-75.

Lee, AY & Chung, SS 1999, 'Contributions of polyol pathway to oxidative stress in diabetic cataract', *FASEB J*, vol. 13, no. 1, pp. 23-30.

Lee, CM, Huxley, RR, Lam, TH, Martiniuk, AL, Ueshema, H, Pan, WH, Welborn, T & Woodward, M 2007, 'Prevalence of diabetes mellitus and population attributable fractions for coronary heart disease and stroke mortality in the WHO South-East Asia and Western Pacific regions', *Asia Pac J Clin Nutr*, vol. 16, no. 1, pp. 187-92.

Leelarasamee, A 1998, '*Burkholderia pseudomallei*: the unbeatable foe?', *Southeast Asian J Trop Med Public Health*, vol. 29, no. 2, pp. 410-5.

Leelarasamee, A 2000, 'Melioidosis in Southeast Asia', *Acta Trop*, vol. 74, no. 2-3, pp. 129-32.

Leelarasamee, A & Bovornkitti, S 1989, 'Melioidosis: review and update', *Rev Infect Dis*, vol. 11, no. 3, pp. 413-25.

Lehrke, M, Reilly, MP, Millington, SC, Iqbal, N, Rader, DJ & Lazar, MA 2004, 'An inflammatory cascade leading to hyperresistinemia in humans', *PLoS Med*, vol. 1, no. 2, p. e45.

Leiter, EH 1989, 'The genetics of diabetes susceptibility in mice', *FASEB J*, vol. 3, no. 11, pp. 2231-41.

Leiter, EH, Coleman, DL & Hummel, KP 1981, 'The influence of genetic background on the expression of mutations at the diabetes locus in the mouse. III. Effect of H-2 haplotype and sex', *Diabetes*, vol. 30, no. 12, pp. 1029-34.

Lenzen, S 2008, 'Oxidative stress: the vulnerable beta-cell', *Biochem Soc Trans*, vol. 36, no. Pt 3, pp. 343-7.

Lenzen, S, Drinkgern, J & Tiedge, M 1996, 'Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues', *Free Radic Biol Med*, vol. 20, no. 3, pp. 463-6.

Leonard, D, McDermott, R, Odea, K, Rowley, KG, Pensio, P, Sambo, E, Twist, A, Toolis, R, Lowson, S & Best, JD 2002, 'Obesity, diabetes and associated cardiovascular risk factors among Torres Strait Islander people', *Aust N Z J Public Health*, vol. 26, no. 2, pp. 144-9.

Lertmemongkolchai, G, Cai, G, Hunter, CA & Bancroft, GJ 2001, 'Bystander activation of CD8+ T cells contributes to the rapid production of IFN-gamma in response to bacterial pathogens', *J Immunol*, vol. 166, no. 2, pp. 1097-105.

Levy, J, Atkinson, AB, Bell, PM, McCance, DR & Hadden, DR 1998, 'Beta-cell deterioration determines the onset and rate of progression of secondary dietary failure in type 2 diabetes mellitus: the 10-year follow-up of the Belfast Diet Study', *Diabet Med*, vol. 15, no. 4, pp. 290-6.

Libermann, TA & Baltimore, D 1990, 'Activation of interleukin-6 gene expression through the NF-kappa B transcription factor', *Mol Cell Biol*, vol. 10, no. 5, pp. 2327-34.

Lillioja, S, Mott, DM, Spraul, M, Ferraro, R, Foley, JE, Ravussin, E, Knowler, WC, Bennett, PH & Bogardus, C 1993, 'Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus. Prospective studies of Pima Indians', *N Engl J Med*, vol. 329, no. 27, pp. 1988-92.

Limmathurotsakul, D, Chaowagul, W, Chantratita, N, Wuthiekanun, V, Biaklang, M, Tumapa, S, White, NJ, Day, NP & Peacock, SJ 2008, 'A simple scoring system to differentiate between relapse and re-infection in patients with recurrent melioidosis', *PLoS Negl Trop Dis*, vol. 2, no. 10, p. e327.

Limmathurotsakul, D, Chaowagul, W, Chierakul, W, Stepniewska, K, Maharjan, B, Wuthiekanun, V, White, NJ, Day, NP & Peacock, SJ 2006, 'Risk factors for recurrent melioidosis in northeast Thailand', *Clin Infect Dis*, vol. 43, no. 8, pp. 979-86.

Limmathurotsakul, D, Dance, DA, Wuthiekanun, V, Kaestli, M, Mayo, M, Warner, J, Wagner, DM, Tuanyok, A, Wertheim, H, Yoke Cheng, T, Mukhopadhyay, C, Puthucheary, S, Day, NP, Steinmetz, I, Currie, BJ & Peacock, SJ 2013, 'Systematic review and consensus guidelines for environmental sampling of *Burkholderia pseudomallei*', *PLoS Negl Trop Dis*, vol. 7, no. 3, p. e2105.

Limmathurotsakul, D, Jamsen, K, Arayawichanont, A, Simpson, JA, White, LJ, Lee, SJ, Wuthiekanun, V, Chantratita, N, Cheng, A, Day, NP, Verzilli, C & Peacock, SJ 2010a, 'Defining the true sensitivity of culture for the diagnosis of melioidosis using Bayesian latent class models', *PLoS One*, vol. 5, no. 8, p. e12485.

Limmathurotsakul, D & Peacock, SJ 2011, 'Melioidosis: a clinical overview', *Br Med Bull*, vol. 99, pp. 125-39.

Limmathurotsakul, D, Wongratanacheewin, S, Teerawattanasook, N, Wongsuvan, G, Chaisuksant, S, Chetchotisakd, P, Chaowagul, W, Day, NP & Peacock, SJ 2010b, 'Increasing incidence of human melioidosis in Northeast Thailand', *Am J Trop Med Hyg*, vol. 82, no. 6, pp. 1113-7.

Linder, K, Arner, P, Flores-Morales, A, Tollet-Egnell, P & Norstedt, G 2004, 'Differentially expressed genes in visceral or subcutaneous adipose tissue of obese men and women', *J Lipid Res*, vol. 45, no. 1, pp. 148-54.

Lindstrom, P 2007, 'The physiology of obese-hyperglycemic mice [*ob/ob* mice]', *ScientificWorldJournal*, vol. 7, pp. 666-85.

Linn, T, Santosa, B, Gronemeyer, D, Aygen, S, Scholz, N, Busch, M & Bretzel, RG 2000, 'Effect of long-term dietary protein intake on glucose metabolism in humans', *Diabetologia*, vol. 43, no. 10, pp. 1257-65.

Liu, B, Koo, GC, Yap, EH, Chua, KL & Gan, YH 2002, 'Model of differential susceptibility to mucosal *Burkholderia pseudomallei* infection', *Infect Immun*, vol. 70, no. 2, pp. 504-11.

Liu, Y, Loh, JP, Aw, LT, Yap, EP, Lee, MA & Ooi, EE 2006, 'Rapid molecular typing of *Burkholderia pseudomallei*, isolated in an outbreak of melioidosis in Singapore in 2004, based on variable-number tandem repeats', *Trans R Soc Trop Med Hyg*, vol. 100, no. 7, pp. 687-92.

Lo, TJ, Ang, LW, James, L & Goh, KT 2009, 'Melioidosis in a tropical city state, Singapore', *Emerg Infect Dis*, vol. 15, no. 10, pp. 1645-7.

Lord, GM, Matarese, G, Howard, JK, Baker, RJ, Bloom, SR & Lechler, RI 1998, 'Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression', *Nature*, vol. 394, no. 6696, pp. 897-901.

Lowe, P, Engler, C & Norton, R 2002, 'Comparison of automated and nonautomated systems for identification of *Burkholderia pseudomallei*', *J Clin Microbiol*, vol. 40, no. 12, pp. 4625-7.

Ludwig, DS, Majzoub, JA, Al-Zahrani, A, Dallal, GE, Blanco, I & Roberts, SB 1999, 'High glycemic index foods, overeating, and obesity', *Pediatrics*, vol. 103, no. 3, p. E26.

Lumeng, CN, Deyoung, SM, Bodzin, JL & Saltiel, AR 2007, 'Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity', *Diabetes*, vol. 56, no. 1, pp. 16-23.

Lumjiaktase, P, Diggle, SP, Loprasert, S, Tungpradabkul, S, Daykin, M, Camara, M, Williams, P & Kunakorn, M 2006, 'Quorum sensing regulates dpsA and the oxidative stress response in *Burkholderia pseudomallei*', *Microbiology*, vol. 152, no. Pt 12, pp. 3651-9.

Ma, RC & Chan, JC 2013, 'Type 2 diabetes in East Asians: similarities and differences with populations in Europe and the United States', *Ann N Y Acad Sci*, vol. 1281, pp. 64-91.

Macia, L, Delacre, M, Abboud, G, Ouk, TS, Delanoye, A, Verwaerde, C, Saule, P & Wolowczuk, I 2006, 'Impairment of dendritic cell functionality and steady-state number in obese mice', *J Immunol*, vol. 177, no. 9, pp. 5997-6006.

Macotela, Y, Boucher, J, Tran, TT & Kahn, CR 2009, 'Sex and depot differences in adipocyte insulin sensitivity and glucose metabolism', *Diabetes*, vol. 58, no. 4, pp. 803-12.

Maharjan, B, Chantratita, N, Vesaratchavest, M, Cheng, A, Wuthiekanun, V, Chierakul, W, Chaowagul, W, Day, NP & Peacock, SJ 2005, 'Recurrent melioidosis in patients in northeast Thailand is frequently due to reinfection rather than relapse', *J Clin Microbiol*, vol. 43, no. 12, pp. 6032-4.

Malczewski, AB, Oman, KM, Norton, RE & Ketheesan, N 2005, 'Clinical presentation of melioidosis in Queensland, Australia', *Trans R Soc Trop Med Hyg*, vol. 99, no. 11, pp. 856-60.

Malecki, MT 2005, 'Genetics of type 2 diabetes mellitus', *Diabetes Res Clin Pract*, vol. 68 Suppl1, pp. S10-21.

Malmezat, T, Breuille, D, Capitan, P, Mirand, PP & Obled, C 2000, 'Glutathione turnover is increased during the acute phase of sepsis in rats', *J Nutr*, vol. 130, no. 5, pp. 1239-46.

Malorni, W, Rivabene, R, Santini, MT, Iosi, F & Donelli, G 1993, 'Cell injury and death induced by free radicals in HIV-susceptible cells', *Cytotechnology*, vol. 11, no. Suppl 1, p. S164.

Mancuso, P, Gottschalk, A, Phare, SM, Peters-Golden, M, Lukacs, NW & Huffnagle, GB 2002, 'Leptin-deficient mice exhibit impaired host defense in Gram-negative pneumonia', *J Immunol*, vol. 168, no. 8, pp. 4018-24.

Marchetti, P, Del Prato, S, Lupi, R & Del Guerra, S 2006, 'The pancreatic beta-cell in human Type 2 diabetes', *Nutr Metab Cardiovasc Dis*, vol. 16 Suppl 1, pp. S3-6.

Marhoffer, W, Stein, M, Maeser, E & Federlin, K 1992, 'Impairment of polymorphonuclear leukocyte function and metabolic control of diabetes', *Diabetes Care*, vol. 15, no. 2, pp. 256-60.

Marshall, JA, Hoag, S, Shetterly, S & Hamman, RF 1994, 'Dietary fat predicts conversion from impaired glucose tolerance to NIDDM. The San Luis Valley Diabetes Study', *Diabetes Care*, vol. 17, no. 1, pp. 50-6.

Martinon, F, Burns, K & Tschopp, J 2002, 'The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta', *Mol Cell*, vol. 10, no. 2, pp. 417-26.

Mastropaolo, MD, Evans, NP, Byrnes, MK, Stevens, AM, Robertson, JL & Melville, SB 2005, 'Synergy in polymicrobial infections in a mouse model of type 2 diabetes', *Infect Immun*, vol. 73, no. 9, pp. 6055-63.

Matarese, G, Moschos, S & Mantzoros, CS 2005, 'Leptin in immunology', *J Immunol*, vol. 174, no. 6, pp. 3137-42.

Matsuura, M, Kawahara, K, Ezaki, T & Nakano, M 1996, 'Biological activities of lipopolysaccharide of *Burkholderia* (*Pseudomonas*) *pseudomallei*', *FEMS Microbiology Letters*, vol. 137, pp. 79-83.

Mayo, M, Kaesti, M, Harrington, G, Cheng, AC, Ward, L, Karp, D, Jolly, P, Godoy, D, Spratt, BG & Currie, BJ 2011, '*Burkholderia pseudomallei* in unchlorinated domestic bore water, Tropical Northern Australia', *Emerg Infect Dis*, vol. 17, no. 7, pp. 1283-5.

McCowen, KC, Malhotra, A & Bistrian, BR 2001, 'Stress-induced hyperglycemia', *Crit Care Clin*, vol. 17, no. 1, pp. 107-24.

McDermott, RA, Li, M & Campbell, SK 2010, 'Incidence of type 2 diabetes in two Indigenous Australian populations: a 6-year follow-up study', *Med J Aust*, vol. 192, no. 10, pp. 562-5.

McGarry, JD 2002, 'Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes', *Diabetes*, vol. 51, no. 1, pp. 7-18.

McGarry, JD & Dobbins, RL 1999, 'Fatty acids, lipotoxicity and insulin secretion', *Diabetologia*, vol. 42, no. 2, pp. 128-38.

Medina-Gomez, G, Virtue, S, Lelliott, C, Boiani, R, Campbell, M, Christodoulides, C, Perrin, C, Jimenez-Linan, M, Blount, M, Dixon, J, Zahn, D, Thresher, RR, Aparicio, S, Carlton, M, Colledge, WH, Kettunen, MI, Seppanen-Laakso, T, Sethi, JK, O'Rahilly, S, Brindle, K, Cinti, S, Oresic, M, Burcelin, R & Vidal-Puig, A 2005, 'The link between nutritional status and insulin sensitivity is dependent on the adipocyte-specific peroxisome proliferator-activated receptor-gamma2 isoform', *Diabetes*, vol. 54, no. 6, pp. 1706-16.

Meduri, GU, Kohler, G, Headley, S, Tolley, E, Stentz, F & Postlethwaite, A 1995, 'Inflammatory cytokines in the BAL of patients with ARDS. Persistent elevation over time predicts poor outcome', *Chest*, vol. 108, no. 5, pp. 1303-14.

Meister, A 1983, 'Selective modification of glutathione metabolism', *Science*, vol. 220, no. 4596, pp. 472-7.

Meister, A & Anderson, ME 1983, 'Glutathione', *Annu Rev Biochem*, vol. 52, pp. 711-60.

Meyer, KA, Kushi, LH, Jacobs, DR, Jr. & Folsom, AR 2001, 'Dietary fat and incidence of type 2 diabetes in older Iowa women', *Diabetes Care*, vol. 24, no. 9, pp. 1528-35.

Miller, WR, Pannell, L & Ingalls, MS 1948, 'Experimental chemotherapy in glanders and melioidosis', *Am J Hyg*, vol. 47, no. 2, pp. 205-13.

Millman, AC, Salman, M, Dayaram, YK, Connell, ND & Venketaraman, V 2008, 'Natural killer cells, glutathione, cytokines, and innate immunity against *Mycobacterium tuberculosis*', *J Interferon Cytokine Res*, vol. 28, no. 3, pp. 153-65.

Misra, A & Khurana, L 2008, 'Obesity and the metabolic syndrome in developing countries', *J Clin Endocrinol Metab*, vol. 93, no. 11 Suppl 1, pp. S9-30.

Mittelman, SD, Van Citters, GW, Kirkman, EL & Bergman, RN 2002, 'Extreme insulin resistance of the central adipose depot in vivo', *Diabetes*, vol. 51, no. 3, pp. 755-61.

Mokdad, AH, Bowman, BA, Ford, ES, Vinicor, F, Marks, JS & Koplan, JP 2001, 'The continuing epidemics of obesity and diabetes in the United States', *JAMA*, vol. 286, no. 10, pp. 1195-200.

Moller, DE 2000, 'Potential role of TNF-alpha in the pathogenesis of insulin resistance and type 2 diabetes', *Trends Endocrinol Metab*, vol. 11, no. 6, pp. 212-7.

Monick, MM, Samavati, L, Butler, NS, Mohning, M, Powers, LS, Yarovinsky, T, Spitz, DR & Hunninghake, GW 2003, 'Intracellular thiols contribute to Th2 function via a positive role in IL-4 production', *J Immunol*, vol. 171, no. 10, pp. 5107-15.

Morise, A, Thomas, C, Landrier, JF, Besnard, P & Hermier, D 2009, 'Hepatic lipid metabolism response to dietary fatty acids is differently modulated by PPARalpha in male and female mice', *Eur J Nutr*, vol. 48, no. 8, pp. 465-73.

Morris, D, Gonzalez, B, Khurasany, M, Kassissa, C, Luong, J, Kasko, S, Pandya, S, Chu, M, Chi, PT, Bui, S, Guerra, C, Chan, J & Venketaraman, V 2013a, 'Characterization of Dendritic Cell and Regulatory T Cell Functions against *Mycobacterium tuberculosis* Infection', *Biomed Res Int*, vol. 2013, p. 402827.

Morris, D, Guerra, C, Donohue, C, Oh, H, Khurasany, M & Venketaraman, V 2012a, 'Unveiling the mechanisms for decreased glutathione in individuals with HIV infection', *Clin Dev Immunol*, vol. 2012, p. 734125.

Morris, D, Guerra, C, Khurasany, M, Guilford, F, Saviola, B, Huang, Y & Venketaraman, V 2013b, 'Glutathione supplementation improves macrophage functions in HIV', *J Interferon Cytokine Res*, vol. 33, no. 5, pp. 270-9.

Morris, D, Khurasany, M, Nguyen, T, Kim, J, Guilford, F, Mehta, R, Gray, D, Saviola, B & Venketaraman, V 2013c, 'Glutathione and infection', *Biochim Biophys Acta*, vol. 1830, no. 5, pp. 3329-49.

Morris, J, Williams, N, Rush, C, Govan, B, Sangla, K, Norton, R & Ketheesan, N 2012b, '*Burkholderia pseudomallei* triggers altered inflammatory profiles in a whole-blood model of type 2 diabetes-melioidosis comorbidity', *Infect Immun*, vol. 80, no. 6, pp. 2089-99.

Morris, JL, Hodgson, KA & Ketheesan, N 2012, 'Section VII.4 Development of protection', in N Ketheesan (ed.), *Melioidosis - A Century of Observation and Research*, First edn, Elsevier B. V., The Netherlands.

Moss, M, Guidot, DM, Steinberg, KP, Duhon, GF, Treece, P, Wolken, R, Hudson, LD & Parsons, PE 2000, 'Diabetic patients have a decreased incidence of acute respiratory distress syndrome', *Crit Care Med*, vol. 28, no. 7, pp. 2187-92.

Muangsombut, V, Suparak, S, Pumirat, P, Damnin, S, Vattanaviboon, P, Thongboonkerd, V & Korbsrisate, S 2008, 'Inactivation of *Burkholderia pseudomallei* bsaQ results in decreased invasion efficiency and delayed escape of bacteria from endocytic vesicles', *Arch Microbiol*, vol. 190, no. 6, pp. 623-31.

Muller, LM, Gorter, KJ, Hak, E, Goudzwaard, WL, Schellevis, FG, Hoepelman, AI & Rutten, GE 2005, 'Increased risk of common infections in patients with type 1 and type 2 diabetes mellitus', *Clin Infect Dis*, vol. 41, no. 3, pp. 281-8.

Muniyappa, R, Srinivas, PR, Ram, JL, Walsh, MF & Sowers, JR 1998, 'Calcium and protein kinase C mediate high-glucose-induced inhibition of inducible nitric oxide synthase in vascular smooth muscle cells', *Hypertension*, vol. 31, no. 1 Pt 2, pp. 289-95.

Murakami, K 1991, '[Glutathione metabolism in erythrocytes from patients with diabetes mellitus]', *Hokkaido Igaku Zasshi*, vol. 66, no. 1, pp. 29-40.

Murakami, K, Kondo, T, Ohtsuka, Y, Fujiwara, Y, Shimada, M & Kawakami, Y 1989, 'Impairment of glutathione metabolism in erythrocytes from patients with diabetes mellitus', *Metabolism*, vol. 38, no. 8, pp. 753-8.

Murata, Y, Amao, M, Yoneda, J & Hamuro, J 2002a, 'Intracellular thiol redox status of macrophages directs the Th1 skewing in thioredoxin transgenic mice during aging', *Mol Immunol*, vol. 38, no. 10, pp. 747-57.

Murata, Y, Ohteki, T, Koyasu, S & Hamuro, J 2002b, 'IFN-gamma and pro-inflammatory cytokine production by antigen-presenting cells is dictated by intracellular thiol redox status regulated by oxygen tension', *Eur J Immunol*, vol. 32, no. 10, pp. 2866-73.

Murata, Y, Shimamura, T & Hamuro, J 2002, 'The polarization of T(h)1/T(h)2 balance is dependent on the intracellular thiol redox status of macrophages due to the distinctive cytokine production', *Int Immunol*, vol. 14, no. 2, pp. 201-12.

Musatov, S, Chen, W, Pfaff, DW, Mobbs, CV, Yang, XJ, Clegg, DJ, Kaplitt, MG & Ogawa, S 2007, 'Silencing of estrogen receptor alpha in the ventromedial nucleus of hypothalamus leads to metabolic syndrome', *Proc Natl Acad Sci U S A*, vol. 104, no. 7, pp. 2501-6.

Must, A, Spadano, J, Coakley, EH, Field, AE, Colditz, G & Dietz, WH 1999, 'The disease burden associated with overweight and obesity', *JAMA*, vol. 282, no. 16, pp. 1523-9.

Muttarak, M, Peh, WC, Euathrongchit, J, Lin, SE, Tan, AG, Lerttumnongtum, P & Sivasomboon, C 2009, 'Spectrum of imaging findings in melioidosis', *Br J Radiol*, vol. 82, no. 978, pp. 514-21.

Naguib, G, Al-Mashat, H, Desta, T & Graves, DT 2004, 'Diabetes prolongs the inflammatory response to a bacterial stimulus through cytokine dysregulation', *J Invest Dermatol*, vol. 123, no. 1, pp. 87-92.

Nakagami, T, Qiao, Q, Carstensen, B, Nhr-Hansen, C, Hu, G, Tuomilehto, J, Balkau, B, Borch-Johnsen, K & Group, D-DS 2003, 'Age, body mass index and Type 2 diabetes-associations modified by ethnicity', *Diabetologia*, vol. 46, no. 8, pp. 1063-70.

Naugler, WE, Sakurai, T, Kim, S, Maeda, S, Kim, K, Elsharkawy, AM & Karin, M 2007, 'Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production', *Science*, vol. 317, no. 5834, pp. 121-4.

Newsholme, P, Haber, EP, Hirabara, SM, Rebelato, EL, Procopio, J, Morgan, D, Oliveira-Emilio, HC, Carpinelli, AR & Curi, R 2007, 'Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity', *J Physiol*, vol. 583, no. Pt 1, pp. 9-24.

Ngauy, V, Lemeshev, Y, Sadkowski, L & Crawford, G 2005, 'Cutaneous melioidosis in a man who was taken as a prisoner of war by the Japanese during World War II', *J Clin Microbiol*, vol. 43, no. 2, pp. 970-2.

Nierman, WC, DeShazer, D, Kim, HS, Tettelin, H, Nelson, KE, Feldblyum, T, Ulrich, RL, Ronning, CM, Brinkac, LM, Daugherty, SC, Davidsen, TD, Deboy, RT, Dimitrov, G, Dodson, RJ, Durkin, AS, Gwinn, ML, Haft, DH, Khouri, H, Kolonay, JF, Madupu, R, Mohammoud, Y, Nelson, WC, Radune, D, Romero, CM, Sarria, S, Selengut, J, Shamblin, C, Sullivan, SA, White, O, Yu, Y, Zafar, N, Zhou, L & Fraser, CM 2004, 'Structural flexibility in the *Burkholderia mallei* genome', *Proc Natl Acad Sci U S A*, vol. 101, no. 39, pp. 14246-51.

Nieto-Vazquez, I, Fernandez-Veledo, S, Kramer, DK, Vila-Bedmar, R, Garcia-Guerra, L & Lorenzo, M 2008, 'Insulin resistance associated to obesity: the link TNF-alpha', *Arch Physiol Biochem*, vol. 114, no. 3, pp. 183-94.

Nishi, Y, Fukushima, M, Suzuki, H, Mitsui, R, Ueda, N, Taniguchi, A, Nakai, Y, Kawakita, T, Kurose, T, Seino, Y & Yamada, Y 2005, 'Insulin secretion and insulin sensitivity in Japanese subjects with impaired fasting glucose and isolated fasting hyperglycemia', *Diabetes Res Clin Pract*, vol. 70, no. 1, pp. 46-52.

Nishizawa, H, Shimomura, I, Kishida, K, Maeda, N, Kuriyama, H, Nagaretani, H, Matsuda, M, Kondo, H, Furuyama, N, Kihara, S, Nakamura, T, Tochino, Y, Funahashi, T & Matsuzawa, Y 2002, 'Androgens decrease plasma adiponectin, an insulin-sensitizing adipocyte-derived protein', *Diabetes*, vol. 51, no. 9, pp. 2734-41.

Noritake, M, Katsura, Y, Shinomiya, N, Kanatani, M, Uwabe, Y, Nagata, N & Tsuru, S 1992, 'Intracellular hydrogen peroxide production by peripheral phagocytes from diabetic patients. Dissociation between polymorphonuclear leucocytes and monocytes', *Clin Exp Immunol*, vol. 88, no. 2, pp. 269-74.

O'Quinn, AL, Wiegand, EM & Jeddeloh, JA 2001, '*Burkholderia pseudomallei* kills the nematode Caenorhabditis elegans using an endotoxin-mediated paralysis', *Cell Microbiol*, vol. 3, no. 6, pp. 381-93.

Olefsky, JM & Glass, CK 2010, 'Macrophages, inflammation, and insulin resistance', *Annu Rev Physiol*, vol. 72, pp. 219-46.

Omar, B, Pacini, G & Ahren, B 2012, 'Differential development of glucose intolerance and pancreatic islet adaptation in multiple diet induced obesity models', *Nutrients*, vol. 4, no. 10, pp. 1367-81.

Osawa, T & Kato, Y 2005, 'Protective role of antioxidative food factors in oxidative stress caused by hyperglycemia', *Ann N Y Acad Sci*, vol. 1043, pp. 440-51.

Ouchi, N, Kihara, S, Funahashi, T, Matsuzawa, Y & Walsh, K 2003, 'Obesity, adiponectin and vascular inflammatory disease', *Curr Opin Lipidol*, vol. 14, no. 6, pp. 561-6.

Ouchi, N, Parker, JL, Lugus, JJ & Walsh, K 2011, 'Adipokines in inflammation and metabolic disease', *Nat Rev Immunol*, vol. 11, no. 2, pp. 85-97.

Pan, DA, Lillioja, S, Milner, MR, Kriketos, AD, Baur, LA, Bogardus, C & Storlien, LH 1995, 'Skeletal muscle membrane lipid composition is related to adiposity and insulin action', *J Clin Invest*, vol. 96, no. 6, pp. 2802-8.

Panchal, SK & Brown, L 2011, 'Rodent models for metabolic syndrome research', *J Biomed Biotechnol*, vol. 2011, p. 351982.

Paolisso, G, D'Amore, A, Di Maro, G, Galzerano, D, Tesauro, P, Varricchio, M & D'Onofrio, F 1993a, 'Evidence for a relationship between free radicals and insulin action in the elderly', *Metabolism*, vol. 42, no. 5, pp. 659-63.

Paolisso, G, D'Amore, A, Giugliano, D, Ceriello, A, Varricchio, M & D'Onofrio, F 1993b, 'Pharmacologic doses of vitamin E improve insulin action in healthy subjects and non-insulin-dependent diabetic patients', *Am J Clin Nutr*, vol. 57, no. 5, pp. 650-6.

Paolisso, G, Di Maro, G, Pizza, G, D'Amore, A, Sgambato, S, Tesauro, P, Varricchio, M & D'Onofrio, F 1992, 'Plasma GSH/GSSG affects glucose homeostasis in healthy subjects and non-insulin-dependent diabetics', *Am J Physiol*, vol. 263, no. 3 Pt 1, pp. E435-40.

Parillo, M & Riccardi, G 2004, 'Diet composition and the risk of type 2 diabetes: epidemiological and clinical evidence', *Br J Nutr*, vol. 92, no. 1, pp. 7-19.

Park, S, Rich, J, Hanses, F & Lee, JC 2009, 'Defects in innate immunity predispose C57BL/6J-Leprdb/Leprdb mice to infection by Staphylococcus aureus', *Infect Immun*, vol. 77, no. 3, pp. 1008-14.

Parmely, MJ, Wang, F & Wright, D 2001, 'Gamma interferon prevents the inhibitory effects of oxidative stress on host responses to *Escherichia coli* infection', *Infect Immun*, vol. 69, no. 4, pp. 2621-9.

Patel, N, Conejero, L, De Reynal, M, Easton, A, Bancroft, GJ & Titball, RW 2011, 'Development of vaccines against *Burkholderia pseudomallei*', *Front Microbiol*, vol. 2, p. 198.

Patsouris, D, Li, PP, Thapar, D, Chapman, J, Olefsky, JM & Neels, JG 2008, 'Ablation of CD11c-positive cells normalizes insulin sensitivity in obese insulin resistant animals', *Cell Metab*, vol. 8, no. 4, pp. 301-9.

Pawlak, DB, Kushner, JA & Ludwig, DS 2004, 'Effects of dietary glycaemic index on adiposity, glucose homoeostasis, and plasma lipids in animals', *Lancet*, vol. 364, no. 9436, pp. 778-85.

Peacock, SJ, Limmathurotsakul, D, Lubell, Y, Koh, GC, White, LJ, Day, NP & Titball, RW 2012, 'Melioidosis vaccines: a systematic review and appraisal of the potential to exploit biodefense vaccines for public health purposes', *PLoS Negl Trop Dis*, vol. 6, no. 1, p. e1488.

Peake, SL, Bailey, M, Bellomo, R, Cameron, PA, Cross, A, Delaney, A, Finfer, S, Higgins, A, Jones, DA, Myburgh, JA, Syres, GA, Webb, SA, Williams, P, Arise Investigators, ftA & New Zealand Intensive Care Society Clinical Trials, G 2009, 'Australasian resuscitation of sepsis evaluation (ARISE): A multi-centre, prospective, inception cohort study', *Resuscitation*, vol. 80, no. 7, pp. 811-8.

Pedrosa, J, Saunders, BM, Appelberg, R, Orme, IM, Silva, MT & Cooper, AM 2000, 'Neutrophils play a protective nonphagocytic role in systemic Mycobacterium tuberculosis infection of mice', *Infect Immun*, vol. 68, no. 2, pp. 577-83.

Petersen, KF & Shulman, GI 2006, 'Etiology of insulin resistance', *Am J Med*, vol. 119, no. 5 Suppl 1, pp. S10-6.

Peterson, JD, Herzenberg, LA, Vasquez, K & Waltenbaugh, C 1998, 'Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns', *Proc Natl Acad Sci U S A*, vol. 95, no. 6, pp. 3071-6.

Petro, AE, Cotter, J, Cooper, DA, Peters, JC, Surwit, SJ & Surwit, RS 2004, 'Fat, carbohydrate, and calories in the development of diabetes and obesity in the C57BL/6J mouse', *Metabolism*, vol. 53, no. 4, pp. 454-7.

Pettersson, US, Walden, TB, Carlsson, PO, Jansson, L & Phillipson, M 2012, 'Female mice are protected against high-fat diet induced metabolic syndrome and increase the regulatory T cell population in adipose tissue', *PLoS One*, vol. 7, no. 9, p. e46057.

Pi-Sunyer, FX 1995, 'Making effective dietary recommendations', *Hosp Food Nutr Focus*, vol. 11, no. 10, pp. 7-8.

Pickup, JC 2004, 'Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes', *Diabetes Care*, vol. 27, no. 3, pp. 813-23.

Pickup, JC & Crook, MA 1998, 'Is type II diabetes mellitus a disease of the innate immune system?', *Diabetologia*, vol. 41, no. 10, pp. 1241-8.

Pirola, L, Johnston, AM & Van Obberghen, E 2004, 'Modulation of insulin action', *Diabetologia*, vol. 47, no. 2, pp. 170-84.

Popkin, BM & Gordon-Larsen, P 2004, 'The nutrition transition: worldwide obesity dynamics and their determinants', *Int J Obes Relat Metab Disord*, vol. 28 Suppl 3, pp. S2-9.

Poretsky, L 2009, *Principles of diabetes mellitus*, vol. 2nd Ed, Springer, New York.

Prescott, LF & Critchley, JA 1983, 'The treatment of acetaminophen poisoning', *Annu Rev Pharmacol Toxicol*, vol. 23, pp. 87-101.

Prodi, E & Obici, S 2006, 'Minireview: the brain as a molecular target for diabetic therapy', *Endocrinology*, vol. 147, no. 6, pp. 2664-9.

Ptak, W, Klimek, M, Bryniarski, K, Ptak, M & Majcher, P 1998, 'Macrophage function in alloxan diabetic mice: expression of adhesion molecules, generation of monokines and oxygen and NO radicals', *Clin Exp Immunol*, vol. 114, no. 1, pp. 13-8.

Puthucheary, SD, Parasakthi, N & Lee, MK 1992, 'Septicaemic melioidosis: a review of 50 cases from Malaysia', *Trans R Soc Trop Med Hyg*, vol. 86, no. 6, pp. 683-5.

Radziuk, J, Pye, S & Zhang, Z 1993, 'Substrates and the regulation of hepatic glycogen metabolism', *Adv Exp Med Biol*, vol. 334, pp. 235-52.

Raitakari, OT, Porkka, KV, Rasanen, L & Viikari, JS 1994, 'Relations of life-style with lipids, blood pressure and insulin in adolescents and young adults. The Cardiovascular Risk in Young Finns Study', *Atherosclerosis*, vol. 111, no. 2, pp. 237-46.

Ramachandran, A, Chamukuttan, S, Immaneni, S, Shanmugam, RM, Vishnu, N, Viswanathan, V & Jaakko, T 2005, 'High incidence of glucose intolerance in Asian-Indian subjects with acute coronary syndrome', *Diabetes Care*, vol. 28, no. 10, pp. 2492-6.

Ramachandran, A, Ma, RC & Snehalatha, C 2010, 'Diabetes in Asia', *Lancet*, vol. 375, no. 9712, pp. 408-18.

Ramachandran, A & Snehalatha, C 2010, 'Rising burden of obesity in Asia', *J Obes*, vol. 2010.

Ranasinghe, P, Jayawardena, R & Katulanda, P 2013, 'Diabetes mellitus in South Asia: scientific evaluation of the research output', *J Diabetes*, vol. 5, no. 1, pp. 34-42.

Ranheim, T, Dumke, C, Schueler, KL, Cartee, GD & Attie, AD 1997, 'Interaction between BTBR and C57BL/6J genomes produces an insulin resistance syndrome in (BTBR x C57BL/6J) F1 mice', *Arterioscler Thromb Vasc Biol*, vol. 17, no. 11, pp. 3286-93.

Rattarasarn, C 2006, 'Physiological and pathophysiological regulation of regional adipose tissue in the development of insulin resistance and type 2 diabetes', *Acta Physiol (Oxf)*, vol. 186, no. 2, pp. 87-101.

Razak, F, Anand, SS, Shannon, H, Vuksan, V, Davis, B, Jacobs, R, Teo, KK, McQueen, M & Yusuf, S 2007, 'Defining obesity cut points in a multiethnic population', *Circulation*, vol. 115, no. 16, pp. 2111-8.

Reaven, G 2005, 'Insulin resistance, type 2 diabetes mellitus, and cardiovascular disease: the end of the beginning', *Circulation*, vol. 112, no. 20, pp. 3030-2.

Reckseidler-Zenteno, SL, DeVinney, R & Woods, DE 2005, 'The capsular polysaccharide of *Burkholderia pseudomallei* contributes to survival in serum by reducing complement factor C3b deposition', *Infect Immun*, vol. 73, no. 2, pp. 1106-15.

Rees, DA & Alcolado, JC 2005, 'Animal models of diabetes mellitus', *Diabet Med*, vol. 22, no. 4, pp. 359-70.

Regitz-Zagrosek, V, Lehmkuhl, E & Weickert, MO 2006, 'Gender differences in the metabolic syndrome and their role for cardiovascular disease', *Clin Res Cardiol*, vol. 95, no. 3, pp. 136-47.

Remais, JV, Zeng, G, Li, G, Tian, L & Engelgau, MM 2013, 'Convergence of non-communicable and infectious diseases in low- and middle-income countries', *Int J Epidemiol*, vol. 42, no. 1, pp. 221-7.

Reynolds, K, Gu, D, Whelton, PK, Wu, X, Duan, X, Mo, J, He, J & Inter, ACG 2007, 'Prevalence and risk factors of overweight and obesity in China', *Obesity (Silver Spring)*, vol. 15, no. 1, pp. 10-8.

Riant, E, Waget, A, Cogo, H, Arnal, JF, Burcelin, R & Gourdy, P 2009, 'Estrogens protect against high-fat diet-induced insulin resistance and glucose intolerance in mice', *Endocrinology*, vol. 150, no. 5, pp. 2109-17.

Riccardi, G, Giacco, R & Rivellese, AA 2004, 'Dietary fat, insulin sensitivity and the metabolic syndrome', *Clin Nutr*, vol. 23, no. 4, pp. 447-56.

Rimington, RA 1962, 'Melioidosis in north Queensland', *Med J Aust*, vol. 49(1), pp. 50-3.

Rittirsch, D, Flierl, MA & Ward, PA 2008, 'Harmful molecular mechanisms in sepsis', *Nat Rev Immunol*, vol. 8, no. 10, pp. 776-87.

Riyapa, D, Buddhisa, S, Korbsrisate, S, Cuccui, J, Wren, BW, Stevens, MP, Ato, M & Lertmemongkolchai, G 2012, 'Neutrophil extracellular traps exhibit antibacterial activity against *Burkholderia pseudomallei* and are influenced by bacterial and host factors', *Infect Immun*, vol. 80, no. 11, pp. 3921-9.

Rode, JW & Webling, DD 1981, 'Melioidosis in the Northern Territory of Australia', *Med J Aust*, vol. 1, no. 4, pp. 181-4.

Rodriguez-Hernandez, H, Simental-Mendia, LE, Rodriguez-Ramirez, G & Reyes-Romero, MA 2013, 'Obesity and inflammation: epidemiology, risk factors, and markers of inflammation', *Int J Endocrinol*, vol. 2013, p. 678159.

Romagnani, S 1997, 'The Th1/Th2 paradigm', *Immunol Today*, vol. 18, no. 6, pp. 263-6.

Rossmeisl, M, Rim, JS, Koza, RA & Kozak, LP 2003, 'Variation in type 2 diabetes--related traits in mouse strains susceptible to diet-induced obesity', *Diabetes*, vol. 52, no. 8, pp. 1958-66.

Rotz, LD, Khan, AS, Lillibridge, SR, Ostroff, SM & Hughes, JM 2002, 'Public health assessment of potential biological terrorism agents', *Emerg Infect Dis*, vol. 8, no. 2, pp. 225-30.

Ruan, H & Lodish, HF 2003, 'Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-alpha', *Cytokine Growth Factor Rev*, vol. 14, no. 5, pp. 447-55.

Ruderman, N, Chisholm, D, Pi-Sunyer, X & Schneider, S 1998, 'The metabolically obese, normal-weight individual revisited', *Diabetes*, vol. 47, no. 5, pp. 699-713.

Ruderman, NB, Schneider, SH & Berchtold, P 1981, 'The "metabolically-obese," normal-weight individual', *Am J Clin Nutr*, vol. 34, no. 8, pp. 1617-21.

Saiki, O, Negoro, S, Tsuyuguchi, I & Yamamura, Y 1980, 'Depressed immunological defence mechanisms in mice with experimentally induced diabetes', *Infect Immun*, vol. 28, no. 1, pp. 127-31.

Salmeron, J, Ascherio, A, Rimm, EB, Colditz, GA, Spiegelman, D, Jenkins, DJ, Stampfer, MJ, Wing, AL & Willett, WC 1997a, 'Dietary fiber, glycemic load, and risk of NIDDM in men', *Diabetes Care*, vol. 20, no. 4, pp. 545-50.

Salmeron, J, Hu, FB, Manson, JE, Stampfer, MJ, Colditz, GA, Rimm, EB & Willett, WC 2001, 'Dietary fat intake and risk of type 2 diabetes in women', *Am J Clin Nutr*, vol. 73, no. 6, pp. 1019-26.

Salmeron, J, Manson, JE, Stampfer, MJ, Colditz, GA, Wing, AL & Willett, WC 1997b, 'Dietary fiber, glycemic load, and risk of non-insulin-dependent diabetes mellitus in women', *JAMA*, vol. 277, no. 6, pp. 472-7.

Saltiel, AR & Kahn, CR 2001, 'Insulin signalling and the regulation of glucose and lipid metabolism', *Nature*, vol. 414, no. 6865, pp. 799-806.

Samaha, FF, Iqbal, N, Seshadri, P, Chicano, KL, Daily, DA, McGrory, J, Williams, T, Williams, M, Gracely, EJ & Stern, L 2003, 'A low-carbohydrate as compared with a low-fat diet in severe obesity', *N Engl J Med*, vol. 348, no. 21, pp. 2074-81.

Samiec, PS, Drews-Botsch, C, Flagg, EW, Kurtz, JC, Sternberg, P, Jr., Reed, RL & Jones, DP 1998, 'Glutathione in human plasma: decline in association with aging, age-related macular degeneration, and diabetes', *Free Radic Biol Med*, vol. 24, no. 5, pp. 699-704.

Samten, B, Wizel, B, Shams, H, Weis, SE, Klucar, P, Wu, S, Vankayalapati, R, Thomas, EK, Okada, S, Krensky, AM & Barnes, PF 2003, 'CD40 ligand trimer enhances the response of CD8+ T cells to *Mycobacterium tuberculosis*', *J Immunol*, vol. 170, no. 6, pp. 3180-6.

Santanirand, P, Harley, VS, Dance, DA, Drasar, BS & Bancroft, GJ 1999, 'Obligatory role of gamma interferon for host survival in a murine model of infection with *Burkholderia pseudomallei*', *Infect Immun*, vol. 67, no. 7, pp. 3593-600.

Saravu, K, Mukhopadhyay, C, Vishwanath, S, Valsalan, R, Docherla, M, Vandana, KE, Shastry, BA, Bairy, I & Rao, SP 2010, 'Melioidosis in southern India: epidemiological and clinical profile', *Southeast Asian J Trop Med Public Health*, vol. 41, no. 2, pp. 401-9.

Sarkar-Tyson, M & Titball, RW 2010, 'Progress toward development of vaccines against melioidosis: A review', *Clin Ther*, vol. 32, no. 8, pp. 1437-45.

Sarovich, DS, Price, EP, Limmathurotsakul, D, Cook, JM, Von Schulze, AT, Wolken, SR, Keim, P, Peacock, SJ & Pearson, T 2012, 'Development of ceftazidime resistance in an acute *Burkholderia pseudomallei* infection', *Infect Drug Resist*, vol. 5, pp. 129-32.

Scatena, R, Nocca, G, De Sole, P, Bottoni, P & Giardina, B 1998, 'Impaired reactive oxygen metabolism of phagocytic leukocytes in NIDDM patients. A role for non-enzymatic glycosylation of collagen', *J Biolumin Chemilumin*, vol. 13, no. 5, pp. 273-8.

Schenk, S, Saberi, M & Olefsky, JM 2008, 'Insulin sensitivity: modulation by nutrients and inflammation', *J Clin Invest*, vol. 118, no. 9, pp. 2992-3002.

Schweizer, HP 2012, 'Mechanisms of antibiotic resistance in *Burkholderia pseudomallei*: implications for treatment of melioidosis', *Future Microbiol*, vol. 7, no. 12, pp. 1389-99.

Scroyen, I, Hemmeryckx, B & Lijnen, HR 2013, 'From mice to men: mouse models in obesity research: What can we learn?', *Thromb Haemost*, vol. 109, no. 5.

Serbina, NV & Pamer, EG 2006, 'Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2', *Nat Immunol*, vol. 7, no. 3, pp. 311-7.

Seres, T, Knickelbein, RG, Warshaw, JB & Johnston, RB, Jr. 2000, 'The phagocytosis-associated respiratory burst in human monocytes is associated with increased uptake of glutathione', *J Immunol*, vol. 165, no. 6, pp. 3333-40.

Shah, BR & Hux, JE 2003, 'Quantifying the risk of infectious diseases for people with diabetes', *Diabetes Care*, vol. 26, no. 2, pp. 510-3.

Shah, PK 2007, 'Innate immune pathway links obesity to insulin resistance', *Circ Res*, vol. 100, no. 11, pp. 1531-3.

Sharma, A, Kharb, S, Chugh, SN, Kakkar, R & Singh, GP 2000, 'Effect of glycemic control and vitamin E supplementation on total glutathione content in non-insulin-dependent diabetes mellitus', *Ann Nutr Metab*, vol. 44, no. 1, pp. 11-3.

Shaw, KA 2013, 'Australia's role in promoting and supporting tuberculosis control in the Western Pacific Region', *N S W Public Health Bull*, vol. 24, no. -1, pp. 43-8.

Sheffner, AL 1963, 'The reduction in vitro in viscosity of mucoprotein solutions by a new mucolytic agent, N-acetyl-L-cysteine', *Ann N Y Acad Sci*, vol. 106, pp. 298-310.

Shen, SW, Reaven, GM & Farquhar, JW 1970, 'Comparison of impedance to insulin-mediated glucose uptake in normal subjects and in subjects with latent diabetes', *J Clin Invest*, vol. 49, no. 12, pp. 2151-60.

Sheng, XQ, Huang, KX & Xu, HB 2005, 'Influence of alloxan-induced diabetes and selenite treatment on blood glucose and glutathione levels in mice', *J Trace Elem Med Biol*, vol. 18, no. 3, pp. 261-7.

Shih, HI, Chuang, YC, Cheung, BM, Yan, JJ, Chang, CM, Chang, K, Lee, NY, Lee, HC, Wu, CJ, Chen, PL, Lee, CC, Wang, LR, Ko, NY & Ko, WC 2009, 'Sporadic and outbreak cases of melioidosis in southern Taiwan: clinical features and antimicrobial susceptibility', *Infection*, vol. 37, no. 1, pp. 9-15.

Shirshev, SV & Orlova, EG 2005, 'Molecular mechanisms of regulation of functional activity of mononuclear phagocytes by leptin', *Biochemistry (Mosc)*, vol. 70, no. 8, pp. 841-7.

Shoelson, SE, Lee, J & Yuan, M 2003, 'Inflammation and the IKK beta/I kappa B/NF-kappa B axis in obesity- and diet-induced insulin resistance', *Int J Obes Relat Metab Disord*, vol. 27 Suppl 3, pp. S49-52.

Sicree, RA, Zimmet, PZ, King, HO & Coventry, JS 1987, 'Plasma insulin response among Nauruans. Prediction of deterioration in glucose tolerance over 6 yr', *Diabetes*, vol. 36, no. 2, pp. 179-86.

Simpson, AJ, Newton, PN, Chierakul, W, Chaowagul, W & White, NJ 2003, 'Diabetes mellitus, insulin, and melioidosis in Thailand', *Clin Infect Dis*, vol. 36, no. 5, pp. e71-2.

Simpson, AJ, Smith, MD, Weverling, GJ, Suputtamongkol, Y, Angus, BJ, Chaowagul, W, White, NJ, van Deventer, SJ & Prins, JM 2000, 'Prognostic value of cytokine concentrations (tumor necrosis factor-alpha, interleukin-6, and interleukin-10) and clinical parameters in severe melioidosis', *J Infect Dis*, vol. 181, no. 2, pp. 621-5.

Simpson, AJ & White, NJ 1999, 'Combination antibiotic therapy for severe melioidosis', *Clin Infect Dis*, vol. 28, no. 2, p. 410.

Sims, EA 2001, 'Are there persons who are obese, but metabolically healthy?', *Metabolism*, vol. 50, no. 12, pp. 1499-504.

Sivalingam, SP, Ulett, GC & Nelson, M 2012, 'Section VII.1 Models of infection', in N Ketheesan (ed.), *Melioidosis - A Century of Observation and Research*, First edn, Elsevier B. V., The Netherlands.

Sjostrom, L 2013, 'Review of the key results from the Swedish Obese Subjects (SOS) trial - a prospective controlled intervention study of bariatric surgery', *J Intern Med*, vol. 273, no. 3, pp. 219-34.

Sjostrom, L, Lindroos, AK, Peltonen, M, Torgerson, J, Bouchard, C, Carlsson, B, Dahlgren, S, Larsson, B, Narbro, K, Sjostrom, CD, Sullivan, M, Wedel, H & Swedish Obese Subjects Study Scientific, G 2004, 'Lifestyle, diabetes, and cardiovascular risk factors 10 years after bariatric surgery', *N Engl J Med*, vol. 351, no. 26, pp. 2683-93.

Smith, CV, Jones, DP, Guenthner, TM, Lash, LH & Lauterburg, BH 1996, 'Compartmentation of glutathione: implications for the study of toxicity and disease', *Toxicol Appl Pharmacol*, vol. 140, no. 1, pp. 1-12.

Sobhakumari, A, Love-Homan, L, Fletcher, EV, Martin, SM, Parsons, AD, Spitz, DR, Knudson, CM & Simons, AL 2012, 'Susceptibility of human head and neck cancer cells to combined inhibition of glutathione and thioredoxin metabolism', *PLoS One*, vol. 7, no. 10, p. e48175.

Spallholz, JE 1987, 'Glutathione: is it an evolutionary vestige of the penicillins?', *Med Hypotheses*, vol. 23, no. 3, pp. 253-7.

Staal, FJ, Roederer, M, Herzenberg, LA & Herzenberg, LA 1990, 'Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus', *Proc Natl Acad Sci U S A*, vol. 87, no. 24, pp. 9943-7.

Stalenhoef, JE, Alisjahbana, B, Nelwan, EJ, van der Ven-Jongekrijg, J, Ottenhoff, TH, van der Meer, JW, Nelwan, RH, Netea, MG & van Crevel, R 2008, 'The role of interferon-gamma in the increased tuberculosis risk in type 2 diabetes mellitus', *Eur J Clin Microbiol Infect Dis*, vol. 27, no. 2, pp. 97-103.

Stanton, AT & Fletcher, W 1921, *Melioidosis, a New Disease of the Tropics*, Studies from the Institute for Medical Research, Federated Malay States.

Stanton, AT & Fletcher, W 1925, 'Melioidosis and its Relation to Glanders', *J Hyg (Lond)*, vol. 23, no. 4, pp. 347-63.

Steinbaum, SR 2004, 'The metabolic syndrome: an emerging health epidemic in women', *Prog Cardiovasc Dis*, vol. 46, no. 4, pp. 321-36.

Steppan, CM & Lazar, MA 2004, 'The current biology of resistin', *J Intern Med*, vol. 255, no. 4, pp. 439-47.

Stevens, MP, Friebel, A, Taylor, LA, Wood, MW, Brown, PJ, Hardt, WD & Galyov, EE 2003, 'A *Burkholderia pseudomallei* type III secreted protein, BopE, facilitates bacterial invasion of epithelial cells and exhibits guanine nucleotide exchange factor activity', *J Bacteriol*, vol. 185, no. 16, pp. 4992-6.

Stevens, MP, Wood, MW, Taylor, LA, Monaghan, P, Hawes, P, Jones, PW, Wallis, TS & Galyov, EE 2002, 'An Inv/Mxi-Spa-like type III protein secretion system in *Burkholderia pseudomallei* modulates intracellular behaviour of the pathogen', *Mol Microbiol*, vol. 46, no. 3, pp. 649-59.

Stolk, RP, Suriyawongpaisal, P, Aekplakorn, W, Woodward, M, Neal, B & Inter, ACG 2005, 'Fat distribution is strongly associated with plasma glucose levels and diabetes in Thai adults-the InterASIA study', *Diabetologia*, vol. 48, no. 4, pp. 657-60.

Stork, S, van der Schouw, YT, Grobbee, DE & Bots, ML 2004, 'Estrogen, inflammation and cardiovascular risk in women: a critical appraisal', *Trends Endocrinol Metab*, vol. 15, no. 2, pp. 66-72.

Storlien, LH, Jenkins, AB, Chisholm, DJ, Pascoe, WS, Khouri, S & Kraegen, EW 1991, 'Influence of dietary fat composition on development of insulin resistance in rats. Relationship to muscle triglyceride and omega-3 fatty acids in muscle phospholipid', *Diabetes*, vol. 40, no. 2, pp. 280-9.

Straub, RH 2007, 'The complex role of estrogens in inflammation', *Endocr Rev*, vol. 28, no. 5, pp. 521-74.

Stubbins, RE, Najjar, K, Holcomb, VB, Hong, J & Nunez, NP 2012, 'Oestrogen alters adipocyte biology and protects female mice from adipocyte inflammation and insulin resistance', *Diabetes Obes Metab*, vol. 14, no. 1, pp. 58-66.

Stumvoll, M, Goldstein, BJ & van Haeften, TW 2005, 'Type 2 diabetes: principles of pathogenesis and therapy', *Lancet*, vol. 365, no. 9467, pp. 1333-46.

Sun, GW, Chen, Y, Liu, Y, Tan, GY, Ong, C, Tan, P & Gan, YH 2010, 'Identification of a regulatory cascade controlling Type III Secretion System 3 gene expression in *Burkholderia pseudomallei*', *Mol Microbiol*, vol. 76, no. 3, pp. 677-89.

Sun, GW, Lu, J, Pervaiz, S, Cao, WP & Gan, YH 2005, 'Caspase-1 dependent macrophage death induced by *Burkholderia pseudomallei*', *Cell Microbiol*, vol. 7, no. 10, pp. 1447-58.

Suparak, S, Kespichayawattana, W, Haque, A, Easton, A, Damnin, S, Lertmemongkolchai, G, Bancroft, GJ & Korbsrisate, S 2005, 'Multinucleated giant cell formation and apoptosis in infected host cells is mediated by *Burkholderia pseudomallei* type III secretion protein BipB', *J Bacteriol*, vol. 187, no. 18, pp. 6556-60.

Suputtamongkol, Y, Chaowagul, W, Chetchotisakd, P, Lertpatanasuwun, N, Intaranongpai, S, Ruchutrakool, T, Budhsarawong, D, Mootsikapun, P, Wuthiekanun, V, Teerawatasook, N & Lulitanond, A 1999, 'Risk factors for melioidosis and bacteremic melioidosis', *Clin Infect Dis*, vol. 29, no. 2, pp. 408-13.

Suputtamongkol, Y, Hall, AJ, Dance, DA, Chaowagul, W, Rajchanuvong, A, Smith, MD & White, NJ 1994, 'The epidemiology of melioidosis in Ubon Ratchatani, northeast Thailand', *Int J Epidemiol*, vol. 23, no. 5, pp. 1082-90.

Suputtamongkol, Y, Kwiatkowski, D, Dance, DA, Chaowagul, W & White, NJ 1992, 'Tumor necrosis factor in septicemic melioidosis', *J Infect Dis*, vol. 165, no. 3, pp. 561-4.

Surwit, RS, Kuhn, CM, Cochrane, C, McCubbin, JA & Feinglos, MN 1988, 'Diet-induced type II diabetes in C57BL/6J mice', *Diabetes*, vol. 37, no. 9, pp. 1163-7.

Takeuchi, O & Akira, S 2010, 'Pattern recognition receptors and inflammation', *Cell*, vol. 140, no. 6, pp. 805-20.

Tan, GY, Liu, Y, Sivalingam, SP, Sim, SH, Wang, D, Paucod, JC, Gauthier, Y & Ooi, EE 2008, '*Burkholderia pseudomallei* aerosol infection results in differential inflammatory responses in BALB/c and C57Bl/6 mice', *J Med Microbiol*, vol. 57, no. Pt 4, pp. 508-15.

Tan, KS, Lee, KO, Low, KC, Gamage, AM, Liu, Y, Tan, GY, Koh, HQ, Alonso, S & Gan, YH 2012, 'Glutathione deficiency in type 2 diabetes impairs cytokine responses and control of intracellular bacteria', *J Clin Invest*, vol. 122, no. 6, pp. 2289-300.

Tan, MH 2000, 'Current treatment of insulin resistance in type 2 diabetes mellitus', *Int J Clin Pract Suppl*, no. 113, pp. 54-62.

Tanriover, MD, Guven, GS, Sen, D, Unal, S & Uzun, O 2006, 'Epidemiology and outcome of sepsis in a tertiary-care hospital in a developing country', *Epidemiol Infect*, vol. 134, no. 2, pp. 315-22.

Tateda, K, Moore, TA, Deng, JC, Newstead, MW, Zeng, X, Matsukawa, A, Swanson, MS, Yamaguchi, K & Standiford, TJ 2001, 'Early recruitment of neutrophils determines subsequent T1/T2 host responses in a murine model of *Legionella pneumophila* pneumonia', *J Immunol*, vol. 166, no. 5, pp. 3355-61.

Tian, Z, Sun, R, Wei, H & Gao, B 2002, 'Impaired natural killer (NK) cell activity in leptin receptor deficient mice: leptin as a critical regulator in NK cell development and activation', *Biochem Biophys Res Commun*, vol. 298, no. 3, pp. 297-302.

Tiedge, M, Lortz, S, Drinkgern, J & Lenzen, S 1997, 'Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells', *Diabetes*, vol. 46, no. 11, pp. 1733-42.

Titball, RW, Russell, P, Cuccui, J, Easton, A, Haque, A, Atkins, T, Sarkar-Tyson, M, Harley, V, Wren, B & Bancroft, GJ 2008, '*Burkholderia pseudomallei*: animal models of infection', *Trans R Soc Trop Med Hyg*, vol. 102 Suppl 1, pp. S111-6.

Tortoriello, DV, McMinn, J & Chua, SC 2004, 'Dietary-induced obesity and hypothalamic infertility in female DBA/2J mice', *Endocrinology*, vol. 145, no. 3, pp. 1238-47.

Townsend, DM, Tew, KD & Tapiero, H 2003, 'The importance of glutathione in human disease', *Biomed Pharmacother*, vol. 57, no. 3-4, pp. 145-55.

Tran, PO, Parker, SM, LeRoy, E, Franklin, CC, Kavanagh, TJ, Zhang, T, Zhou, H, Vliet, P, Oseid, E, Harmon, JS & Robertson, RP 2004, 'Adenoviral overexpression of the glutamylcysteine ligase catalytic subunit protects pancreatic islets against oxidative stress', *J Biol Chem*, vol. 279, no. 52, pp. 53988-93.

Trinchieri, G 1998, 'Interleukin-12: a cytokine at the interface of inflammation and immunity', *Adv Immunol*, vol. 70, pp. 83-243.

Tseng, CC, Hattori, Y, Kasai, K, Nakanishi, N & Shimoda, S 1997, 'Decreased production of nitric oxide by LPS-treated J774 macrophages in high-glucose medium', *Life Sci*, vol. 60, no. 7, pp. PL99-106.

Tsou, CL, Peters, W, Si, Y, Slaymaker, S, Aslanian, AM, Weisberg, SP, Mack, M & Charo, IF 2007, 'Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites', *J Clin Invest*, vol. 117, no. 4, pp. 902-9.

Tsukaguchi, K, Okamura, H, Ikuno, M, Kobayashi, A, Fukuoka, A, Takenaka, H, Yamamoto, C, Tokuyama, T, Okamoto, Y, Fu, A, Yoshikawa, M, Yoneda, T & Narita, N 1997, '[The relation between diabetes mellitus and IFN-gamma, IL-12 and IL-10 productions by CD4+ alpha beta T cells and monocytes in patients with pulmonary tuberculosis]', *Kekkaku*, vol. 72, no. 11, pp. 617-22.

Tsukaguchi, K, Okamura, H, Matsuzawa, K, Tamura, M, Miyazaki, R, Tamaki, S & Kimura, H 2002, '[Longitudinal assessment of IFN-gamma production in patients with pulmonary tuberculosis complicated with diabetes mellitus]', *Kekkaku*, vol. 77, no. 5, pp. 409-13.

Tuomilehto, J, Lindström, J, Eriksson, JG, Valle, TT, Hämäläinen, H, Ilanne-Parikka, P, Keinänen-Kiukaanniemi, S, Laakso, M, Louheranta, A, Rastas, M, Salminen, V, Uusitupa, M & Group), FDPS 2001, 'Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance', *N Engl J Med*, vol. 344, no. 18, pp. 1343-50.

Turk, Z, Ljubic, S, Turk, N & Benko, B 2001, 'Detection of autoantibodies against advanced glycation endproducts and AGE-immune complexes in serum of patients with diabetes mellitus', *Clin Chim Acta*, vol. 303, no. 1-2, pp. 105-15.

Twisk, JW, Boreham, C, Cran, G, Savage, JM, Strain, J & van Mechelen, W 1999, 'Clustering of biological risk factors for cardiovascular disease and the longitudinal relationship with lifestyle of an adolescent population: the Northern Ireland Young Hearts Project', *J Cardiovasc Risk*, vol. 6, no. 6, pp. 355-62.

Ulett, GC, Currie, BJ, Clair, TW, Mayo, M, Ketheesan, N, Labrooy, J, Gal, D, Norton, R, Smith, CA, Barnes, J, Warner, J & Hirst, RG 2001, '*Burkholderia pseudomallei* virulence: definition, stability and association with clonality', *Microbes Infect*, vol. 3, no. 8, pp. 621-31.

Ulett, GC, Ketheesan, N & Hirst, RG 1998, 'Macrophage-lymphocyte interactions mediate anti-*Burkholderia pseudomallei* activity', *FEMS Immunol Med Microbiol*, vol. 21, no. 4, pp. 283-6.

Ulett, GC, Ketheesan, N & Hirst, RG 2000a, 'Cytokine gene expression in innately susceptible BALB/c mice and relatively resistant C57BL/6 mice during infection with virulent *Burkholderia pseudomallei*', *Infect Immun*, vol. 68, no. 4, pp. 2034-42.

Ulett, GC, Ketheesan, N & Hirst, RG 2000b, 'Proinflammatory cytokine mRNA responses in experimental *Burkholderia pseudomallei* infection in mice', *Acta Trop*, vol. 74, no. 2-3, pp. 229-34.

Ulett, GC, Labrooy, JT, Currie, BJ, Barnes, JL & Ketheesan, N 2005, 'A model of immunity to *Burkholderia pseudomallei*: unique responses following immunization and acute lethal infection', *Microbes Infect*, vol. 7, no. 11-12, pp. 1263-75.

Unger, RH 2002, 'Lipotoxic diseases', *Annu Rev Med*, vol. 53, pp. 319-36.

United Kingdom Prospective Diabetes Study Group 1995, 'U.K. prospective diabetes study 16. Overview of 6 years' therapy of type II diabetes: a progressive disease. U.K. Prospective Diabetes Study Group', *Diabetes*, vol. 44, no. 11, pp. 1249-58.

Unlucerci, Y, Bekpinar, S & Kocak, H 2000, 'Testis glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase activities in aminoguanidine-treated diabetic rats', *Arch Biochem Biophys*, vol. 379, no. 2, pp. 217-20.

Utaisincharoen, P, Arjcharoen, S, Limposuwan, K, Tungpradabkul, S & Sirisinha, S 2006, '*Burkholderia pseudomallei* RpoS regulates multinucleated giant cell formation and inducible nitric oxide synthase expression in mouse macrophage cell line (RAW 264.7)', *Microb Pathog*, vol. 40, no. 4, pp. 184-9.

Utaisincharoen, P, Tangthawornchaikul, N, Kespichayawattana, W, Chaisuriya, P & Sirisinha, S 2001, '*Burkholderia pseudomallei* interferes with inducible nitric oxide synthase (iNOS) production: a possible mechanism of evading macrophage killing', *Microbiol Immunol*, vol. 45, no. 4, pp. 307-13.

Uusitupa, M, Schwab, U, Makimattila, S, Karhapaa, P, Sarkkinen, E, Maliranta, H, Agren, J & Penttila, I 1994, 'Effects of two high-fat diets with different fatty acid compositions on glucose and lipid metabolism in healthy young women', *Am J Clin Nutr*, vol. 59, no. 6, pp. 1310-6.

Valade, E, Thibault, FM, Gauthier, YP, Palencia, M, Popoff, MY & Vidal, DR 2004, 'The PmlI-PmlR quorum-sensing system in *Burkholderia pseudomallei* plays a key role in virulence and modulates production of the MprA protease', *J Bacteriol*, vol. 186, no. 8, pp. 2288-94.

Vallerskog, T, Martens, GW & Kornfeld, H 2010, 'Diabetic mice display a delayed adaptive immune response to *Mycobacterium tuberculosis*', *J Immunol*, vol. 184, no. 11, pp. 6275-82.

Vasu, C, Vadivelu, J & Puthucheary, SD 2003, 'The humoral immune response in melioidosis patients during therapy', *Infection*, vol. 31, no. 1, pp. 24-30.

Velazquez-Lopez, L, Gonzalez-Figueroa, E, Medina-Bravo, P, Pineda-Del Aguila, I, Avila-Jimenez, L, Ramos-Hernandez, R, Klunder-Klunder, M & Escobedo-de la Pena, J 2013, 'Low calorie and carbohydrate diet: to improve the cardiovascular risk indicators in overweight or obese adults with prediabetes', *Endocrine*, vol. 43, no. 3, pp. 593-602.

Vendrame, F & Gottlieb, PA 2004, 'Prediabetes: prediction and prevention trials', *Endocrinol Metab Clin North Am*, vol. 33, no. 1, pp. 75-92, ix.

Venketaraman, V, Dayaram, YK, Amin, AG, Ngo, R, Green, RM, Talaue, MT, Mann, J & Connell, ND 2003, 'Role of glutathione in macrophage control of mycobacteria', *Infect Immun*, vol. 71, no. 4, pp. 1864-71.

Venketaraman, V, Dayaram, YK, Talaue, MT & Connell, ND 2005, 'Glutathione and nitrosoglutathione in macrophage defense against *Mycobacterium tuberculosis*', *Infect Immun*, vol. 73, no. 3, pp. 1886-9.

Venketaraman, V, Millman, A, Salman, M, Swaminathan, S, Goetz, M, Lardizabal, A, David, H & Connell, ND 2008, 'Glutathione levels and immune responses in tuberculosis patients', *Microb Pathog*, vol. 44, no. 3, pp. 255-61.

Venketaraman, V, Rodgers, T, Linares, R, Reilly, N, Swaminathan, S, Hom, D, Millman, AC, Wallis, R & Connell, ND 2006, 'Glutathione and growth inhibition of *Mycobacterium tuberculosis* in healthy and HIV infected subjects', *AIDS Res Ther*, vol. 3, p. 5.

Vessby, B 2000, 'Dietary fat and insulin action in humans', *Br J Nutr*, vol. 83 Suppl 1, pp. S91-6.

Vessby, B, Uusitupa, M, Hermansen, K, Riccardi, G, Rivellese, AA, Tapsell, LC, Nalsen, C, Berglund, L, Louheranta, A, Rasmussen, BM, Calvert, GD, Maffetone, A, Pedersen, E, Gustafsson, IB, Storlien, LH & Study, K 2001, 'Substituting dietary saturated for monounsaturated fat impairs insulin sensitivity in healthy men and women: The KANWU Study', *Diabetologia*, vol. 44, no. 3, pp. 312-9.

Vidyalakshmi, K, Lipika, S, Vishal, S, Damodar, S & Chakrapani, M 2012, 'Emerging clinico-epidemiological trends in melioidosis: analysis of 95 cases from western coastal India', *Int J Infect Dis*, vol. 16, no. 7, pp. e491-7.

Vidyalakshmi, K, Shrikala, B, Bharathi, B & Suchitra, U 2007, 'Melioidosis: an under-diagnosed entity in western coastal India: a clinico-microbiological analysis', *Indian J Med Microbiol*, vol. 25, no. 3, pp. 245-8.

Vishnu Prasad, NR, Balasubramaniam, G, Karthikeyan, VS, Ramesh, CK & Srinivasan, K 2012, 'Melioidosis of chest wall masquerading as a tubercular cold abscess', *J Surg Tech Case Rep*, vol. 4, no. 2, pp. 115-7.

Vital, P, Larrieta, E & Hiriart, M 2006, 'Sexual dimorphism in insulin sensitivity and susceptibility to develop diabetes in rats', *J Endocrinol*, vol. 190, no. 2, pp. 425-32.

Vlassara, H, Cai, W, Crandall, J, Goldberg, T, Oberstein, R, Dardaine, V, Peppa, M & Rayfield, EJ 2002, 'Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy', *Proc Natl Acad Sci U S A*, vol. 99, no. 24, pp. 15596-601.

Vuddhakul, V, Tharavichitkul, P, Na-Ngam, N, Jitsurong, S, Kunthawa, B, Noimay, P, Noimay, P, Binla, A & Thamlikitkul, V 1999, 'Epidemiology of *Burkholderia pseudomallei* in Thailand', *Am J Trop Med Hyg*, vol. 60, no. 3, pp. 458-61.

Wagner, AH, Schroeter, MR & Hecker, M 2001, '17beta-estradiol inhibition of NADPH oxidase expression in human endothelial cells', *FASEB J*, vol. 15, no. 12, pp. 2121-30.

Wang, G, Bainbridge, D, Martin, J & Cheng, D 2011, 'N-acetylcysteine in cardiac surgery: do the benefits outweigh the risks? A meta-analytic reappraisal', *J Cardiothorac Vasc Anesth*, vol. 25, no. 2, pp. 268-75.

Wei, M, Gaskill, SP, Haffner, SM & Stern, MP 1998, 'Effects of diabetes and level of glycemia on all-cause and cardiovascular mortality. The San Antonio Heart Study', *Diabetes Care*, vol. 21, no. 7, pp. 1167-72.

Weisberg, SP, McCann, D, Desai, M, Rosenbaum, M, Leibel, RL & Ferrante, AW, Jr. 2003, 'Obesity is associated with macrophage accumulation in adipose tissue', *J Clin Invest*, vol. 112, no. 12, pp. 1796-808.

Wellen, KE & Hotamisligil, GS 2003, 'Obesity-induced inflammatory changes in adipose tissue', *J Clin Invest*, vol. 112, no. 12, pp. 1785-8.

West, DB, Boozer, CN, Moody, DL & Atkinson, RL 1992, 'Dietary obesity in nine inbred mouse strains', *Am J Physiol*, vol. 262, no. 6 Pt 2, pp. R1025-32.

West, DB, Waguespack, J & McCollister, S 1995, 'Dietary obesity in the mouse: interaction of strain with diet composition', *Am J Physiol*, vol. 268, no. 3 Pt 2, pp. R658-65.

West, TE, Ernst, RK, Jansson-Hutson, MJ & Skerrett, SJ 2008, 'Activation of Toll-like receptors by *Burkholderia pseudomallei*', *BMC Immunol*, vol. 9, p. 46.

White, NJ 2003, 'Melioidosis', *Lancet*, vol. 361, no. 9370, pp. 1715-22.

White, NJ, Dance, DA, Chaowagul, W, Wattanagoon, Y, Wuthiekanun, V & Pitakwatchara, N 1989, 'Halving of mortality of severe melioidosis by ceftazidime', *Lancet*, vol. 2, no. 8665, pp. 697-701.

Whitmore, A & Krishnaswami, CS 1912, 'An account of the discovery of a hitherto undescribed infective disease occurring among the population of Rangoon', *Indian Med Gaz*, vol. July, pp. 262-7.

WHO 2003, *Diet, nutrition and the prevention of chronic diseases: report of a joint WHO/FAO expert consultation, Geneva, 28 January - 1 February 2002.*, World Health Organisation, Geneva, Switzerland.

WHO/IDF 2006, 'Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia', viewed 30 July 2013.

Wiersinga, WJ, Currie, BJ & Peacock, SJ 2012, 'Melioidosis', *N Engl J Med*, vol. 367, no. 11, pp. 1035-44.

Wiersinga, WJ, de Vos, AF, de Beer, R, Wieland, CW, Roelofs, JJ, Woods, DE & van der Poll, T 2008a, 'Inflammation patterns induced by different *Burkholderia* species in mice', *Cell Microbiol*, vol. 10, no. 1, pp. 81-7.

Wiersinga, WJ, de Vos, AF, Wieland, CW, Leendertse, M, Roelofs, JJ & van der Poll, T 2008b, 'CD14 impairs host defense against gram-negative sepsis caused by *Burkholderia pseudomallei* in mice', *J Infect Dis*, vol. 198, no. 9, pp. 1388-97.

Wiersinga, WJ, Dessing, MC, Kager, PA, Cheng, AC, Limmathurotsakul, D, Day, NP, Dondorp, AM, van der Poll, T & Peacock, SJ 2007a, 'High-throughput mRNA profiling characterizes the expression of inflammatory molecules in sepsis caused by *Burkholderia pseudomallei*', *Infect Immun*, vol. 75, no. 6, pp. 3074-9.

Wiersinga, WJ, Meijers, JC, Levi, M, Van 't Veer, C, Day, NP, Peacock, SJ & van der Poll, T 2008c, 'Activation of coagulation with concurrent impairment of anticoagulant mechanisms correlates with a poor outcome in severe melioidosis', *J Thromb Haemost*, vol. 6, no. 1, pp. 32-9.

Wiersinga, WJ, van der Poll, T, White, NJ, Day, NP & Peacock, SJ 2006, 'Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*', *Nat Rev Microbiol*, vol. 4, no. 4, pp. 272-82.

Wiersinga, WJ, Wieland, CW, Dessing, MC, Chantratita, N, Cheng, AC, Limmathurotsakul, D, Chierakul, W, Leendertse, M, Florquin, S, de Vos, AF, White, N, Dondorp, AM, Day, NP, Peacock, SJ & van der Poll, T 2007b, 'Toll-like receptor 2 impairs host defense in gram-negative sepsis caused by *Burkholderia pseudomallei* (Melioidosis)', *PLoS Med*, vol. 4, no. 7, p. e248.

Willett, W, Manson, J & Liu, S 2002, 'Glycemic index, glycemic load, and risk of type 2 diabetes', *Am J Clin Nutr*, vol. 76, no. 1, pp. 274S-80S.

Williams, NL, Morris, JL, Rush, C, Govan, BL & Ketheesan, N 2011, 'Impact of streptozotocin-induced diabetes on functional responses of dendritic cells and macrophages towards *Burkholderia pseudomallei*', *FEMS Immunol Med Microbiol*, vol. 61, no. 2, pp. 218-27.

Winzell, MS & Ahren, B 2004, 'The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes', *Diabetes*, vol. 53 Suppl 3, pp. S215-9.

Witschi, A, Reddy, S, Stofer, B & Lauterburg, BH 1992, 'The systemic availability of oral glutathione', *Eur J Clin Pharmacol*, vol. 43, no. 6, pp. 667-9.

Wolever, TM, Jenkins, DJ, Vuksan, V, Jenkins, AL, Buckley, GC, Wong, GS & Josse, RG 1992, 'Beneficial effect of a low glycaemic index diet in type 2 diabetes', *Diabet Med*, vol. 9, no. 5, pp. 451-8.

Wong, KT, Puthucheary, SD & Vadivelu, J 1995, 'The histopathology of human melioidosis', *Histopathology*, vol. 26, no. 1, pp. 51-5.

Woods, DE 2002, 'The use of animal infection models to study the pathogenesis of melioidosis and glanders', *Trends Microbiol*, vol. 10, no. 11, pp. 483-4; discussion 4-5.

Wuthiekanun, V & Peacock, SJ 2006, 'Management of melioidosis', *Expert Rev Anti Infect Ther*, vol. 4, no. 3, pp. 445-55.

Wuthiekanun, V, Pheaktra, N, Putchhat, H, Sin, L, Sen, B, Kumar, V, Langla, S, Peacock, SJ & Day, NP 2008, '*Burkholderia pseudomallei* antibodies in children, Cambodia', *Emerg Infect Dis*, vol. 14, no. 2, pp. 301-3.

Wuthiekanun, V, Smith, MD & White, NJ 1995, 'Survival of *Burkholderia pseudomallei* in the absence of nutrients', *Trans R Soc Trop Med Hyg*, vol. 89, no. 5, p. 491.

Xu, H, Barnes, GT, Yang, Q, Tan, G, Yang, D, Chou, CJ, Sole, J, Nichols, A, Ross, JS, Tartaglia, LA & Chen, H 2003, 'Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance', *J Clin Invest*, vol. 112, no. 12, pp. 1821-30.

Xu, LL, Warren, MK, Rose, WL, Gong, W & Wang, JM 1996, 'Human recombinant monocyte chemotactic protein and other C-C chemokines bind and induce directional migration of dendritic cells in vitro', *J Leukoc Biol*, vol. 60, no. 3, pp. 365-71.

Yabuuchi, E, Kosako, Y, Oyaizu, H, Yano, I, Hotta, H, Hashimoto, Y, Ezaki, T & Arakawa, M 1992, 'Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov', *Microbiol Immunol*, vol. 36, no. 12, pp. 1251-75.

Yakar, S, Nunez, NP, Pennisi, P, Brodt, P, Sun, H, Fallavollita, L, Zhao, H, Scavo, L, Novosyadlyy, R, Kurshan, N, Stannard, B, East-Palmer, J, Smith, NC, Perkins, SN, Fuchs-Young, R, Barrett, JC, Hursting, SD & LeRoith, D 2006, 'Increased tumor growth in mice with diet-induced obesity: impact of ovarian hormones', *Endocrinology*, vol. 147, no. 12, pp. 5826-34.

Yamashiro, S, Kawakami, K, Uezu, K, Kinjo, T, Miyagi, K, Nakamura, K & Saito, A 2005, 'Lower expression of Th1-related cytokines and inducible nitric oxide synthase in mice with streptozotocin-induced diabetes mellitus infected with *Mycobacterium tuberculosis*', *Clin Exp Immunol*, vol. 139, no. 1, pp. 57-64.

Yan, J, Meng, X, Wancket, LM, Lintner, K, Nelin, LD, Chen, B, Francis, KP, Smith, CV, Rogers, LK & Liu, Y 2012, 'Glutathione reductase facilitates host defense by sustaining phagocytic oxidative burst and promoting the development of neutrophil extracellular traps', *J Immunol*, vol. 188, no. 5, pp. 2316-27.

Yan, J, Ralston, MM, Meng, X, Bongiovanni, KD, Jones, AL, Benndorf, R, Nelin, LD, Joshua Frazier, W, Rogers, LK, Smith, CV & Liu, Y 2013, 'Glutathione reductase is essential for host defense against bacterial infection', *Free Radic Biol Med*, vol. 61C, pp. 320-32.

Yap, EH, Chan, YC, Goh, KT, Chao, TC, Heng, BH, Thong, TW, Tan, HC, Thong, KT, Jacob, E & Singh, M 1991, 'Sudden unexplained death syndrome--a new manifestation in melioidosis?', *Epidemiol Infect*, vol. 107, no. 3, pp. 577-84.

Yeni-Komshian, H, Carantoni, M, Abbasi, F & Reaven, GM 2000, 'Relationship between several surrogate estimates of insulin resistance and quantification of insulin-mediated glucose disposal in 490 healthy nondiabetic volunteers', *Diabetes Care*, vol. 23, no. 2, pp. 171-5.

Yoon, KH, Lee, JH, Kim, JW, Cho, JH, Choi, YH, Ko, SH, Zimmet, P & Son, HY 2006, 'Epidemic obesity and type 2 diabetes in Asia', *Lancet*, vol. 368, no. 9548, pp. 1681-8.

Yoshida, K, Hirokawa, J, Tagami, S, Kawakami, Y, Urata, Y & Kondo, T 1995, 'Weakened cellular scavenging activity against oxidative stress in diabetes mellitus: regulation of glutathione synthesis and efflux', *Diabetologia*, vol. 38, no. 2, pp. 201-10.

Zafarullah, M, Li, WQ, Sylvester, J & Ahmad, M 2003, 'Molecular mechanisms of N-acetylcysteine actions', *Cell Mol Life Sci*, vol. 60, no. 1, pp. 6-20.

Zarkesh-Esfahani, H, Pockley, AG, Wu, Z, Hellewell, PG, Weetman, AP & Ross, RJ 2004, 'Leptin indirectly activates human neutrophils via induction of TNF-alpha', *J Immunol*, vol. 172, no. 3, pp. 1809-14.

Zarkesh-Esfahani, H, Pockley, G, Metcalfe, RA, Bidlingmaier, M, Wu, Z, Ajami, A, Weetman, AP, Strasburger, CJ & Ross, RJ 2001, 'High-dose leptin activates human leukocytes via receptor expression on monocytes', *J Immunol*, vol. 167, no. 8, pp. 4593-9.

Zeyda, M, Huber, J, Prager, G & Stulnig, TM 2010, 'Inflammation Correlates With Markers of T-Cell Subsets Including Regulatory T Cells in Adipose Tissue From Obese Patients', *Obesity (Silver Spring)*.

Zhang, H, Forman, HJ & Choi, J 2005, 'Gamma-glutamyl transpeptidase in glutathione biosynthesis', *Methods Enzymol*, vol. 401, pp. 468-83.

Zoccali, C, Mallamaci, F, Tripepi, G, Benedetto, FA, Cutrupi, S, Parlongo, S, Malatino, LS, Bonanno, G, Seminara, G, Rapisarda, F, Fatuzzo, P, Buemi, M, Nicocia, G, Tanaka, S, Ouchi, N, Kihara, S, Funahashi, T & Matsuzawa, Y 2002, 'Adiponectin, metabolic risk factors, and cardiovascular events among patients with end-stage renal disease', *J Am Soc Nephrol*, vol. 13, no. 1, pp. 134-41.

Zykova, SN, Jenssen, TG, Berdal, M, Olsen, R, Myklebust, R & Seljelid, R 2000, 'Altered cytokine and nitric oxide secretion in vitro by macrophages from diabetic type II-like *db/db* mice', *Diabetes*, vol. 49, no. 9, pp. 1451-8.

Zykova, SN, Svartberg, J, Seljelid, R, Iversen, H, Lund, A, Svistounov, DN & Jenssen, TG 2004, 'Release of TNF-alpha from *in vitro*-stimulated monocytes is negatively associated with serum levels of apolipoprotein B in patients with type 2 diabetes', *Scand J Immunol*, vol. 60, no. 5, pp. 535-42.

APPENDIX 1
MEDIA AND REAGENTS

A1.1 General Solutions and Agars

A1.1.1 Phosphate buffered saline (PBS), pH 7.2

NaCl	8g
Na_2PO_4	0.64g
KCl	0.2g
KH_2PO_4	0.16g

Make up to 950ml with single distilled water and adjust pH to 7.2, before bringing volume to 1000ml. Autoclave at 121^{o}C for 15 minutes.

A1.1.2 Haematoxylin and eosin staining technique

Paraffin sections were dewaxed in xylene, treated with graded ethanol (descending concentrations; 100% to 70% v/v), washed in running tap water (1 minute) and stained with haematoxylin (8 minutes). Slides were washed in running tap water for 30 seconds, immersed in Scott's tap water for 30 seconds and washed again in running tap water for 2 minutes. Slides were then stained with eosin (4 minutes), washed in tap water and dehydrated through graded ethanol (70% to 100%) and xylene. Slides were mounted in DPX.

A1.1.2.1 Mayer's haematoxylin

Haematoxylin	2g
Sodium iodate	4g
Aluminium ammonium sulphate	100g
Citric acid	2g
Chlorol hydrate	100g

Combine ingredients with 2L of distilled water.

A1.1.2.2 Eosin

Eosin	15g
Erythrosin	5g
Calcium chloride	5g

Combine ingredients with 2L of distilled water.

A1.1.2.3 Scott's tap water

Sodium carbonate	8.75g
Magnesium sulphate	50g

Combine ingredients with 2.5L of distilled water.

A1.1.3 Herxheimer's staining technique

Stain frozen sections with Mayer's haematoxylin as previously described. Rinse in 70% ethanol and stain in Sudan IV Herxheimer's solution (Thermo Fisher Scientific, Australia) for 1 minute. Rinse in 70% ethanol, followed by water and mount in an aqueous mounting medium.

A1.1.4 Ashdown agar

Agar Technical No. 3	12g
Tryptone	12g
Glycerol	32ml
Crystal violet – 0.1% aqueous	4ml
Neutral red – 1% aqueous	4ml
Single distilled water	800ml

Combine ingredients and boil for 15 minutes. Autoclave at 121^{o}C for 15 minutes, cool to 50^{o}C and add 6.4ml of filter sterilised 1000µg/ml gentamicin sulphate.

A1.1.5 Sheep's blood agar

Agar Technical No. 3	12g
Sheep's blood	5%
Single distilled water	800ml

Combine agar with water and boil for 15 minutes. Autoclave at 121°C for 15 minutes, cool to 50°C and add sheep's blood.

A1.2 Cell Culture Media and Reagents

A1.2.1 Single strength culture medium

RPMI 1640	1L
Penicillin	100,000 Units
Streptomycin	100mg
HEPES Buffer	20mM

L-Glutamine	2mM
Heat-inactivated foetal bovine serum (HIFBS)	100ml

NB: Double concentration of HEPES Buffer and L-Glutamine for double strength culture medium.

A1.3.2.1 L-glutamine stock solution

FCC grade L-glutamine	15g
RPMI 1640	1000ml

Combine ingredients and filter sterilise (0.22µm). Dispense into 2ml aliquots and store at -70^{o}C until use.

A1.3.2.2 Heat-inactivated foetal bovine serum (HIFBS)

Heat 400ml of FBS (Life Technologies) at 56^{o}C for 25 minutes. Cool to room temperature. Aliquot into 10ml plastic tubes and store at -20^{o}C until use.

A1.2.2 Heparin-PBS (lavage medium)

PBS, pH 7.2	100ml
Porcine heparin	1000 IU

A1.3 Glutathione Modulators and Reagents

A1.3.1 Buthionine sulfoximine

Buthionine sulfoximine	338mg
PBS, pH 7.2	5ml

Dissolve buthionine sulfoximine (BSO) in PBS.

A1.3.2 N-acetyl cysteine

N-acetyl cysteine	513mg
PBS, pH 7.2	5ml

Dissolve N-acetyl cysteine (NAC) in PBS and adjust pH to 7.2 with NaOH.

A1.3.3 2-Vinylpyridine

2-vinylpyridine	27µl
Ethanol	98µl

Use immediately after mixing.

APPENDIX 2

SUPPLEMENTARY DATA

A2.1 Bacterial loads in C57BL/6 *Lept*^{db} mice (Chapter 4)

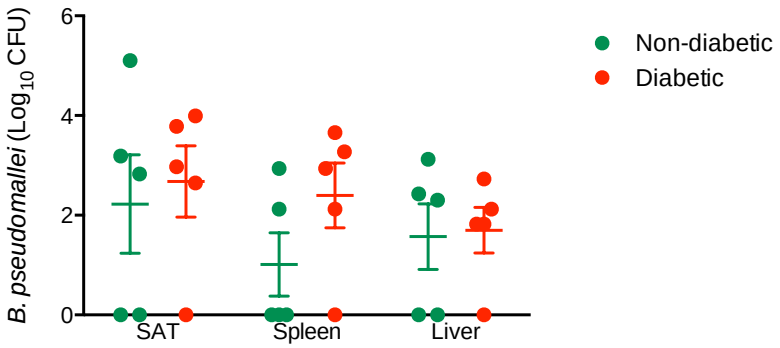

Figure A2.1 Bacterial loads in C57BL/6 *Lept*^{db} mice at 24 hours post-infection
C57BL/6 diabetic (*db/db*) and non-diabetic (*db/+*) mice were infected subcutaneously with 4.5×10^5 CFU of *B. pseudomallei*. At 24 hours post-infection, *B. pseudomallei* levels were comparable in the SAT, spleen and liver of diabetic and non-diabetic mice. *n* =5 per group; mean Log_{10} CFU ± SEM.

A2.2 Glucose tolerance of B6D2F1 mice at 5 weeks (Chapter 5)

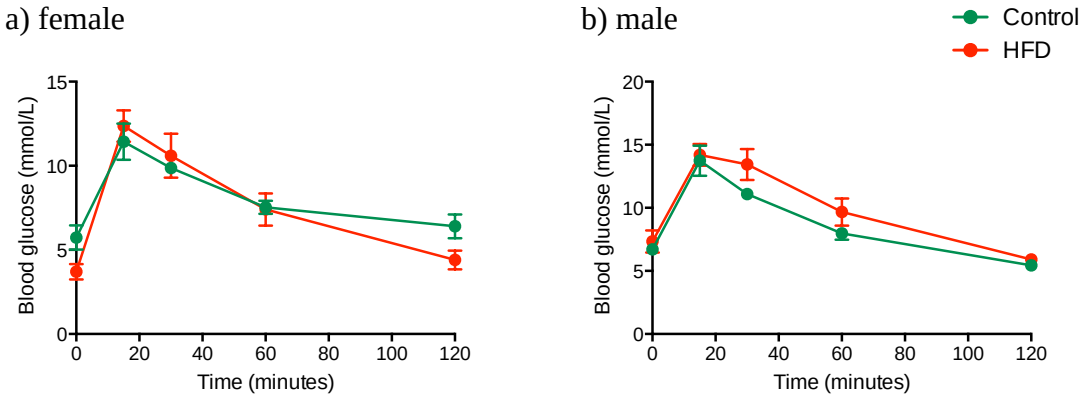

Figure A2.2 Glucose tolerance of B6D2F1 mice after 5 weeks of diet intervention
Glucose tolerance of a) female and b) male mice was comparable between control and HFD-fed mice after 5 weeks of diet intervention. *n* = 5 per group; mean ± SEM.

A2.3 Glucose tolerance of B6D2F1 mice at 10 weeks (Chapter 5)

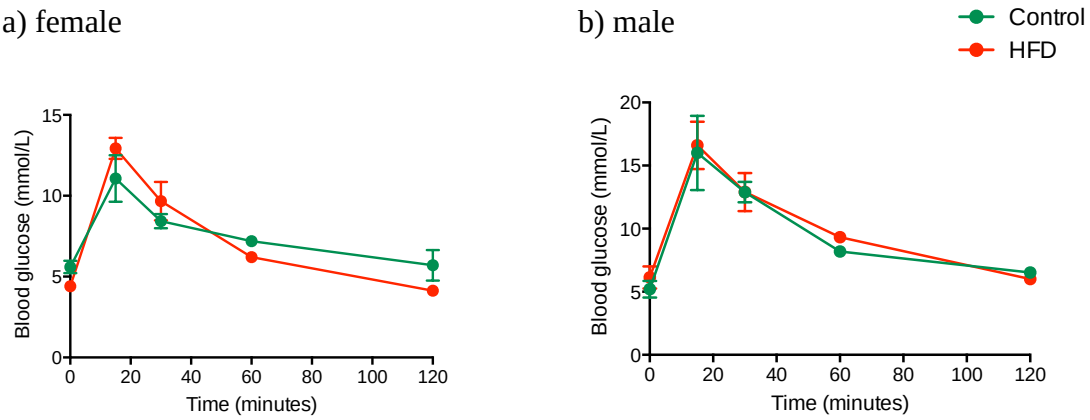

Figure A2.3 Glucose tolerance of B6D2F1 mice after 10 weeks of diet intervention

Glucose tolerance of a) female and b) male mice was comparable between control and HFD-fed mice after 10 weeks of diet intervention. *n* = 5 per group; mean ± SEM.

A2.4 Dietary intake of B6D2F1 mice (Chapter 5)

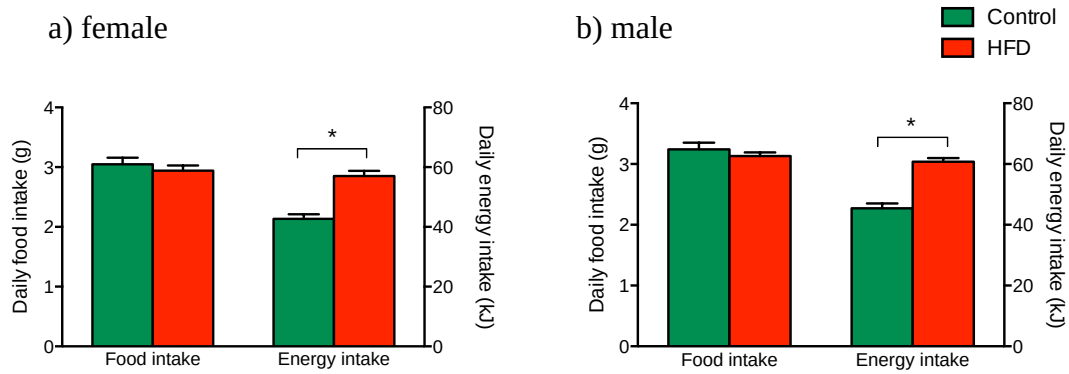

Figure A2.4 Daily dietary intakes of female and male B6D2F1 mice on an isometrically controlled dietary regime

Food intake was measure bi-weekly for 10 weeks of diet intervention (*n* = 20 per group). Energy consumption was significantly higher in a) female and b) male mice consuming a HFD, due to the higher energy density of the diet, compared to control mice. * $P < 0.05$; mean ± SEM.

A2.5 Glucose tolerance of B6D2F1 mice at 15 weeks (Chapter 5)

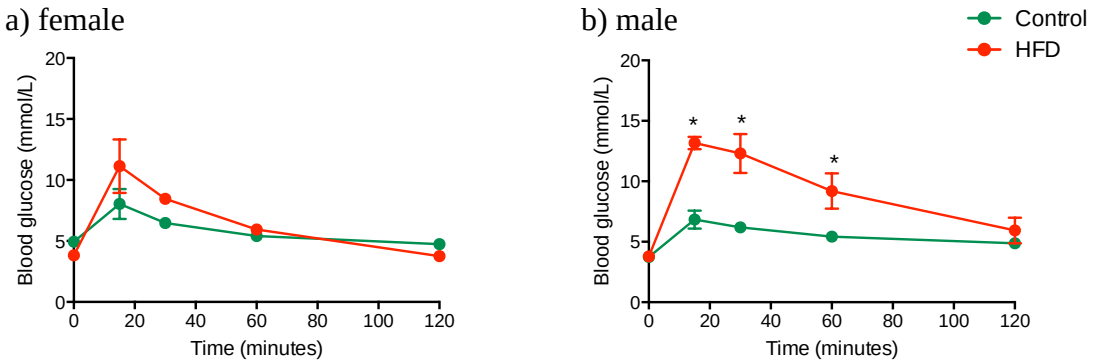

Figure A2.5 Glucose tolerance of B6D2F1 mice after 15 weeks of diet intervention

Glucose tolerance of a) female mice was comparable between dietary groups after 15 weeks of isometrically controlled diet intervention. b) Glucose tolerance of male mice was significantly impaired in mice consuming a HFD compared to an isometric control diet at 15 weeks of diet intervention. *n* = 5 per group; * *P* < 0.05; mean ± SEM.

A2.6 Glucose tolerance of B6D2F1 and C57BL/6 mice (Chapter 6)

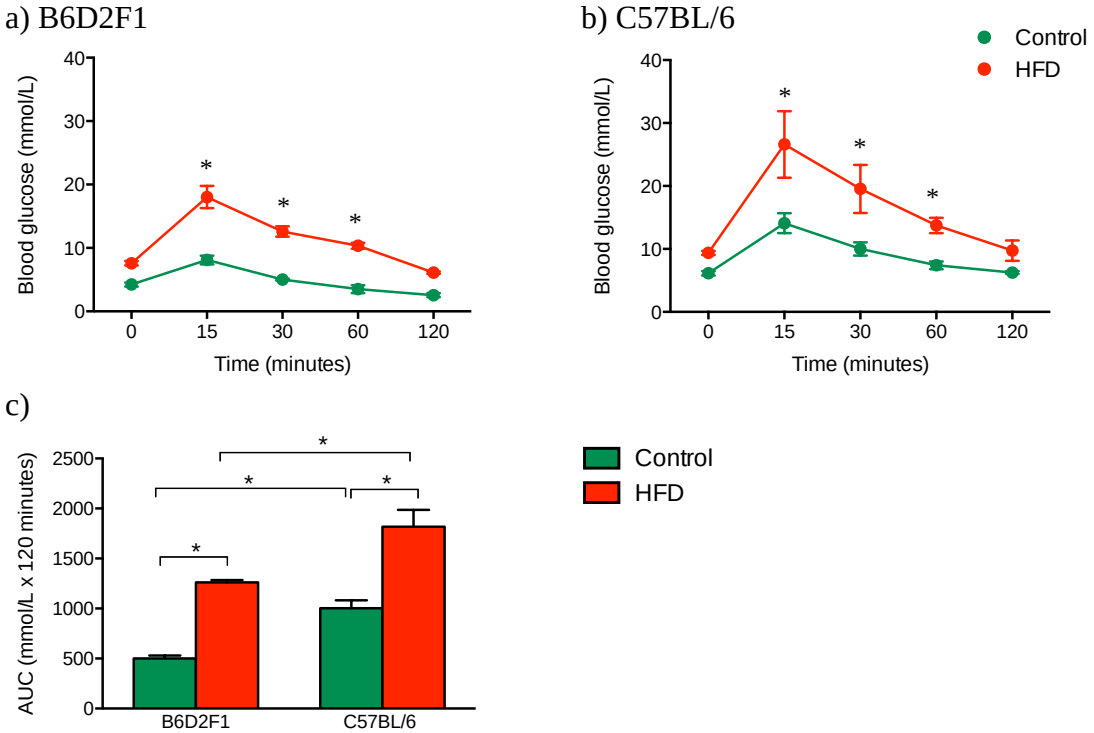

Figure A2.6 Glucose tolerance of B6D2F1 and C57BL/6 mice after diet intervention

After 10 weeks of HFD feeding, glucose tolerance was impaired in a) B6D2F1 and b) C57BL/6 mice compared to control littermates. Glucose intolerance measured as c) AUC, was more severe in C57BL/6 mice compared to B6D2F1 mice regardless of dietary group. *n* = 5 per group; * *P* < 0.05; mean ± SEM.

A2.7 Baseline levels of IL-10 in C57BL/6 mice (Chapter 7)

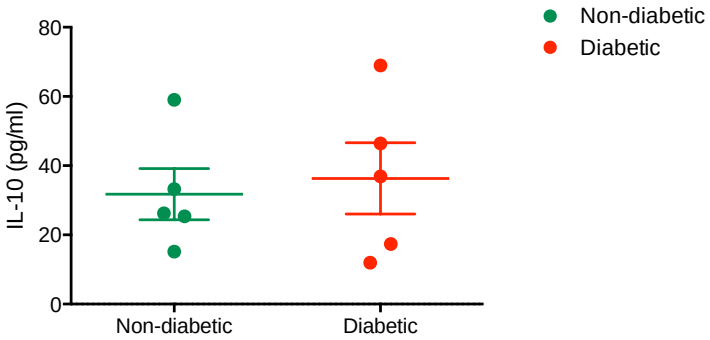

Figure A2.7 Baseline levels of IL-10 in spleen of C57BL/6 mice after 15 weeks of consuming a HF/HGD (diabetic) or control diet (non-diabetic)
After 15 weeks of diet intervention, concentrations of IL-10 were comparable in the spleen of diabetic and non-diabetic mice. *n* = 5 per group; mean ± SEM.

APPENDIX 3
PUBLICATIONS

A3.1 Publication 1

Hodgson, K. A., Govan, B. L., Walduck, A. K., Ketheesan, N., and Morris, J. L. (2013). Impaired early cytokine responses at the site of infection in a murine model of type 2 diabetes and melioidosis comorbidity. *Infection and Immunity, 81*(2), 470-477.

A3.2 Publication 2

Hodgson, K., Govan, B., Ketheesan, N., and Morris, J. (2013). Dietary composition of carbohydrates contributes to the development of experimental type 2 diabetes. *Endocrine, 43*(2), 447-451.

A3.3 Publication 3

Hodgson, K. A., Morris, J. L., Feterl, M. L., Govan, B. L., and Ketheesan, N. (2011). Altered macrophage function is associated with severe Burkholderia pseudomallei infection in a murine model of type 2 diabetes. *Microbes and Infection / Institut Pasteur, 13*(14-15), 1177-1184.

A3.4 Publication 4

Morris, J. L., Hodgson, K. A., and Ketheesan N. (2012). Section VII.4 Development of protection. In N. Ketheesan (Ed.), *Melioidosis - A Century of Observation and Research*. The Netherlands: Elsevier B. V., pp 282-299.