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Optimization of Burkholderia Glycoconjugate Vaccines: A Reverse Vaccinology Approach

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Optimization of *Burkholderia* Glycoconjugate Vaccines:

A Reverse Vaccinology Approach

by

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Dedication

To my husband Tony, whose constant love, support and encouragement inspire me to follow my dreams, no matter how big they might be.

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Optimization of *Burkholderia* Glycoconjugate Vaccines: A Reverse Vaccinology Approach

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Burkholderia pseudomallei is a Gram-negative, intracellular pathogen and the etiological agent of melioidosis. Because of intrinsic multi-drug resistance, lack of effective treatment and high case-fatality rates, this organism is classified as a Tier 1 Select Agent and considered a priority for vaccine development. Previous studies have shown that glycoconjugate vaccines can provide enhanced protection against lethal *B. pseudomallei* challenge. However, the limited pool of *Burkholderia* antigens hinders continued optimization of these vaccines. In this study, we used a reverse vaccinology approach to identify outer membrane and secreted *Burkholderia* proteins. These proteins were ranked according to predicted immunogenicity, and top vaccine candidates were selected based on the number and affinity of Major Histocompatibility Complex (MHC) epitopes. To confirm the *in silico* immunogenicity predictions, the top seven proteins were purified and evaluated for seroreactivity against convalescent human and experimental murine melioidosis sera. All proteins were shown to exhibit varying reactivity with convalescent

sera. To evaluate immunogenicity *in vivo*, a series of vaccination studies were performed in mice. Recombinant proteins were shown to be immunogenic in mice, generating high antibody titers irrespective of administration route, concentration or adjuvant. Despite the ability to induce a strong humoral immune response, vaccination did not protect animals from lethal *B. pseudomallei* challenge. To evaluate whether immunogenic proteins could enhance the immunogenicity of a glycoconjugate vaccine, we optimized a method for the construction of a gold-nanoparticle (AuNP) glycoconjugate vaccines and evaluated immunogenicity in mice. Subcutaneous administration of AuNP-glycoconjugate vaccines resulted in high anti-lipopolysaccharide (LPS) responses, a correlate of protection in human and animal melioidosis. Additionally, immune sera were shown to facilitate uptake of *B. pseudomallei* by murine macrophages *in vitro*. While AuNP-glycoconjugate vaccination did not afford protection against lethal challenge, the ability to induce high antibody titers confirms immunogenicity and provides a strong rationale for continued optimization of this platform.

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List of Abbreviations

ABSL	Animal biosafety lab
APC	Antigen presenting cell
AuNP	Gold nanoparticle
BCA	Bicinchoninic assay
BCR	B-cell receptor
Bpm	*Burkholderia pseudomallei*
Bm	*Burkholderia mallei*
Bth	*Burkholderia thailandensis*
BSA	Bovine Serum Albumin
BSL	Biosafety level
CFU	Colony forming units
CpG	Cytosine-guanine dinucleotides
CNS	Central nervous system
CPS	Capsular Polysaccharide
CRM197	Cross-reactive material 197
DC	Dendritic cell
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle's Medium
DMTMM	4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol

ECL	Enhanced chemiluminescence
EDC	N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMCH	6-maleimidocaproic acid hydrazide
EtOH	Ethanol
FBS	Fetal bovine serum
G-CSF	Granulocyte colony stimulate factor
HBSS	Hank's balanced salt solution
Hcp	Hemolysin-coregulatory protein
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IHA	Indirect hemagglutinin assay
IL	Interleukin
IM	Intramuscular
IN	Intranasal
IP	Intraperitoneal
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IV	Intravenous
Km	Kanamycin
LB	Luria Bertani
LBG	Luria Bertani with 4% glycerol

LD50	Median Lethal Dose
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MALDI-ToF	Matrix-assisted laser desorption/ionisation time-of-flight
MCP-1	Monocyte chemotactic protein-1
MED	Mean epitope density
MES	2-(N-morpholino) ethanesulfonic acid
MLD	Mean lethal dose
MeOH	Methanol
MHC	Major histocompatibility complex
MHDA	16-mercaptohexadecanoic acid
MNGC	Multinucleated giant cell
MOI	Multiplicity of Infection
MWCO	Molecular weight cut off
NGS	Normal goat serum
NHP	Non-human primate
NHS	N-hydroxysuccinimide
NO	Nitric oxide
NP	Nanoparticle
OMP	Outer membrane protein
OMV	Outer membrane vesicles
ORF	Open reading frame
PBS	Phosphate buffered saline

PMN	Polymorphonuclear
PVDF	Polyvinylidene fluoride
SATA	S-acetylthioglycolic acid N-hydroxysuccinimide ester
SC	Subcutaneous
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
Tc	Cytotoxic T-cell
TEM	Transmission Electron Microscopy
TetHc	Tetanus toxin heavy chain
Th	T-helper cell
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TMP-SMX	Trimethoprim-sulfamethoxazole
TNF-α	Tumour necrosis factor – alpha
T3SS	Type III Secretion System
T6SS	Type VI Secretion System
UTMB	University of Texas Medical Branch
UV/Vis	Ultraviolet/Visible

INTRODUCTION

Chapter 1 *Burkholderia pseudomallei*

THE PATHOGEN

First described by Whitmore in 1912, *Burkholderia pseudomallei* has received many classifications throughout the last century, including *Bacillus whitmori, Malleomyces pseudomallei* and *Pseudomonas pseudomallei* (Winston R. Miller, 1948). The *Burkholderia* genus is comprised of facultatively aerobic, Gram negative bacilli. When it was first proposed in 1992, this genus consisted of only seven species, including *Burkholderia pseudomallei*, *Burkholderia mallei*, and *Burkholderia cenocepacia* (Eberl and Vandamme, 2016). Since then, nearly 100 different *Burkholderia* species have been described, the majority of which are non-pathogenic and comprise diverse environmental niches.

B. pseudomallei and *B. mallei*, the most pathogenic members of the *Burkholderia* genus, are the etiological agents of the diseases melioidosis and glanders, respectively. Together with *B. thailandensis*, these species represent genetically similar bacteria that occupy different reservoirs. *B. mallei* – a host-restricted bacterium—is believed to have evolved from *B. pseudomallei* via reductive evolution, a process by which it lost the ability to adapt and survive in the environment. Because *B. mallei* shares 99% genetic identity with *B. pseudomallei*, it retained some defining characteristics, including virulence factors, intracellular lifestyle, routes of infection and similar disease manifestations (Nierman et al., 2004).

Despite its environmental reservoir and low virulence, *B. thailandensis* still retains 93% genetic similarity with *B. pseudomallei* (Kim et al., 2005) and has served as an invaluable tool for elucidating the predicted pathogenesis of *B. pseudomallei* under Biosafety Level 2 conditions.

First sequenced in 2004, the *B. pseudomallei* K96243 genome is one of the most complex bacterial genomes described to date. The 7.25 Mb genome is comprised of two large chromosomes, encoding >5,000 proteins. Interestingly, each chromosome contains distinct gene signatures. The larger chromosome (4.1 Mb) is considered the "housekeeping chromosome" and encodes genes necessary for bacterial metabolism and growth. In contrast, the smaller chromosome (3.17 M) encodes many genes involved in bacterial adaptation (Holden et al., 2004). Chromosome 2 is thought to be less conserved between species and has been shown to display varied expression in response to bacterial growth conditions (Ong et al., 2004; Ooi et al., 2013). The sheer size of these chromosomes, together with the diversity and redundancy of adaptive and metabolic genes, provides *B. pseudomallei* with sufficient genetic material to thrive in numerous ecological niches.

B. pseudomallei displays an astounding ability to adapt and survive in diverse environments, and has been isolated from a wide range of conditions, including wound irrigation fluid (Merritt et al., 2016), hand wash detergent (Gal et al., 2004), water pH ranging from 2-9 (Finkelstein et al., 2000) and chlorinated water up to 1000 ppm (Howard and Inglis, 2003). Remarkably, this organism is capable of utilizing more than 85 different compounds as sole carbon sources (Smith CJ, 1987) and has been shown to survive for many years in distilled water (Wuthiekanun et al., 1995). Existing naturally in

the soil and water, *B. pseudomallei* displays a proclivity for tropical and subtropical environments. In addition to the endemic "hot spots" of Thailand and northern Australia, *B. pseuodomallei* has also been isolated in India, Cambodia, Malaysia and nearly 40 other countries (Limmathurotsakul et al., 2016). The widespread endemicity of *B. pseudomallei* comprises several South American and Caribbean countries, including Brazil, El Salvador, Puerto Rico and Haiti (Dance, 1991, 2015; Doker et al., 2015). In North America, nearly all of the reported human melioidosis cases occur in laboratory personnel or persons with recent travel to endemic places (Benoit et al., 2015). Although *B. pseudomallei* has never been definitively isolated from North American soil, recent reports suggest that southern Florida, Texas and Louisiana are environmentally suitable for *B. pseudomallei* colonization, highlighting the potential for global spread (Limmathurotsakul et al., 2016).

B. pseudomallei comprises a heterogenous bacterial species. This heterogeneity has presented a challenge to researchers, as inherent differences in bacterial strains make comparative analyses difficult. *B. pseudomallei* isolates, both clinical and environmental, have been shown to exhibit vastly different metabolic profiles, antibiotic susceptibility, genomic content and virulence (Anuntagool et al., 2006; Challacombe et al., 2014; Chantratita et al., 2008; DeShazer, 2004; Thibault et al., 2004). However, certain major virulence factors appear to be conserved, including lipopolysaccharide (LPS) (Arjcharoen et al., 2007; Wikraiphat et al., 2009), capsular polysaccharide (CPS) (Atkins et al., 2002a; Reckseidler-Zenteno et al., 2005; Wikraiphat et al., 2009; Woodman et al., 2012), Type 3 Secretion System (T3SS) (Burtnick et al., 2008; Stevens et al., 2002; Warawa and Woods, 2005), and the Type 6 Secretion System (T6SS) (Burtnick et al., 2011; Schell et

al., 2007). Because *B. pseudomallei* is a heterogenous pathogen, selection of conserved candidates is essential for vaccine development against this pathogen.

Chapter 2 Melioidosis

DISEASE MANIFESTATIONS, TREATMENT AND DIAGNOSIS

Melioidosis can be acquired through cutaneous inoculation, inhalation or ingestion of the organism *B. pseudomallei*. Inhalation of this bacterium results in the most rapid and fulminant disease whereas percutaneous inoculation is slower to progress and is often limited to a cutaneous lesion (Currie et al., 2010; Yeager et al., 2012). While subcutaneous inoculation is thought to be the most common route for infection, there is a shift towards inhalational exposure during rainy monsoon seasons, as more than 75% of melioidosis cases in Thailand and Australia occur during periods of intense rainfall (Currie et al., 2000; Currie and Jacups, 2003).

B. pseudomallei can cause asymptomatic infections in healthy individuals, or can induce an acute, chronic or latent disease. The incubation period is thought to last between 1-21 days; however, the onset of symptoms and severity of disease are thought to arise from the number and virulence of inoculating organisms, the route of infection and the competency of the host immune response (Ngauy et al., 2005). This disease most commonly affects individuals with underlying conditions, including those with a history of type II diabetes, excessive alcohol consumption or chronic lung disease (Churuangsuk et al., 2016; Currie et al., 2010; Suputtamongkol et al., 1999). Called "the great mimicker", *B. pseudomallei* infections can present with a wide variety of symptoms ranging from localized skin lesions to pneumonia, bacteremia and sepsis (Currie et al., 2010). Target organs commonly include the lung, spleen and prostate, but *B. pseudomallei* has also been shown to establish infections in the bone marrow, central

nervous system (CNS), kidneys, and gastrointestinal tract (Currie, 2015). Soft tissue abscesses involving the spleen, prostate, liver and kidneys are also common findings.

While the majority of human meliodiosis cases present as acute infections, this bacterium is also able to induce chronic infections. In the most extreme example of chronic *B. pseudomallei* infection, a patient presented with symptoms more than 60 years following initial exposure (Ngauy et al., 2005). This potential for reactivation has led to particular concern for military personnel stationed in endemic areas. This phenomenon, called the "Vietnamese Time Bomb", became apparent after the Vietnam War. Military personnel stationed in Vietnam were exposed to *B. pseudomallei* through the contaminated water and dirt aerated by helicopter blades (Howe et al., 1971; Sanford and Moore, 1971). Serological studies after the war revealed that as many as 20% of returning military personnel were seropositive (as determined by Indirect Hemagglutinin Assay, or IHA) post-deployment to Vietnam (Clayton et al., 1973; Sanford and Moore, 1971)

Treatment of this disease is limited, as *B. pseudomallei* is intrinsically resistant to major antibiotic classes, including many β-lactams, macrolides and aminoglycosides (Wiersinga et al., 2012). Treatment regimens are usually biphasic, including an intensive phase (2-8 weeks) of intravenous (IV) antibiotics, followed by an eradication phase (3-6 months) of oral antibiotics. Commonly used antibiotics include IV Ceftaxidime (intensive phase) and oral Trimethoprim-sulfamethoxazole (TMP-SMX, eradication phase) (Currie, 2015; Wiersinga et al., 2012). Misdiagnosis and subsequent mistreatment of the disease can cause case fatality rates to exceed 70% (White et al., 1989). Even with appropriate antibiotic therapy, relapse occurs in 12% of patients and case-fatality rates approach 40% in certain regions of Thailand (Limmathurotsakul et al., 2010).

The widespread resistance of *B. pseudomallei*, together with the high morbidity and mortality it causes, emphasizes the urgency for quick and accurate diagnosis. The current diagnostic "gold standard" is to culture *B. pseudomallei* from commonly collected samples like blood, sputum, pus or urine (Currie, 2015). However, this process is slow and error prone. To address this, recent research has focused on developing more rapid and accurate diagnostic tests, including latex agglutination, PCR, lateral flow and ELISA (Duval et al., 2014; Houghton et al., 2014; Hsu et al., 2013; Janse et al., 2013; Sorenson et al., 2013). While these diagnostic tests are highly promising, commercialization and distribution to resource-poor areas remains a major challenge.

Because of its high morbidity and mortality, limited treatment options and severity of disease upon inhalation, *B. pseudomallei* is classified by the U.S. Department of Health and Human Services (HHS) as a Tier 1 Select Agent, and is currently considered a priority for vaccine development.

Chapter 3 Immune Response

INNATE IMMUNE RESPONSE

The importance of the innate immune response for the initial detection and killing of *B. pseudomallei* is well understood. Macrophages and neutrophils are essential in controlling bacterial spread and inhibition of these cells in rodent models results in severely exacerbated disease (Barnes et al., 2008; Breitbach et al., 2006; Easton et al., 2007). For this reason, the innate immune dysregulation observed in type II diabetes is thought to account for the severe susceptibility of this population subset (Chanchamroen et al., 2009; Hodgson et al., 2011; Krishnananthasivam et al., 2017; Morris et al., 2012; Riyapa et al., 2012; Saengmuang et al., 2014)

Once inside the host, *B. pseudomallei* infects a variety of host cells and colonizes distant sites via hematogenous or lymphatic spread. *B. pseudomallei* possesses the ability to survive and replicate not only within the extracellular milieu, but also within a wide variety of host cells, including: epithelial cells, macrophages, neutrophils and dendritic cells (Chanchamroen et al., 2009; Pruksachartvuthi et al., 1990; Williams et al., 2014). Additionally, *B. pseudomallei*-infected dendritic cells (DCs) have been shown to facilitate spread of this bacterium to lymphoid tissues in a mouse model (Williams et al., 2014). This intracellular lifestyle is a distinct advantage for this bacterium, as it allows evasion of immune detection and antibiotic killing. To survive within these innate cells, *B. pseudomallei* has evolved elaborate mechanisms to suppress innate immune cell functions. Inside the cell, *B. pseudomallei* is able to inhibit the MyD88 pathway, suppress autophagy, and reduce expression of reactive nitrogen intermediates (e.g., nitric oxide) (Devenish and Lai, 2015; Pudla et al., 2011; Utaisincharoen et al., 2001) *B. pseudomallei*

is also capable of rearranging host cell actin to form multi-nucleated giant cells (MNGC), allowing cell-to-cell spread without exiting the cell (Burtnick et al., 2011).

B. pseudomallei LPS has been shown to play an important role in the modulating the innate immune response during infection. Like many other Gram negative bacteria, the lipid A moiety from *B. pseudomallei* LPS activates Toll-like Receptor 4 (TLR4). Interestingly, TLR2 has also been shown to play a role in the recognition of *B. pseudomallei* LPS during human and murine infection (Weehuizen et al., 2015; Wiersinga et al., 2007). Initial studies showed that *B. pseudomallei* LPS is weakly inflammatory in mice and approximately 30 times less toxic when compared to LPS from *Staphylococcus aureus* (Matsuura et al., 1996). Additionally, stimulation of RAW 264.7 macrophages with *B. pseudomallei* LPS resulted in a delayed and overall decreased production of nitric oxide (NO) and tumor necrosis factor-α (TNFα) compared to *Escherichia coli* or *Salmonella enterica* Typhi LPS (Utaisincharoen et al., 2000). However, in Thailand, stimulation of whole human blood with *B. pseudomallei* LPS was shown to stimulate significant TNF-α, interleukin-10 (IL-10), monocyte chemotactic protein 1 (MCP-1), granulocyte-colony stimulating factor (G-CSF), and IL-1β responses. Importantly, these cytokine responses were similar to those induced by whole cell *B. pseudomallei* stimulation, suggesting that *B. pseudomallei* LPS plays a major role in innate immune recognition, at least in endemic areas (Chantratita et al., 2008). However, the role of *B. pseudomallei* LPS in the innate immune response of seronegative humans has not been determined.

ADAPTIVE IMMUNE RESPONSE

The role of adaptive immunity to *B. pseudomallei* pathogenesis remains poorly understood and oftentimes controversial. Heterogeneity among *B. pseudomallei* species, together with differences in challenge route and dose, likely account for discrepancies between studies. Dendritic cells – which bridge the gap between innate and adaptive immunity – have been shown to upregulate expression of IL-12, Major Histocompatibility Complex (MHC) II and co-stimulatory molecule CD86 upon infection with *B. pseudomallei ex vivo* (Williams et al., 2014).

$CD4^+$ T-lymphocytes are thought to be important for adaptive immunity against *B. pseudomallei*, particularly in the later stages of infection. Studies have demonstrated that deletion of $CD4^+$ T-cells in rodent models results in decreased survival following challenge (Haque et al., 2006a; Haque et al., 2006b). In contrast, Silva *et al* demonstrated that deletion of $CD4^+$ and/or $CD8^+$ T-cells had no effect on vaccine-induced immunity to *B. pseudomallei* (Silva et al., 2013). However, these studies used different bacterial strains and routes, which may account for these incongruent findings.

The role of $CD8^+$ T-lymphocytes remains poorly understood. Several studies have shown that deletion of $CD8^+$ T-cells does not affect survival against *B. pseudomallei* in rodent models (Haque et al., 2006a; Haque et al., 2006b; Silva et al., 2013). However, other studies have shown that human melioidosis patients develop $CD8^+$ T-cell memory against *Burkholderia* antigens. Specifically, antigen-pulsed DCs were shown to induce Granzyme B production from $CD8^+$ T-cells from seropositive donors (Tippayawat et al., 2011). Additionally, recent studies have demonstrated that both $CD4^+$ and $CD8^+$ T-cells from surviving melioidosis patients produced increased interferon-γ (IFNγ) compared to

T-cells from deceased patients (Jenjaroen et al., 2015). Despite conflicting murine studies, human responses seem to support the idea that $CD4^+$ and $CD8^+$ cells are important in protection against *B. pseudomallei*.

Chapter 4 Vaccine Development Against *B. pseudomallei*

WHOLE CELL VACCINES

For the last two decades there has been considerable effort towards developing a vaccine against *B. pseudomallei.* Various vaccine candidates have been tested, including killed/irradiated, live attenuated, and subunit vaccines. While many of these studies have been able to provide protection against *B. pseudomallei*, no vaccine has been able to achieve sterilizing immunity. Early vaccine studies focused on evaluating the protective capacity of killed or irradiated *B. pseudomallei* on disease outcome. Immunization with heat killed *B. pseudomallei* was able to provide up to 90% protection post-challenge when administered intradermally via infected DCs; however, significantly lower protection was observed when heat killed *B. pseudomallei* was administered alone (Barnes and Ketheesan, 2007; Elvin et al., 2006; Healey et al., 2005; Sarkar-Tyson et al., 2009).

Live-attenuated vaccines have provided the most significant protection against *B. pseudomallei*, namely because of their ability to mimic natural infection. Numerous studies have examined the protective capacity of various live attenuated vaccines, including mutant strains deficient in biosynthesis pathways (Atkins et al., 2002a; Atkins et al., 2002b; Breitbach et al., 2008; Haque et al., 2006a; Srilunchang et al., 2009) and virulence factors (Stevens et al., 2004). The most significant protection has been achieved with a *B. pseudomallei* strain deficient in biosynthesis of branched chain amino acids. A single intraperitoneal (i.p.) immunization with this strain provided up to 100% protection against acute challenge (Atkins et al., 2002b). This strain did not persist in the murine host beyond 30 days post-immunization, and was shown to induce T-cell mediated

responses (Haque et al., 2006a). Despite its inability to induce sterilizing immunity, the efficacy of this vaccine has led it to be considered the 'gold standard' against which new vaccine formulations should be compared (Limmathurotsakul et al., 2015).

SUBUNIT VACCINES

Subunit vaccines are preferred over live-attenuated vaccines due to their ability to be administered to a more diverse population (e.g., immunocompromised) without fear of reactivation or dormancy. This is an important consideration for *B. pseudomallei* vaccine development, as immunocompromised patients are considered highly susceptible to infection. However, subunit vaccines are often poorly immunogenic – a fact likely attributed to their rapid degradation inside the mammalian host. To address this need, many groups have explored the use of novel adjuvants or platforms in order to extend vaccine half-life and improve immunogenicity. Because many parameters can be altered to achieve increased efficacy, subunit vaccines often require extensive optimization in order to elicit a robust immune response. Subunit vaccines for Biosafety Level (BSL) 3 Select Agent pathogens like *B. pseudomallei* can be manufactured under BSL2 conditions without potential hazards to laboratory personnel. For these reasons, there is a particular interest in developing subunit vaccines against *B. pseudomallei.*

Initial studies focused on elucidating the protective capacity of various *Burkholderia* proteins. In these studies, protein antigens were most commonly selected because of similarity with other known antigens or association with known virulence factors. Immunization with individual proteins has been shown to provide partial protection against lethal challenge in rodent models but does not protect from bacterial colonization (Table 1). In an effort to generate a more robust immune response, recent

Table 1. Subunit vaccine approaches against *B. pseudomallei*

		Immunization						Challenge		
Reference	**Subunit (s)**	**Adjuvant(s)**	**Dose**	**Duration**	**Route**	**Animal model**	**Strain**	**Route**	**Dose**	**% Survival**
(Nelson et al., 2004)	CPS	---	25 ug/ml	3 doses in 28 days	i.p.	BALB/c	NCTC 4845	i.p.	$2x10^4$	0% at day 28
		RAS								10% at day 35
	LPS	---								50% at day 35
		RAS								10% at day 35
(Chen et al., 2006b)	Flagellin	CFA	50 ug	3 doses in 28 days	i.m.	BALB/c	mixed isolates	i.v.	$1x10^5$	50% at day 7
(Harland et al., 2007)	LolC	MPL+TDM	10ug	3 doses in 28 days	i.p.	BALB/c	K96243	i.p.	$4x10^4$	80% at day 42
									$7x10^4$	0% at day 40
	PotF3	ISCOM-CpG		3 doses in 49 days					$7x10^4$	40% at day 50
	OppA	MPL+TDM		3 doses in 28 days					$4x10^4$	50% at day 42
									$4x10^4$	22% at day 42
(Legutki et al., 2007)	Peptide mimotopes of exopolysaccharide conjugated to thyroglobulin	Alum	50ug	3 doses in 28 days	i.p.	BALB/c	NCTC 4845	i.p.	250 MLD	0% at day 28
(Druar et al., 2008)	BipB, BipC, and BipD	CFA	100ug	Single dose	s.c.	BALB/c	Ashdown	i.p.	970	0% at day 5
(Hara et al., 2009)	Omp3 andOmp7	CFA (prime) and IFA (both boosts)	50ug	3 doses in 9 weeks	i.p.	BALB/c	D286	i.p.	$1x10^6$	50% at day 21
(Su et al., 2010)	Omp85	CFA (prime) and IFA (both boosts)	20ug	3 doses in 42 days	i.p.	BALB/c	D286	i.p	$1x10^6$	70% at day 15
(Ngugi et al., 2010)	LPS	---	10ug	3 doses in 28 days	i.p.	BALB/c	K96243	i.p	$2x10^4$	50% at day 35
(Whitlock et al., 2010)	BopA	CpG	2ug	3 doses in 28 days	i.n.	BALB/c	1026b	i.p.	2 LD_{50}	60% at day 55
	BimA									20% at day 55
	LolC									0% at day 53

(Burtnick et al., 2011)	Hcp1 Hcp2 Hcp3 Hcp4 Hcp5 Hcp6	Sigma Adjuvant System	10µg	3 doses in 14 days	i.p.	BALB/c	K96243	i.p.	$5x10^4$	50% at day 42 80% at day 42 50% at day 42 33% at day 42 0% at day 42 50% at day 42
(Nieves et al., 2011)	OMVs	---	2.5µg	3 doses in 42 days	i.n. s.c.	BALB/c	1026b	aerosol	$1x10^3$	20% at day 14 60% at day 14
(Nieves et al., 2014)	OMVs	---	5µg	3 doses in 42 days	s.c.	BALB/c	K96243	i.p.	$2x10^4$ $8x10^5$	100% at day 21 67% at day 21
(Scott et al., 2014b)	LPS-TetHc conjugate LPS only LPS + TetHc	---	10µg	3 doses in 28 days	i.p.	BALB/c	K96243	i.p.	$4x10^4$	81% at day 29 62% at day 29 75% at day 29
(Scott et al., 2014a)	OPS-BSA CPS-BSA CPS+BSA with 10µg	Alhydrogel + CpG	5µg	3 doses in 35 days	s.c.	BALB/c	K96243	i.p.	$4x10^4$ $1x10^4$ $8x10^4$ $8x10^4$	0% at day 13 90% at day 21 50% at day 35 75% at day 35
(Schully et al., 2015)	Lysate/MP + Resiq/MP Lysate + Resiq/MP Lysate + Resiq/MP	--- Alum ---	10µg	2 doses in 7 days	s.c.	BALB/c	1026b	i.p.	$1x10^6$	12% at day 26 8% at day 26 16% at day 26
(Champion et al., 2016)	Chronic *Bpm* antigens LolC+Chronic CPS+Chronic CPS	SAS (25ug)	5µg protein +/- 10µg CPS	3 doses in 28 days	i.p.	BALB/c	K96243	i.p.	$7x10^4$	16% at day 26 30% at day 50 40% at day 50 50% at day 50 10% at day 50

Table adapted from source (Choh et al., 2013)

subunit vaccine efforts have focused on developing multivalent vaccines.

Significant protection has been achieved with outer membrane vesicles (OMVs). BALB/c administered a prime and two boosts (5 μg each) of OMVs s.c. demonstrated 100% protection 21 days post-challenge with 5 x LD_{50} of *B. pseudomallei* K96243. This protection appeared to be mediated strongly by humoral response, as passive immunization protected 80% of the animals against 50 x LD_{50} *B. pseudomallei* challenge (Nieves et al., 2014).

The most protective and immunogenic *Burkholderia* antigens to date include LPS and CPS. These molecules are essential to *B. pseudomallei* virulence, and appear to be conserved within this highly heterogenous bacterial population. In a study by Anuntagool *et al*, 99% of 1,327 clinical and environmental *B. pseudomallei* isolates expressed smooth LPS, with 97% expressing serotype A (Anuntagool et al., 2006). Importantly, *B. thailandensis* E264 also expresses a smooth, Type A LPS and shares an identical O-antigen structure with *B. pseudomallei* (Heiss et al., 2013; Titball et al., 2017). Therefore, *B. thailandensis* LPS is often used as a surrogate for *B. pseudomallei* in *in vitro* and *in vivo* studies.

Anti-LPS and anti-CPS antibodies are associated with protection against *B. pseudomallei* (Charuchaimontri et al., 1999; Ho et al., 1997; Silva and Dow, 2013; Titball et al., 2017). In experimental rodent models, monoclonal antibodies (mAbs) against LPS and CPS have been shown to provide up to 100% protection against challenge with 15 x LD_{50} *B. pseudomallei* (AuCoin et al., 2012). In melioidosis patients, anti-LPS antibodies are associated with survival (Charuchaimontri et al., 1999). Additionally, polysaccharide vaccination has been shown to provide significant protection in rodent models of

melioidosis and glanders (Burtnick et al., 2012; Gregory et al., 2015; Nelson et al., 2004; Scott et al., 2014a; Scott et al., 2014b; Torres et al., 2014). Importantly, because of its structural similarity, *B. thailandensis* LPS has also been utilized in vaccination studies and has been shown to provide protection against melioidosis in murine models (Ngugi et al., 2010).

Despite the antigenicity of these *Burkholderia* polysaccharides, they remain T-cell independent antigens. Because polysaccharides are large molecules, they can induce antibody production from B-cells by crosslinking B-cell receptors (BCRs) on the surface of the cell (Vinuesa and Chang, 2013). However, the antibodies produced in this T-cell independent manner often lack affinity, and do not undergo the isotype switching and affinity maturation needed for immunological memory responses (Avci et al., 2011; Mond et al., 1995). Groundbreaking work by Schneerson *et al* in the early 1980's addressed this deficiency by indiscriminately conjugating *Haemophilus influenzae* Type B polysaccharide with a diphtheria toxin protein (Schneerson et al., 1980). This protein-polysaccharide conjugate vaccine was shown to be immunogenic in rodent models, generating high anti-polysaccharide antibody titers capable of bacterial killing *in vitro*. This vaccine work led to the licensing of the first glycoconjugate vaccine by Connaught Laboratories (now Sanofi Pasteur). Currently, FDA-approved glycoconjugate vaccines exist against several bacterial pathogens, including *Haemophilus influenzae, Streptococcus pneumoniae* and *Neisseria meningococcus* Type *B.*

The success of glycoconjugate vaccines has generated significant interest in developing similar platforms against other bacterial pathogens. Experimental studies in rodent models have shown that vaccines containing *Burkholderia* LPS or CPS conjugated

to immunogenic protein can provide enhanced protection compared to unconjugated counterparts (Burtnick et al., 2012; Gregory et al., 2015; Scott et al., 2014a; Scott et al., 2014b; Torres et al., 2014). However, while these studies are highly promising, complete protection and sterilizing immunity has not yet been achieved. Therefore, there is a need to optimize these glycoconjugate vaccines in order to achieve increased immunogenicity and protection against challenge. Incorporation of *Burkholderia* proteins into these glycoconjugate formulations has the potential to increase immunogenicity by generating protein-specific antibodies. However, few *Burkholderia* proteins have been well-characterized, and many have not provided significant protection against challenge when used in a vaccine formulation (Table 1). Additionally, very few studies have examined the effects of combining multiple proteins into a single vaccine formulation. Therefore, there is currently a need to identify novel, immunogenic *Burkholderia* proteins and evaluate whether combinations of antigens could provide increased efficacy (Titball et al., 2017).

Chapter 5 Role of Informatics in Vaccine Development

TRADITIONAL VACCINOLOGY

In a conventional vaccinology approach, the microorganism is cultured and analyzed using a variety of microbiological, biochemical and molecular techniques. These analyses allow identification of virulence factors that can be targeted for mutagenesis or incorporated into a subunit vaccine. Such vaccine approaches have been successful in generating highly efficacious vaccines such as those against smallpox, polio, yellow fever, and tetanus. However, the majority of the licensed vaccines today are generated against relatively invariable pathogens (Rappuoli, 2007). It has been shown that antibody responses are the most important contributor of protection against these pathogens. In contrast, complex pathogens that exhibit considerable antigen drift (e.g., HIV) are able to evade the antibody responses generated via conventional vaccine approaches (Rappuoli, 2007). Importantly, *B. pseudomallei* is able to evade host immune responses by entering and replicating within host cells. This intracellular lifestyle and subsequent immune evasion suggests that a complex immune response will be required for protection.

Traditional vaccine development is a lengthy process, and often requires extensive knowledge of the pathogen in order to select genes for mutagenesis or antigens for isolation. The challenge here lies in the fact that many emerging or neglected pathogens are poorly understood. The emergence of alternative vaccine development approaches like reverse vaccinology has allowed for more rapid antigen identification, an approach particularly useful for neglected infectious agents.

REVERSE VACCINOLOGY

In 2001, Rino Rappuoli coined the term "reverse vaccinology" to describe the process of rational vaccine design by exploitation of the pathogen genome (Rappuoli, 2001). This process was first described in a groundbreaking study by Pizza and colleagues, in which novel *Neisseria meningitidis* antigens were identified by screening open reading frames (ORFs) against various computational prediction programs (Pizza et al., 2000). These researchers selected outer membrane or exported proteins based on structural characteristics such as transmembrane domains and similarity to known surface antigens. Recombinant proteins were administered to mice and the resulting immune sera were screened for bactericidal activity. Following these analyses, the seven most promising candidates were selected for downstream vaccination studies (Pizza et al., 2000). One of these candidates was later incorporated into Bexsero®, an FDA-approved vaccine against *N. meningiditis* Serogroup B (*Men*B).

The ability to screen for desired physiochemical (e.g., subcellular localization) and immunological (e.g., T-cell epitopes) properties allows for rational vaccine design against complex pathogens. Indeed, the wide-applicability of reverse vaccinology has led to the identification of novel antigens for many other pathogens, including the Gram positive bacteria *Streptococcus pnuemoniae* and *Staphylococcus aureus* and the Gram negative bacteria *Clostridium difficile* and *Escherichia coli* [reviewed in (Delany et al., 2013)].

Previous studies have employed *in silico* methodologies to identify immunogenic proteins and epitopes from *Burkholderia* in an attempt to improve vaccine efficacy. Felgner *et al* first utilized a microarray to identify 170 seroreactive antigens (Felgner et

al., 2009). Using informatics programs, these authors were able to predict subcellular localization, presence of a signal peptide and protein function. From these findings, the authors concluded that many of the identified antigens could serve not only as potential vaccine antigens, but also as serodiagnostic markers.

DeGroot *et al* proposed the use of EpiVax, a novel informatics program, to identify 54,010 conserved HLA Class II epitopes between 31 species of *B. pseudomallei*, *B. mallei* and *B. cepacia* (De Groot et al., 2011). These authors validated their findings via HLA binding assays *in vitro.* However, these epitopes have not yet been evaluated for efficacy *in vitro* or *in vivo*, and the potential role of these T-cell epitopes in protective immunity remains indeterminate.

Other groups have focused on identifying immunogenic epitopes within known *Burkholderia* antigens. Musson *et al* found that certain Human Leukocyte Antigen (HLA) Class II epitopes within the FliC protein are conserved between *B. pseudomallei*, *B. multivorans,* and *B. cenocepacia*. The conservation of these epitopes was validated by T-cell hybridomas that recognized various FliC homologues between these species (Musson et al., 2014). Further work by Nithichanon *et al* utilized *in silico* methods to identify additional B- and T-cell epitopes within the FliC protein that demonstrated reactivity with human sera and elicited an IFNγ response from human peripheral blood mononuclear cells (PBMCs). Additionally, antibodies raised against these peptides in mice were shown to increase phagocytosis of *B. pseudomallei* by neutrophils, resulting in increased oxidative burst and increased bacterial killing (Nithichanon et al., 2015).

Computational and structural analyses have also led to the identification of additional immunogenic epitopes. Lassaux *et al* crystallized the structure of OppA and

used epitope mapping to identify immunogenic peptides within the seroreactive *B. pseudomallei* OppA protein (Lassaux et al., 2013; Suwannasaen et al., 2011). These peptides were shown to react with convalescent human melioidosis sera. However, previous *in vivo* studies have shown that immunization with the OppA protein does not afford significant protection against challenge (Harland et al., 2007).

Gourlay *et al* used a similar approach to examine conserved structural components of the flagella associated protein FlgK (Gourlay et al., 2013). Interestingly, this seroreactive antigen was shown to be cytotoxic to murine macrophages *in vitro*, suggesting a role in *B. pseudomallei* pathogenesis.

Taken together, these studies support the use of computational predictions for identifying novel proteins and epitopes for continued vaccine development and pathogenicity studies.

Chapter 6 Use of Gold Nanoparticles as a Vaccine Platform

HISTORICAL USE OF GOLD FOR THERAPEUTIC PURPOSES

The use of gold for medicinal purposes is believed to date back to Ancient Chinese culture (2500 BC) (Thakor et al., 2011). Many ancient and medieval cultures embraced gold therapies on the premise of superstition. However, it wasn't until 1890 that gold therapy was actually founded on scientific repute. In a series of experiments, Robert Koch discovered that 0.5 ppm gold cyanide inhibited the growth of *Mycobacterium tuberculosis in vitro.* Despite unsuccessful clinical trials for *M. tuberculosis*, Robert Koch is largely credited with the current use of gold in the treatment of rheumatoid arthritis (Higby, 1982). Due to its misconceived association with *M. tuberculosis,* rheumatoid arthritis was also treated with gold at this time and symptoms were shown to improve with gold therapy. Two gold-based therapies for rheumatoid arthritis are still in use today – injectable Aurolate® (gold sodium thiomalate) and the orally-administered Ridaura® (auranofin). Studies from these treatments have demonstrated that high concentrations of gold can be well-tolerated in humans without overt toxicity (Gottlieb et al., 1972). Gold has an extensive history of safe use in humans. For centuries, gold has been used for jewelry and dental purposes, with allergic responses occurring very rarely. Small quantities of gold can be also found in healthy human tissue and blood, and is occasionally ingested in food products (Thakor et al., 2011).

GOLD NANOPARTICLES IN VACCINE DEVELOPMENT

Nanostructures are defined as particles between 1-100 nm in size. The particle size is an important consideration for the development of human therapies, as size has been shown to affect cellular uptake mechanisms, cellular retention and accumulation of the particles, as well as unintended toxicity and inflammatory responses. Uptake of 14 nm and 50 nm gold nanoparticles (AuNPs) have been shown to occur through Scavenger Receptor A on

RAW 264.7 macrophages, in addition to clathrin- and caveolin-mediated endocytosis (Chithrani and Chan, 2007; Franca et al., 2011; Jiang et al., 2008). Importantly, exocytosis of gold nanoparticles was also shown to be size-dependent, with small (< 20 nm) AuNPs exhibiting more rapid and complete exocytosis than larger particles (30 nm – 100 nm) (Chithrani and Chan, 2007; Niikura et al., 2013). The rapid exocytosis of AuNPs allows excretion of these particles through the urine and feces, a process that is important in order to avoid accumulation of these non-biodegradable particles in host tissues (Thakor et al., 2011).

While considered relatively inert, the size and shape of AuNPs have also been shown to influence antibody and cytokine production. High concentrations of AuNPs can induce inflammatory cytokine production in a size and shape-dependent mechanism (Niikura et al., 2013). Spherical AuNPs appear to be the most inert, possibly attributed to their rapid exocytosis from cells. Additionally, 15 nm AuNPs have been shown to be non-toxic in a variety of *in vitro* cell lines even at high concentrations; whereas smaller particles (1-2 nm) have been shown to induce cytotoxicity (Pan et al., 2007).

Despite these findings, the immunogenicity of AuNPs remains highly dependent on their cargo ("protein corona"). Surface modifications and association of AuNPs with serum proteins can have major implications on cellular uptake and immunogenicity. Because they rapidly associate with thiol-containing ligands, AuNPs can be modified to carry a variety of biomolecules, including polysaccharides, proteins and antibodies. Because of their ease of synthesis, low immunogenicity and history of safe use in humans, AuNPs provide an attractive platform for the delivery of vaccines and therapeutics. Importantly, because AuNPs can function as a non-biodegradable vaccine

carrier, they have the capacity to stabilize their cargo and prolong exposure to the immune response.

Several groups have shown that inclusion of AuNPs in various formulations can increase vaccine immunogenicity. Many studies have exploited the chemistry of gold nanoparticles by expressing antigens containing cysteine residues (Dakterzada et al., 2016; McCoy et al., 2013; Tao et al., 2017; Vetro et al., 2017). Thiol groups spontaneously form strong bonds with gold; therefore, thiol-containing compounds can be easily attached to the gold surface. In order to control the concentration of antigen on the AuNP surface, other groups have conjugated antigens to thiol-containg ligands bound to the AuNP surface (Gregory et al., 2015; Niikura et al., 2013; Rodriguez-Del Rio et al., 2015; Torres et al., 2014).

Previous research by Gregory *et al* and Torres *et al* demonstrated that AuNP-glycoconjugate vaccines could provide protection in murine and non-human primate (NHP) models of inhalational *B. mallei* infection (Gregory et al., 2015; Torres et al., 2014). In these studies, 15 nm spherical AuNPs were first modified with 16-mercaptohexadecanoic acid (16-MHDA). Using a carbodiimide approach, immunogenic proteins (Tetanus toxin heavy chain (TetHc), or *Burkholderia* proteins Hcp1 and FliC) and *B. thailandensis* LPS were conjugated to the AuNP surface. Mice were intranasally (i.n.) administered a prime and two boosts of AuNP-glycoconjugate vaccine formulation containing 0.93 μg protein, 0.93 μg LPS and 0.26% Alhydrogel (Gregory et al., 2015). Similarly, NHPs received a prime and two boosts [30 day intervals, delivered intramuscularly (i.m.)] of 20 μg AuNP-FliC-LPS formulated with 0.26% Alhydrogel (Torres et al., 2014). Upon lethal respiratory challenge with *B. mallei* ATCC 23344,

vaccinated mice and NHPs demonstrated increased survival and decreased splenic colonization. These studies are highly promising and demonstrate the immunogenicity of AuNP-glycoconjugate vaccines in mammals. However, vaccinated animals remained colonized post-challenge, indicating that optimization is needed to further enhance the immunogenicity of this vaccine platform.

Taken together, this study and others demonstrate that inclusion of AuNPs into various vaccine formulations can enhance immunogenicity of subunit vaccines and induce IgG antibody responses. Importantly, other research has shown that AuNP inclusion can also stimulate cellular responses, including B- and T-cell activation and DC maturation (Rodriguez-Del Rio et al., 2015; Tao et al., 2017). Taken together, these studies indicate that AuNPs are safe and well-tolerated in mammalian hosts, and are a promising platform for the optimization of *Burkholderia* glycoconjugate vaccines.

Objectives of this dissertation study

Burkholderia pseudomallei is a Gram negative bacterium that causes melioidosis, a disease characterized by high morbidity and mortality in humans and other mammals. Inhalation of the bacterium results in severe disease, including pneumonia, bacteremia, sepsis and death (Currie, 2015; Yeager et al., 2012). Because this disease manifests as a febrile illness, it is often mistaken for more common bacterial infections such as *M. tuberculosis.* Rapid and accurate diagnosis is key, as mistreatment can lead to mortality in > 70% of patients (White et al., 1989). Because *B. pseudomallei is* inherently resistant to numerous classes of antibiotics, limited treatment options are available. Despite appropriate antibiotic therapy, approximately 12% of patients will relapse and up to 40% will succumb to infection (Currie et al., 2010; Limmathurotsakul et al., 2010).

Because of the high morbidity and mortality associated with respiratory cases and lack of efficient treatment, *B. pseudomallei* is considered a potential bioweapon and has been classified as a Tier 1 Select Agent by the US Department of Health and Human Services. The public health threat associated with this bacterium emphasizes the need for an effective vaccine that can be administered to military personnel, humanitarian workers and susceptible populations living in endemic areas.

During the past two decades, the significant interest in *Burkholderia* vaccine development has led to a more comprehensive understanding of pathogenesis and immunity. However, as our knowledge of this pathogen increases, so does its complexity. The ability to achieve sterilizing immunity through vaccination remains elusive, suggesting that unique vaccine approaches may be needed in order to achieve full protection.

Subunit vaccines are advantageous because they can be manufactured under BSL2 conditions and have potential to be administered to immunocompromised individuals living in endemic areas. Immunization with protein or polysaccharide alone can protect animals from acute lethal infection; however, surviving animals remain heavily colonized with bacteria. The need to generate more robust immunological responses has led to the development of glycoconjugate vaccines against *Burkholderia*. Glycoconjugate vaccines are known to induce strong humoral and cell-mediate immunity against bacterial polysaccharides (Avci et al., 2011). Because LPS antibodies are associated with protection in animals and humans, it represents an important antigen for glycoconjugate inclusion (Titball et al., 2017). Conjugation of *Burkholderia* polysaccharides to immunogenic proteins has been shown to generate increased protection against *B. pseudomallei* challenge, as evidenced by increased antibody titers, decreased splenic colonization and increased survival following challenge (Gregory et al., 2015; Scott et al., 2014a; Scott et al., 2014b; Torres et al., 2014). However, bacteria were still able to colonize the spleen following challenge, indicating that the immunogenicity generated by current glycoconjugate vaccines is not sufficient to provide sterilizing immunity. Incorporation of immunogenic proteins has potential to increase vaccine efficacy by generating protein-specific antibodies. Reverse vaccinology approaches have been able to successfully identify novel antigens from a variety of pathogens (Delany et al., 2013); therefore, I hypothesized that *in silico* screening would identify immunoreactive proteins and incorporation of these proteins into various vaccine formulations would enhance efficacy and increase protection against challenge. This hypothesis was tested by completion of the following two aims: 1) Purify highly ranked,

immunogenic proteins and confirm recognition by convalescent sera and 2) Evaluate the protective capacity of protein candidates *in vivo* when administered alone and in a glycoconjugate platform.

MATERIALS AND METHODS

In vitro studies

BACTERIAL STRAINS AND PLASMIDS

The bacterial strains used in this study are included in Table 2. All manipulations of *B. pseudomallei* and *B. mallei* strains were conducted in Centers for Disease Control and Prevention (CDC)-approved and registered BSL3 or CDC/ U.S. Department of Agriculture (USDA)-approved and registered animal biosafety level (ABSL) 3 facilities at the University of Texas Medical Branch (UTMB) and experiments were performed in accordance with Select Agent standard operating practices. For *in vitro* assays and *in vivo* challenges, freezer stocks of *B. pseudomallei* K96243 were streaked onto Luria Bertani (LB) agar and allowed to grow at 37°C for 36 hrs. For liquid cultures, 3-5 colonies were inoculated into LB broth and incubated at 37°C with agitation (200 rpm) for 12 hrs. Similarly, freezer stocks of *B. mallei lux* were streaked onto LB agar containing 4% glycerol (LBG) and incubated for 60 hrs at 37°C. For liquid cultures, 3-5 colonies were inoculated into LBG broth, and incubated for 16 hrs at 37°C with agitation (200 rpm). For animal challenge, overnight *B. pseudomallei* or *B. mallei* cultures were diluted in phosphate buffered saline (PBS) and 50 µl administered i.n. to mice.

Glycerol stocks of *E. coli* strains were stored at -80°C. Freezer stocks were streaked onto LB agar plates or directly inoculated into LB broth and incubated overnight at 37°C. As needed, media was supplemented with 50 µg/ml kanamycin (Km, Sigma).

Table 2. Bacterial strains and plasmids used in this study

Bacterial Strain	Relevant features	Use	Reference or Source
Bacterial Strains			
B. pseudomallei K96243	Human clinical isolate; Km^R Gm^R Pb^R	*In vitro* assays and animal challenge	(Holden et al., 2004)
B. mallei CSM001	*B. mallei* ATCC 23344 with a mini-Tn*5*::*lux*Km2; Km^RPb^R	Animal challenge	(Massey et al., 2011)
B. mallei ATCC 23344	Human clinical isolate; Km^SPb^R	DNA extraction	(Yabuuchi et al., 1992)
B. thailandensis E264	Environmental isolate; Km^R Gm^R Pb^R	LPS extraction	(Brett et al., 1998)
E. coli BL21 (DE3)	*E.coli B* strain deficient in proteases Lon and OmpT	Protein expression	New England BioLabs
E. coli DH5α	*E. coli* K-12 strain with high transformation efficiency	Plasmid propagation	New England BioLabs
Plasmids			
pVAX-1	PMV promoter, BGH polyadenylation signal, Km^R	*In vivo* immunization	Invitrogen
pET30a(+)	T7 promoter, 6x histidine tag, Km^R	Protein expression	Novagen

MAINTENANCE OF IMMORTAL CELL LINES

The cell lines used in this study include RAW 264.7 (ATCC® Tib-71™) murine macrophage-like cells, HeLa (ATCC® CCL-2™) human epithelial cells and LA-4 (ATCC® CCL-196™) murine lung epithelial cells. RAW 264.7 and HeLa cells were maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, Gibco), 1% non-essential amino acids (Sigma) and 1% penicillin-streptomycin (Gibco). Additionally, HeLa media was supplemented with 1% sodium pyruvate. LA-4 cells were maintained in Ham's F-12K (Kaighn's) Medium (Gibco) supplemented with 15% FBS and 1% penicillin-streptomycin (Gibco). All cells were maintained in humidified chamber at 37°C with an atmosphere of 5% CO_2.

BIO- AND IMMUNO- INFORMATICS

All informatics analyses were performed using the *B. mallei* ATCC 23344 proteome (4,806 proteins) obtained from the *Burkholderia* Genome Database (Winsor et al., 2008). To identify potential vaccine candidates, BLAST (Altschul et al., 1990) analyses were used to screen for *B. mallei* proteins exhibiting > 98% identity with *B. pseudomallei* K96243. Using the Vaxign (He et al., 2010) program, proteins exhibiting sequence homology with human and/or mouse proteins were eliminated. Next, proteins were screened through pSORTb 3.0 (Yu et al., 2010) to identify those with outer membrane or extracellular subcellular localization (threshold ≥ 9.5). The transmembrane prediction programs TMHMM (Krogh et al., 2001), Phobius (Kall et al., 2004) and HMMTOP (Tusnady and Simon, 2001) were used to select for proteins with ≤ 1 transmembrane domains. Following these analyses, proteins were screened for predicted antigenicity

(threshold = 0.4) and adhesive properties via VaxiJen (Doytchinova and Flower, 2007) and Vaxign (He et al., 2010), respectively. The predicted stability of each protein was also assessed via ProtParam (Gasteiger E., 2005).

Following these physiochemical analyses, proteins were screened for predicted immunogenicity based on binding to MHC class I and class II molecules. Potential MHCI and MHCII epitopes and their corresponding affinities were evaluated using NetCTL 1.2 (Larsen et al., 2007) and NetMHCII 2.2 (Nielsen and Lund, 2009) programs, respectively. Additionally, a Mature Epitope Density (MED) score was used to assess the number and average affinity of MHC epitopes irrespective of HLA allele, by utilizing the following algorithm (Santos et al., 2013):

$$\text{MED} = \frac{\text{Predictions}}{\text{Chances}} = \frac{\text{Predicted epitopes} * (50 - (\text{Average (MHC affinity)}))}{\text{Aminoacids length} - \text{epitope length} + 1}$$

Finally, to downselect to the most ideal vaccine candidates, proteins were ranked against each other based on adhesion probability and predicted antigenicity, as well as the number and affinity of MHC epitopes. The top seven protein candidates (together with known *Burkholderia* antigen Hcp1) were selected for further validation studies (Table 5).

CLONING

Gene sequences were obtained from the *Burkholderia* Genome Database (Winsor et al., 2008). For pVAX-1, primers were designed to flank genes of interest in order to amplify the entire ORF, incorporating the KpnI and PstI restriction sites, together with a Kozak sequence for enhanced transcription. For incorporation into the pET30a(+) plasmid, primers were designed to flank the entire ORF and incorporate NdeI and XhoI or HindIII

restriction enzyme sites (Table 4). Following amplification via Phusion® High Fidelity DNA polymerase (New England BioLabs), PCR products were run on 1% agarose gel at 100V for 30 min. Upon visualization of one band of expected size, PCR products were purified via QIAquick PCR purification kit (Qiagen) according to manufacturer's directions. However, in the instances that more than one band was visible, the gel fragment containing the band of interest was excised and digested via QIAquick Gel Extraction kit (Qiagen), according to manufacturer's directions. Amplified DNA and pET30a(+) or pVAX-1 plasmid were digested with respective restriction endonucleases (Table 4) for 16 hrs at 16°C. Following digestion, PCR products were purified via QIAquick PCR purification kit (Qiagen), and ligated into a pET30a(+) expression vector via T4 ligase (New England BioLabs). Transformation of *E. coli* DH5α (New England BioLabs) was performed via heat shock at 42°C for 30 sec, after which SOC outgrowth media (New England BioLabs) was added and bacteria allowed to grow for 1 hr at 37°C with agitation (200 rpm). At this time, bacteria were plated on LB agar, and allowed to grow overnight at 37°C. To confirm the presence of insert, transformed colonies were selected and inoculated into a PCR reaction in addition to 5 ml LB broth for plasmid isolation. Upon PCR confirmation, plasmids were isolated and purified via Plasmid purification kit (Qiagen). Directional gene sequencing was performed at the UTMB Sequencing Core.

Purified pET30a(+) plasmid was transformed into BL21 (DE3) competent cells, a protease-deficient *E. coli* strain commonly used for expression of recombinant proteins. BL21 cells were transformed via heat shock at 42°C for 10 sec. Transformed isolates were inoculated into 10 ml LB broth and incubated overnight at 37°C. After incubation,

bacteria were pelleted via centrifugation (4,000 x g for 10 min) and resuspended in 50% glycerol for freezer (-80°C) storage.

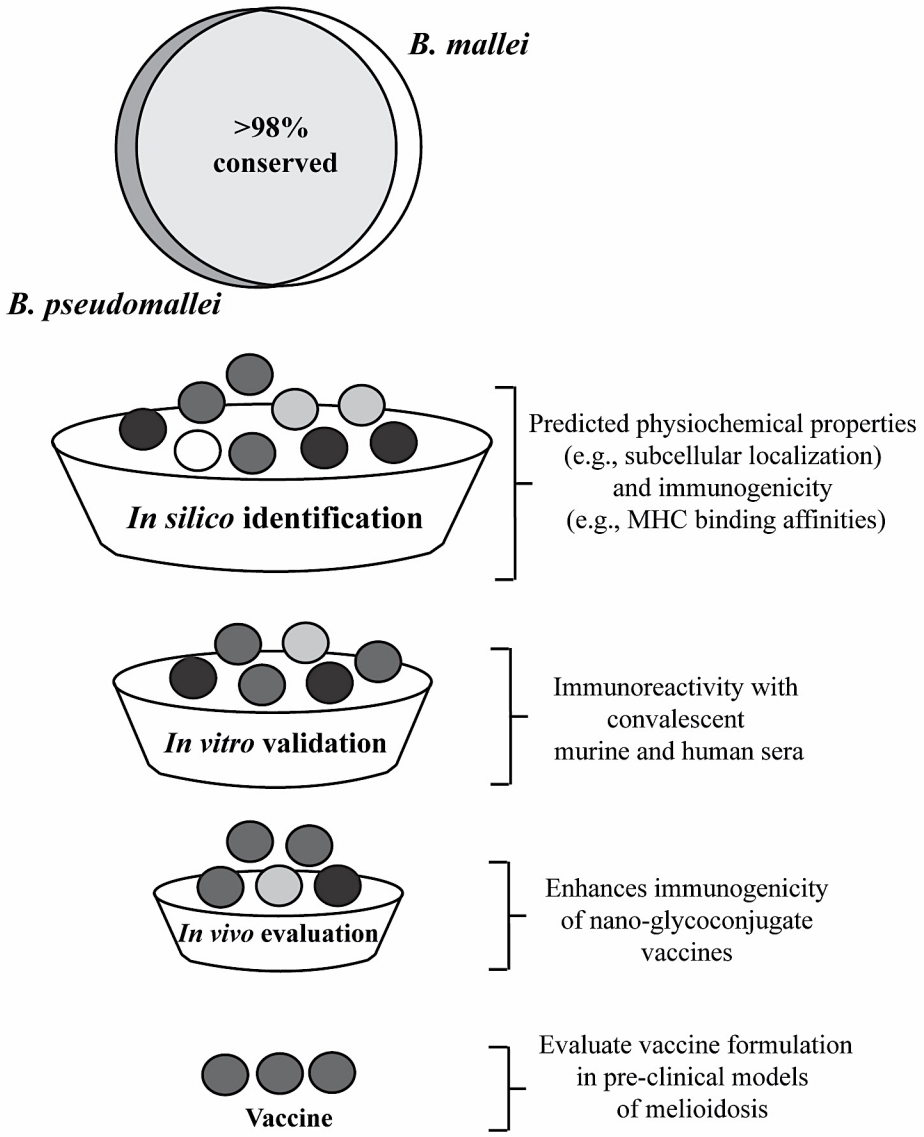


Illustration 1: Schematic representation of reverse vaccinology approach. Proteins were selected based on predicted subcellular localization, antigenicity, adhesive properties and affinity to MHCI and MHCII. Top seven candidates, together with known antigen Hcp1, were screened for seroreactivity and evaluated in vivo for immunogenicity.

Table 3. Criteria for selection and ranking of potential vaccine candidates

Criteria for selection/ranking	Program	Rationale	References
Conserved; > 98% identity between *B. mallei* and *B. pseudomallei*	BLAST	Increase likelihood of involvement in mammalian adaptation; increase possibility of cross-protection	(Whitlock et al 2010)
Non-homology with murine and human proteins	Vaxign	Avoid risk of autoimmunity	(Garcia-Angulo et al., 2014; Singh et al., 2016)
Secreted or outer membrane subcellular localization	pSORTb	Increase chance of encountering host immune system	(Pizza et al 2000; Moriel et al., 2010; Garcia-Angulo et al., 2014)
≤1 transmembrane domain	TMHMM Phobius HMMTOP	High rate of failure when expressing recombinant proteins with >1 transmembrane domain	(Garcia-Angulo et al., 2014; Pizza et al., 2000)
Adhesin probability > 0.4	Vaxign	Potential to generate antibodies that block bacterial attachment	(Garcia-Angulo et al., 2014; Singh et al., 2016; Wizemann et al., 1999)
Antigen probability > 0.50	VaxiJen	Similarity to known antigens	(Garcia-Angulo et al., 2014; Monterrubio-Lopez et al., 2015)
Number and affinity of MHCI epitopes	NetCTL	Evaluate potential MHCI epitopes	(Garcia-Angulo et al., 2014; Lund et al., 2011; Singh et al., 2016)
Number and affinity of MHCII epitopes	NetMHCII	Evaluate potential MHCII epitopes	(Garcia-Angulo et al., 2014; Singh et al., 2016)
Mature epitope density (MED)	----	Assess immunogenic potential across all HLA alleles	(Garcia-Angulo et al., 2014; Santos et al., 2013; Tapia et al., 2016)

Table 4. Top ranking proteins identified through informatics analyses

Protein	Bpm locus	Bm locus	Length (bp)	Weight (kDa)	MHCI rank	MHCII rank
Hemagglutinin*	**BPSS0908**	**BMAA1324**	**2493 bp**	**79.6 kDa**	**20**	**1**
FliK	BPSL0225	BMA3276	466 bp	44.2 kDa	---	2
Lipoprotein	BPSL0892a	BMA2255	512 bp	40.8 kDa	15	3
FlgD*	**BPSL0272**	**BMA3327**	**834 bp**	**27.5 kDa**	**---**	**4**
OmpW*	**BPSL2704**	**BMA2010**	**822 bp**	**28.7 kDa**	**7**	**5**
FlgE	BPSL0273	BMA3328	1242 bp	42.5 kDa	---	6
Lipoprotein	BPSL0019	BMA2773	538 bp	56.0 kDa	17	7
FlgL*	**BPSL0281**	**BMA3336**	**1233 bp**	**42.1 kDa**	**21**	**8**
OpcP1 porin*	**BPSS0708**	**BMAA1122**	**1200 bp**	**41.6 kDa**	**3**	**9**
Porin	BPSS1679	BMAA1698	1155 bp	40.2 kDa	9	10
Porin	BPSS0265	BMAA1502	837bp	29.2 kDa	5	11
Porin*	**BPSS0757**	**BMAA0599**	**1155 bp**	**40.4 kDa**	**4**	**12**
Hcp1*	**BPSS1498**	**BMAA0742**	**510 bp**	**18.7 kDa**	**---**	**13**
BtuB	BPSL0976	BMA0685	2058 bp	72.5 kDa	---	14
Hypothetical	BPSL3105	BMAA0405	492 bp	17.1 kDa	---	15
FliD-1	BPSL3320	BMA2874	1521 bp	49.7 kDa	11	16
Porin	BPSL1029	BMA0743	1089 bp	38.4 kDa	2	---
Porin	BPSS0783	BMAA0633	1140 bp	39.4	6	---
Porin OpcP	BPSL1728	BMA1125	1224 bp	42.5 ka	10	---
TonB-dependent siderophore receptor	BPSS1204, BPSL1775, BPSS1029	BMA1178	2226 bp	81.0 kDa	12	---
Hypothetical	BPSL1957	BMA1111	1359 bp	48.4 kDa	13	---
Hypothetical	BPSS1260	BMAA1111	1815 bp	63.3 kDa	14	---
Type-1 fimbrial protein, A subunit	BPSL1629	BMA1024	615 bp	20.7 kDa	8	---
OpcP porin*	**BPSS0879**	**BMAA1353**	**1131 bp**	**39.4 kDa**	**1**	**---**

*Proteins selected for downstream validation and immunogenicity studies.

Table 5. Primers used in this study

Name	Sequence 5'-3'	RE sites	Product length (bp)
pVAX-2010F	ACCGGTACCACCATGGATGAAGAAGCTGATTGC (Forward primer to amplify BMA2010)	KpnI	822 bp
pVAX-2010R	ACCCTGCAGTCAGAACTTGTATGAAATCCCGACGAACGT (Reverse primer to amplify BMA2010)	PstI	
pVAX-1122F	ACCGGTACCACCATGGATGAAAAAGCGCACTGC (Forward primer to amplify BMAA1122)	KpnI	1200 bp
pVAX-1122R	ACCCTGCAGTCAGAAACGGTGGATCAGGCC (Reverse primer to amplify BMAA1122)	PstI	
pVAX-1324F	ACCGGTACCACCATGGATGAACAAAATCTACAATGTGGTTTG (Forward primer to amplify BMAA1324)	KpnI	2493 bp
pVAX-1324R	ACCCTGCAGTCACCACTGATAACCGGC (Reverse primer to amplify BMAA1324)	PstI	
pET-2010F	TAGTATCATATGAAGAAGCTGATTGCCG (Forward primer to amplify BMA2010)	NdeI	822 bp
pET-2010R	TAGTATCTCGAGGAACTTGTATGAAATCCC (Reverse primer to amplify BMA2010)	XhoI	
pET-1122F	TAGTATCATATGAAAAAGCGCACTGCG (Forward primer to amplify BMAA1122)	NdeI	1200 bp
pET-1122R	TAGTATCTCGAGGAAACGGTGGATCA (Reverse primer to amplify BMAA1122)	XhoI	
pET-1324F	TAGTATCATATGAACAAAATCTACAATGTGGTTT (Forward primer to amplify BMAA1324)	NdeI	2493 bp
pET-1324R	TAGTATAAGCTTCCACTGATAACCGGC (Reverse primer to amplify BMAA1324)	HindIII	
pET-0742F	TAGTATCATATGCTGGCCGGAATATATCTCAAG (Forward primer to amplify BMAA0742)	NdeI	510 bp
pET-0742R	TAGTATAAGCTTGCCATTCGTCCAGTTTG (Reverse primer to amplify BMAA0742)	XhoI	
pET-1353F	TAGTATCATATGAACAAGACTCTGATTGTTGC (Forward primer to amplify BMAA1353)	NdeI	1131 bp
pET-1353R	TAGTATAAGCTTGAAGCGGTGACG (Forward primer to amplify BMAA1353)	XhoI	
pET-0599F	TAGTATCATATGAAAAACATCCGGTTTGCAT (Forward primer to amplify BMAA0599)	NdeI	1155 bp
pET-0599R	TAGTATAAGCTTGAAGCGCGTGCG (Reverse primer to amplify BMAA0599)	XhoI	
pET-3327F	TAGTATCATATGACATCCTCCTTCACCACC (Forward primer to amplify BMA3327)	NdeI	834 bp
pET-3327R	TAGTATAAGCTTGTTGGTGGAAGACGAGG (Reverse primer to amplify BMA3327)	XhoI	
pET-3336F	TAGTATCATATGCGCATTTCCAGC (Forward primer to amplify BMA3336)	NdeI	1233 bp
pET-3336R	TAGTATAAGCTTCGGGTTCAGATACTGG (Reverse primer to amplify BMA3336)	XhoI	

CONFIRMATION OF PVAX-1 TRANSCRIPTION

To confirm the transcriptional activity of pVAX-1, HeLa cells were first seeded in 12-well plates at a density of $5x10^5$ cells/well and incubated at 37°C overnight to allow adherence. To prepare transfection reactions, 2500 ng pVAX-1 or pMAX-GFP DNA (transfection control) was mixed with 15 µl Lipofectamine® (Invitrogen) diluted in 300 µl Opti-Mem™ (Gibco). Transfection reaction was incubated at room temperature for 5 min, and 150 µl added to cells containing 850 µl Opti-Mem™, for a final volume of 1 ml. At 24 and 48 hrs post-transfection, cells were washed 2 times with PBS prior to addition of 350 µl RLT cell lysis buffer (Qiagen), and RNA extraction according to RNeasy RNA isolation kit (Qiagen) according to manufacturer's directions. Resulting RNA was quantified via Epoch Nanospectrophotometer (BioTek) and stored at -20°C until use. Complementary DNA (cDNA) was prepared via QuantiTect Reverse Transcription kit (Qiagen), and PCR performed with gene-specific primers (Table 4). Confirmation of pVAX-1 transcription was assessed via gel electrophoresis.

PROTEIN EXPRESSION

To confirm protein expression, freezer stocks of *E. coli* BL21 were inoculated into LB broth and grown overnight at 37°C with agitation (200 rpm). Overnight cultures were diluted 1:100 in 40 ml of LB broth, grown to OD_{600} of ~0.5, and induced with 1 mM final concentration of Isopropyl β-D-1-thiogalactopyranoside (IPTG). At 3 hrs post-induction, cultures were centrifuged (4000 x g for 15 min) and the resulting bacterial pellet frozen at -20°C. In order to confirm protein expression, the bacterial pellet was re-suspended in cold 20 mM Tris-HCl [pH 7.5] and sonicated with 750 W Ultrasonic Processor on ice for 10 pulses (30 sec on, 30 sec off). The resulting material was centrifuged (14,000 x g for

10 min) to separate soluble and insoluble fractions. Following addition of 2X Laemmli Buffer (Bio-Rad), samples were heated at 95°C for 5 min and run on 10% SDS-PAGE (Bio-Rad). Protein bands were cut out, digested and analyzed via MALDI-TOF (UTMB Mass Spectrometry Core) to confirm protein identity.

INCLUSION BODY PURIFICATION AND REFOLDING

To induce expression of protein inclusion bodies, freezer stocks of *E. coli* BL21 were inoculated into LB broth and grown overnight at 37°C with agitation (200 rpm). Overnight cultures were diluted 1:100 in LB broth, and grown to mid-log phase (OD_{600} ~0.5). At this time, 1 mM final concentration of IPTG was added to the culture, and incubated at 37°C with agitation (200 rpm) an additional 3 hrs. Bacteria were pelleted by centrifugation (6,000 x g for 15 min) and frozen at -20°C until use. To purify inclusion bodies, 1 g of cell pellet was re-suspended in 10 ml 1X Cell Lytic™ B (Sigma) in PBS, together with 0.2 mg/ml Lysozyme and 1 pellet EDTA-free protease inhibitor cocktail (Roche Diagnostics). This solution was incubated with shaking for 15 min and centrifuged (16,000 x g for 15 min) to pellet insoluble material. The insoluble pellet was re-suspended in 10 ml 1X Cell Lytic™ B (Sigma) and vortexed for 2 min prior to addition of 0.2 mg/ml lysozyme (Sigma). This solution was incubated for 10 min at room temperature and centrifuged (16,000 x g for 5 min). In order to fully remove all soluble material from the inclusion bodies, the insoluble pellet was washed 10 times with Cell Lytic™ B (Sigma) diluted 1:100 in PBS, centrifuging (16,000 x g for 5 min) between washes. To solubilize inclusion bodies, 1 g of insoluble material was re-suspended in 8 ml of Cell Lytic™ IB (Sigma), and incubated with shaking for 30 min at room temperature. After incubation, solution was centrifuged (16,000 x g for 15 min) to pellet

cell debris. Supernatant containing solubilized inclusion bodies was assayed via BCA (Pierce) to determine protein concentration and stored at -20°C until use.

In order to determine refolding conditions, small scale refolding was performed using 15 different buffers provided by the QuickFold™ Protein Refolding Kit (AthenaES). Briefly, inclusion bodies were diluted to 1 mg/ml in buffer containing 8 M Urea, 100 mM NaH_2PO_4 and 10 mM Tris-HCl [pH 7.3], and 50 µl of this solution were slowly added to 950 µl refolding buffer, with gentle vortexing. Solution was allowed to incubate at 4°C for 1 hour prior to reading at 280 nm (Epoch Spectrophotometer). The buffer exhibiting the lowest precipitation (e.g., reads similar to denatured protein diluted in 8 M Urea, 100 mM NaH_2PO_4 and 10 mM Tris-HCl [pH 7.3]) was selected for use in large scale refolding. All proteins were able to be solubilized without precipitation into a buffer containing 50 mM Tris-HCl pH 8.5, 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.5 M Arginine, 0.75 M Guanidine HCl, 0.5% Triton-X 100, and 1 mM DTT (Athena ES). Therefore, this buffer was selected for all refolding purposes.

For large scale purification and refolding, solubilized inclusion bodies were adjusted to 1 mg/ml in buffer containing 8 M Urea, 100 mM NaH_2PO_4 and 10 mM Tris-HCl [pH 7.3]. Adjusted protein was added drop wise to 20X volume of refolding buffer and incubated for 1 hour at 4°C with stirring. The protein solution was then added to equilibrated dialysis tubing [SpectraPor® molecular weight cut off (MWCO) of 10kDa] and dialyzed against 4-6 L of 2X PBS for 2 hrs, 1X PBS for 2 hrs, followed by eight exchanges with 1X PBS performed at 8 hour intervals. All proteins were dialyzed against PBS, with the exception of Hcp1 (BMAA0742/BPSS1498), which was dialyzed against decreasing concentrations of Tris-HCl [pH 8.0] from 50 mM to 20 mM with exchanges

performed as described above. The resulting protein solution was concentrated via filter centrifugation (EMD Millipore Amicon™ Ultra-15, 10 kDa MWCO) and centrifuged at 5,000 *x g* for 5 min to remove any precipitates. Concentrated protein was assayed via BCA to determine protein concentration, adjusted to concentration of 1 mg/ml and stored at -80°C until use. Protein purity was assessed via SDS-PAGE analysis (Figure 1)

WESTERN BLOTS AND ELISAS WITH CONVALESCENT SERA

To validate our *in silico* predictions, we confirmed seroreactivity with both anti-*B. pseudomallei* sera and convalescent human melioidosis sera. Convalescent sera were obtained from seropositive volunteers (Northeastern Thailand) with informed written consent according to the Khon Kaen University Ethics Committee for Human Research. Anti-*B. pseudomallei* sera were obtained from C57BL/6 mice 35 days following sublethal infection with *B. pseudomallei* K96243.

ELISAs were performed as previously described, with modifications (Hatcher et al., 2016). To evaluate seroreactivity against anti-*B. pseudomallei* sera, Corning™ high binding polystyrene plates (Fisher) were coated overnight at 4°C with 10 µg/ml recombinant protein diluted in PBS. The following morning, plates were washed twice with PBS containing 0.05% Tween-20 (Sigma) and blocked for 2 hrs at room temperature with a PBS solution containing 0.1% Tween-20 and 2% Bovine Serum Albumin (BSA). After blocking, plates were washed twice prior to the addition of serum samples. Sera were diluted 1:25 in PBS solution containing 0.01% Tween-20 and 1% BSA, added to the plate in triplicate and serially diluted. After addition of sera, 50 µl of goat anti-mouse IgG polyclonal antibody (1:500, Abcam) was added to the plate and incubated for 3 hrs at room temperature with shaking. After incubation, the plate was washed four times with

wash solution prior to addition of Tetramethylbenzidine (TMB, eBioscience) substrate for 15 min at room temperature. Reaction was stopped with 2 N H_2SO_4 and read at 450-570nm (Epoch Nanospectrophotometer, BioTek). Endpoint titers were determined to be the $OD_{450-570}$ value equivalent to twice the standard deviation of naïve sera

To evaluate seroreactivity with convalescent human sera, 1-2 µg of recombinant proteins were diluted in Laemmli Buffer (Bio-Rad) and heated at 95°C for 5 min. Samples were added to SDS-PAGE (10% or 4-20%, Bio-Rad) and run at 100V for 1.5 hrs. Proteins were transferred to Immobilon PVDF membrane (EMD Millipore) using Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad) at 15V for 30 min. Membrane was blocked for 1 hour at room temperature in TBST (TBS containing 0.001% Tween-20) solution containing 5% powdered milk and 1% BSA. After blocking, membrane was incubated with either sera (diluted 1:500-1:1000) or mouse-anti-his antibody (Abcam, diluted 1:10000) in TBST containing 0.01% powdered milk and 0.01% BSA and incubated with membrane overnight at 4°C with rocking. The following morning, the PVDF membrane was washed three times with TBST (10 min each) prior to addition of goat-anti-mouse or goat-anti-human IgG antibody (Abcam) diluted 1:5000-1:10000 for 1 hour at room temperature. Membrane was washed three times with TBST prior to addition of ECL 2 Chemiluminescent substrate (Pierce) and imaged with ImageQuant™ LAS4000 (GE Healthcare Life Sciences).

LIPOPOLYSACCHARIDE PURIFICATION

LPS was purified from *B. thailandensis* E264 via modified hot-phenol extraction method, as described previously (Burtnick et al., 2012). First, freezer stocks were inoculated into 25 mls LB broth and allowed to incubate for ~20 hrs at 37°C with agitation (200 rpm).

The next day, the culture was diluted 1:100 into 2.5 L of LB broth, and allowed to grow for an additional 20 hrs at 37°C with agitation (200 rpm). Bacterial pellet was obtained via centrifugation (6,000 x g for 15 min) and resuspended in 100 mls molecular grade water (Corning). Upon the addition of 100 mls 90% phenol (Fisher), solution was heated with stirring to 80°C and allowed to cool to room temperature. Solution was then added to equilibrated dialysis tubing (SpectraPor® MWCO 3kDa) and dialyzed against 4-5 exchanges of water for complete phenol removal. Dialysate was clarified via centrifugation (6,000 x g for 15 min) and lyophilized. The resulting dry material was resuspended in an aqueous solution containing 10 mM Tris-HCl [pH 7.5], 1 mM $MgCl_2$, 1 mM $CaCl_2$ and digested with RNase and DNase I (50 µg/ml each, Sigma) at 37°C with agitation (200 rpm) for 2-3 hrs. At this time, Proteinase K (50 µg/ml, Sigma) was added to the solution and incubated at 60°C for an additional 2-3 hrs. Insoluble material was removed via centrifugation (16,000 x g for 20 min), and the supernatant stored at 4°C overnight. To obtain LPS pellet, supernatant was ultracentrifuged 3 times (100,000 *x g* for 1 hour), washing with 25 mls molecular grade water between centrifugations. After lyophilization, the dry, purified carbohydrate preparation was washed 4-5 times with 90% ethanol and lyophilized a further time to obtain purified LPS. Dry LPS was weighed, resuspended to 1 mg/ml in PBS and stored at -80°C until use. To assess purity, 1 µg LPS was diluted into 2X Laemmli buffer (BioRad), heated at 95°C for 5 min and run on 12% SDS-PAGE (BioRad). To visualize LPS, gel was either transferred to PVDF for western blotting or stained with Silver Stain kit (Pierce) according to manufacturer's directions. For western blot, PVDF membrane was probed with 1 µg/ml *B. pseudomallei* O-antigen mAb C5A and detected via addition of HRP-conjugated goat-anti-mouse antibody.

CONSTRUCTION OF GOLD-NANOPARTICLE GLYCOCONJUGATES

AuNP-glycoconjugates were constructed as previously described (Gregory et al., 2015), with modifications. First, 15 nm spherical AuNPs were synthesized by heating 1 mM gold (III) chloride trihydrate (Sigma) to 90°C with stirring, followed by rapid reduction with 90 mM sodium citrate dehydrate (Sigma), according to the Turkevich method (Turkevich J, 1951). The solution was allowed to cool to room temperature and stored away from light until use. Particle size and shape were confirmed via transmission electron microscopy (TEM). In order to immobilize antigens to the AuNP surface, 0.1 mM 16-mercaptohexadecanoic acid (16-MHDA) and 0.1% Triton-X 100 were added to a solution of AuNPs. After a 2 hour incubation away from light, this solution was filter centrifuged (EMD Millipore Amicon™ Ultra-15, 3 kDa MWCO) at 4,000 x g for ~20 min and addition of 16-MHDA repeated to ensure complete coverage. Attachment of 16-MHDA was confirmed by measuring plasmon resonance via UV-Vis (Epoch Spectrophotometer).

To optimize conditions for protein conjugation, 1 ml of 16-MHDA functionalized AuNPs was centrifuged (16,000 *x g* for 10 min) and resuspended to 800 µl final volume in a variety of buffers (including PBS at pH 6.5, 7.5 and 8.5, MES at pH 4.5 and 5.5, Tris-HCl at pH 7.5 and 8.5, and 0.1M Borate at pH 7.5 and 8.5). From this solution, 80 µl of AuNPs were added to each well of a 96-well plate, followed by addition of 2 µl 5% Triton™ X-100 (v/v), 10 µl EDC/NHS (0.15 mM and 0.6 mM, respectively) and protein (concentration ranging from 0 µg to 50 µg per well) to final volume of 100 µl. Plate was incubated overnight at room temperature with shaking. The following day, the plate was examined visually for aggregation. Additionally, to further assess stability of protein-

conjugated AuNPs, 100 µl of 10% (w/v) NaCl solution was added to each well. Aggregation in the presence of high salt concentrations was visualized by the color change from red to blue. Solutions that did not undergo a color change demonstrated increased stability as a result of protein saturation. The wells demonstrating the least aggregation were selected for SDS-PAGE confirmation.

Briefly, the conjugation reaction (100 µl) was removed from the plate, and centrifuged (16,000 x g for 10 min). After discarding the supernatant containing unconjugated fractions, 5 µl of 1mM 11-mercapto-1-undecanol was added to cleave the protein, and the solution allowed to incubate at room temperature for 30 min. At this time, 2X Laemmli buffer (BioRad) was added prior to heating samples at 95°C for 5 min. Denatured samples were loaded on 4-20% SDS-PAGE (BioRad) at 100V for 1 hour. Gel was removed, washed with ultrapure water and stained with BioSafe (BioRad) protein stain.

In addition to the parameters described above, we also assessed the effect of additional parameters on their ability to prevent/reduce aggregation, including addition of BSA (ranging from 0.1, 0.5 and 1% (w/v) BSA), use of Tween®-20, concentration of surfactant (0.05, 0.1 or 0.5% TritonX-100 or Tween-20), length of conjugation (1.5-18 hrs) and alternative activating reagents (EDC/NHS vs DMTMM).

Using the optimized parameters above, 0.1 mM 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, Sigma), 0.1% Tween-20 and 20 µg recombinant protein were added to a 1 ml solution of gold nanoparticles (AuNPs), and allowed to incubate overnight at room temperature with agitation. To prevent aggregation of AuNPs, conjugation reactions were performed in either 1X PBS [pH 7.5] or 0.1 M

Borate [pH 8.6] buffer. To confirm efficacy of protein conjugation, 100 µl of AuNP-protein conjugates were centrifuged (16,000 *x g* for 5 min), and protein cleaved with 1 mM 11-mercapto-1-undecanol (Sigma), as described above. After heating, the AuNP-protein solution was added to an SDS-PAGE gel (4-20%, BioRad) and visualized with Bio-Safe stain (BioRad).

LPS was covalently attached to AuNP-protein conjugates using a modified thiol-malemide coupling approach. First, 4.3 µl of 40 mM N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and 17.3 µl of 10 mM N-hydroxysuccinimide (NHS) were combined with 0.2 mg LPS in 50 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer and allowed to incubate for 15 min. Next, 10.9 µl of 800 µM EMCH was added and incubated an additional 15 min at room temperature. The pH of the LPS solution was then adjusted to 7.0 with 0.5 M NaOH, and incubated for 1 hour at room temperature with rocking, followed by filter centrifugation and desalting into 5 mM EDTA. In the meantime, 25.4 µl of 250 µM S- acetylthioglycolic acid N-hydroxysuccinimide ester (SATA, Sigma) was added to protein-conjugated AuNPs and incubated for 1 hour at room temperature. The reaction was quenched with 50 µl of 50% (w/v) hydroxylamine and 50 µl of 5 mM EDTA. At this time, the protein-conjugated AuNPs were filter centrifuged and re-suspended into LPS/EDTA solution for 4 hrs, at which time the reaction was quenched with 10 µl of 5 mM N-ethylmaleimide. Prior to immunization, the AuNP glycoconjugates were washed twice with PBS (filter centrifuging between washes) and re-suspended to final desired volume. Conjugation was confirmed by SDS-PAGE and dynamic light scattering (DLS, UTMB Sealy Center for Structural Biology and Molecular Biophysics).

In vivo studies

ANIMAL STUDIES

All *in vivo* studies were performed on 6-8 week-old female BALB/c mice obtained from Charles River (Wilmington, MA, USA). Animals were housed in microisolator cages under pathogen-free conditions, provided with rodent feed and water *ad libitum*, and maintained on 12 hour light cycle, as previously described (Hatcher et al., 2016; Mott et al., 2015). To allow adequate acclimation, mice were housed within the animal facility for 1 week prior to experimentation. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Medical Branch (Protocol Number 0503014B/D).

DNA VACCINATION STUDIES

To collect DNA for vaccination studies, *E. coli* DH5α freezer stocks were inoculated into 10 ml LB broth and incubated overnight at 37°C with agitation (200 rpm). The following day, cultures were diluted 1:1000 into 2.5 liters LB broth, and grown for an additional 16 hrs. Bacteria were pelleted via centrifugation (6,000 x g for 15 min) and frozen at -20°C until use. Plasmid DNA was harvested via Plasmid Giga Prep kit (Qiagen), according to manufacturer's directions. Final DNA pellet was resuspended in TE buffer [pH 8.0] and quantified via Epoch Spectrophotometer (BioTek). For immunization studies, 6-8 week old BALB/c mice were i.n. vaccinated with a prime and two boosts of 60 μg pVAX-1 DNA. Prime vaccination was administered with 1 μg Cholera toxin B adjuvant. Two

weeks following the last boost and one week prior to challenge, mice were bled retro-orbitally. For sera collection, blood was allowed to clot at room temperature for 30 min prior to centrifugation (10,000 x g for 10 min). Sera was removed and stored at -20°C until use. Mice were challenged with ~3 x LD_{50} *B. pseudomallei* K96243, and survival and weights were monitored for 21 days post-challenge.

PROTEIN VACCINATION STUDIES

In order to assess the protective capacity of purified protein alone, 6-8 week old female BALB/c mice were administered a prime and two boosts of purified protein (10 µg or 20 µg), together with 200 µg Alhydrogel® adjuvant or 10 µg VacciGrade™ CpG ODN 2395 (Invitrogen) through various routes [i.p., and subcutaneous (s.c.)]. Immunization groups included recombinant hemagglutinin, Hcp1, Porin OpcP1, FlgL, and Porin OpcP. Two weeks following last boost and one week prior to challenge, anesthetized animals were bled retro-orbitally for sera collection. Mice were challenged with ~3 x LD_{50} *B. pseudomallei* K96243, and survival and weights were monitored for 21 days post-challenge.

AUNP-GLYCOCONJUGATE VACCINE STUDIES

To assess the immunogenicity and protective capacity of nano-glycoconjuates in animals, BALB/c mice were immunized s.c. with a prime and two boosts of nanoglycoconjugate formulation. Each immunization was formulated to contain 10 µg LPS and 10 µg protein (BMAA1324, BMAA0742, BMA3336, or combination), together with 500 µg Alhydrogel® and 30 µg VacciGrade™ Poly(1:C). Two weeks post-boost and one week prior to challenge, anesthetized mice were bled retro-orbitally for sera collection. Mice

were challenged with ~3 x LD_{50} *B. pseudomallei* K96243, and survival and weights were monitored for 21 days post-challenge.

DETERMINATION OF SERUM ANTIBODY TITERS

Total serum IgG was determined via Ready-set-go!™ (eBioscience), according to manufacturer's directions. ELISAs were performed as previously described (Hatcher et al., 2016), with modifications. Briefly, to determine protein-specific antibody titers, Corning high binding polystyrene plates (Fisher) were coated overnight at 4°C with 10 µg/ml recombinant protein diluted in PBS. The following morning, plates were washed twice with PBS containing 0.05% Tween-20 (Sigma) and blocked for 2 hrs at room temperature with PBS solution containing 0.1% Tween-20 and 2% BSA. After blocking, plates were washed twice prior to the addition of serum samples. Sera were diluted 1:25 in a PBS solution containing 0.01% Tween-20 and 1% BSA, added to the plate in triplicate and serially diluted. After addition of sera, 50 µl of goat anti-mouse IgG polyclonal antibody (diluted 1:500, Abcam) were added to the plate and incubated for 3 hrs at room temperature with shaking. After incubation, the plate was washed four times with wash solution prior to addition of Tetramethylbenzidine (TMB, eBioscience) substrate for 15 min at room temperature. Reaction was stopped with 2 N H_2SO_4 and plate read at 450-570 nm (Epoch Nanospectrophotometer, BioTek). Positive endpoint titer was determined to be twice the standard deviation of the mean of naïve murine sera.

OPSONOPHAGOCYTOSIS ASSAY

Opsonophagocytsois assays were performed to evaluate uptake of *B. pseudomallei* in the presence of immune sera, as described previously with minor modifications (Burtnick et

al., 2012). RAW264.7 cells were seeded in a 24 well plate at a density of $5x10^5$ cells/well and incubated overnight to allow adherence. Overnight (12 hour) cultures of *B. pseudomallei* K96243 were diluted 1:10 in LB broth and allowed to grow to mid-log phase (~3 hrs). Bacteria were adjusted to $2.5x10^7$ CFU/ml in DMEM and incubated with 10% pooled (n=10), heat inactivated (30 min at 56°C) sera for 30 min at 37°C with agitation (200 rpm). After incubation, bacteria were adjusted to $1x10^6$ CFU/ml with DMEM, and 500 µl administered to cells to achieve a multiplicity of infection (MOI) = 1. At this time, bacterial suspensions were plated on LB agar in order to determine input. After addition of bacteria, cells were centrifuged (800 rpm for 3 min) to facilitate adhesion and incubated at 37°C for 1 hour. After incubation, cells were washed twice with Hank's Balanced Salt Solution (HBSS, Corning) prior to addition of DMEM containing 250 µg/ml kanamycin (Sigma) for 1 hour at 37°C. After extracellular killing, cells were washed twice with HBSS and lysed with 0.2% Triton X-100 (Sigma). Cell lysates were serially diluted in PBS and plated on LB agar for bacterial enumeration. The percentage of bacterial uptake was determined by input/output.

ADHESION AND INVASION ASSAYS

Adhesion and invasion assays were performed as described previously, with modifications (Tapia et al., 2016). To evaluate bacterial adhesion and invasion in the presence of immune sera, LA-4 or HeLa cells were seeded in a 24 well plate at a density of $5x10^5$ and incubated overnight to allow adherence. Overnight (12 hour) cultures of *B. pseudomallei* K96243 were diluted 1:10 in LB broth and allowed to grow to mid-log phase (~3 hrs). Bacteria were adjusted to $2.5x10^7$ CFU/ml in DMEM (HeLa cells) or F12K media (LA-4 cells) and incubated with 10% pooled (n=10), heat inactivated (30

min at 56°C) sera for 30 min at 37°C with agitation (200 rpm). After incubation, bacteria were adjusted to $1x10^6$ CFU/ml with DMEM of F12K media, and 500 µl administered to cells to achieve an MOI of 1. At this time, bacterial suspensions were plated on LB agar in order to determine input. After addition of bacteria, cells were incubated at 37°C for 3 hrs. After incubation, cells were washed twice with HBSS and lysed with 0.2% Triton X-100. Cell lysates were serially diluted in PBS and plated on LB agar for bacterial enumeration. The percentage of bacteria adhesion was determined by output/intput.

To determine bacterial invasion, experiment was performed as described above. At 3 hrs post-infection, cells were washed twice with HBSS, followed by addition of DMEM or F12K containing 250 µg/ml Km for 1 hour. After incubation, cells were washed twice, lysed with 0.2% Triton X-100 and plated on LB agar for CFU enumeration. The percentage of bacterial invasion was determined by output/intput.

Chapter 7 Selection of potential vaccine candidates

INTRODUCTION

The current pool of characterized *Burkholderia* proteins is limited, thereby hindering progress towards the optimization of various vaccine platforms. To address this need, we optimized a reverse vaccinology approach to identify immunogenic proteins from the host-adapted pathogen *B. mallei*. This pathogen was selected for informatics analyses based on its smaller genome size, 99% genetic identity with *B. pseudomallei* (increase probability of cross-protection) and its mammalian reservoir (loss of genes involved only in environmental adaptation). A variety of well-recognized, previously validated bio- and immuno-informatics programs were utilized to select for outer membrane or secreted proteins with a high number and affinity of MHC epitopes (Table 3). Proteins were ranked against themselves according to predicted immunogenicity, and the seven highest-ranking proteins were selected for downstream expression and validation studies. All selected proteins were cloned into pET30a(+) expression vectors and protein expression induced with IPTG. Protein identity was confirmed via MALDI TOF (UTMB Mass Spectrometry Core). Purified recombinant proteins were evaluated for reactivity against experimental murine and convalescent human sera using western blots and ELISAs, respectively.

RESULTS

Bio- and Immuno- informatics

Through bioinformatics predictions, the *B. mallei* ATCC 23344 proteome (>4500 proteins) was first downselected to 160 outer membrane and secreted proteins (pSORTb threshold ≥9.5). The remaining proteins were comprised of approximately 3%

periplasmic, 18% inner membrane and 44% cytoplasmic proteins, with 32% of proteins possessing unknown or multiple localizations.

From the 160 outer membrane and secreted proteins, 56 were eliminated on the basis of > 1 transmembrane domain, < 98% conservation between *B. mallei* and *B. pseudomallei*, homology with host (mouse and human) proteins, high instability index or non-antigenicity. The remaining 104 proteins were highly enriched with flagellar and porin proteins, comprising 9% and 17% of the proteins identified, respectively. Additionally, approximately 11% of these proteins lacked classification and were considered "hypothetical".

The remaining 104 outer membrane/secreted proteins were evaluated for the number of high binding (IC_{50} < 50 nM) MHCI and MHCII epitopes via NetCTL 1.2 and NetMHCII 2.2 programs, respectively. Following these analyses, proteins were ranked against themselves based on predicted antigenicity (VaxiJen threshold > 0.4), adhesive properties, MED score and % high binding MHC epitopes. The top seven proteins were selected for downstream immunogenicity studies (Table 5). Additionally, Hcp1 – an immunogenic *Burkholderia* protein associated with T6SS cluster 1 – was selected for comparison in *in vitro* and *in vivo* studies.

Cloning and purification of vaccine candidates

Genes of interest were cloned into a pET30a(+) expression vector and sequenced (UTMB Genomics Core) to confirm 100% gene identity. Upon IPTG induction, all proteins, with the exception of FlgD, were expressed in high yields as inclusion bodies. Interestingly, despite sufficient bacterial growth, FlgD was expressed in very low yields. Additionally, little to no soluble protein was produced from any of the pET30a(+) constructs, even when bacteria were grown at low temperatures (e.g., 30°C, data not shown).

All proteins were able to be solubilized into a refolding buffer containing 50 mM Tris-HCl pH [8.5], 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.5 M Arginine, 0.75 M Guanidine HCl, 0.5% Triton-X 100, and 1 mM DTT. All proteins, with the exception of Hcp1, were dialyzed and stored in PBS at 1 mg/ml concentrations. However, Hcp1 precipitated upon dialysis with PBS, and was therefore dialyzed and stored in 20 mM Tris-HCl [pH 8.0]. Once refolded, Hcp1 could be diluted into PBS without precipitation. Protein identities were confirmed via MALDI-TOF (UTMB Mass Spectrometry Core), and purity assessed via SDS-PAGE (Figure 1).

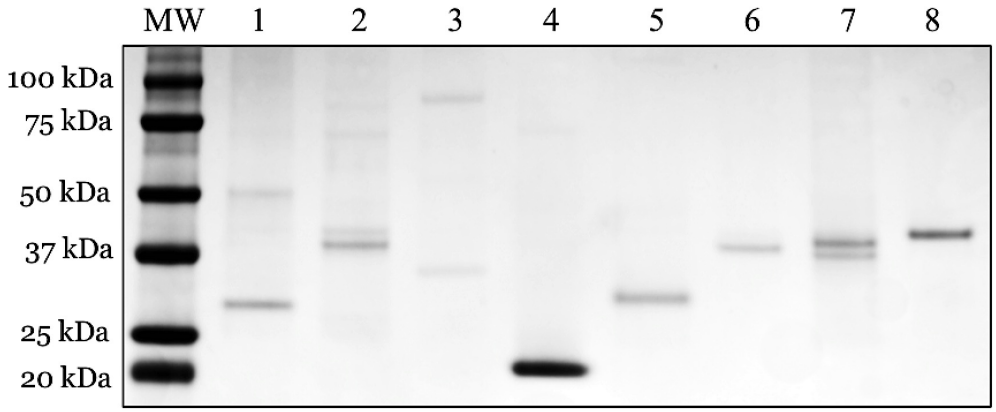
Validation of *in silico* predictions

Indirect ELISAs were used to determine serum reactivity with experimental murine melioidosis sera. All proteins exhibited reactivity at serum dilutions of 1:50, with endpoint titer determined to be $OD_{450-570}$ value equivalent to two standard deviations from the mean of naïve murine sera.

Because these proteins are conserved between *B. pseudomallei* and *B. mallei,* we wanted to evaluate cross-seroreactivity with melioidosis and glanders sera, respectively. To evaluate seroreactivity with convalescent human melioidosis and equine glanders sera, proteins were transferred to PVDF membrane and probed with individual sera (n=13, 1:400-1:1000 dilution) or goat-anti-his primary antibody (1:10,000 dilution). All proteins exhibited varying reactivity with convalescent human melioidosis sera (n=13, Figure 2-3), but were unreactive with convalescent human glanders sera (n=1), as well as naïve human sera (n=1) (data not shown). When proteins were evaluated against convalescent equine glanders sera, only the hemagglutinin protein (BMAA1324)

demonstrated significant reactivity. Interestingly, this protein was shown to react with both seropositive and seronegative equine sera (Figure 4).

A



B

Protein	Protein yield per liter
OmpW	52.5 mg
OpcP1	19.6 mg
Hemagglutinin	57.3 mg
Hcp1	60 mg
FlgD	0.1 mg
Porin OpcP	40.8 mg
Porin	21 mg
FlgL	60 mg

Figure 1. Expression and purification of top vaccine candidates. For SDS-PAGE analysis, 1 µg of recombinant protein was diluted into Laemmli buffer and heated at 95° C for 5 min prior to addition to 4-20% polyacrylamide gel. A) SDS-PAGE of top candidates indicating molecular weight and purity of recombinant proteins. From L to R: MW- Ladder, 1- OmpW, 2- OpcP1, 3- Hemagglutinin, 4- Hcp1, 5- FlgD, 6- OpcP porin, 7- Porin, 8- FlgL (B) Protein yields per liter bacterial culture.

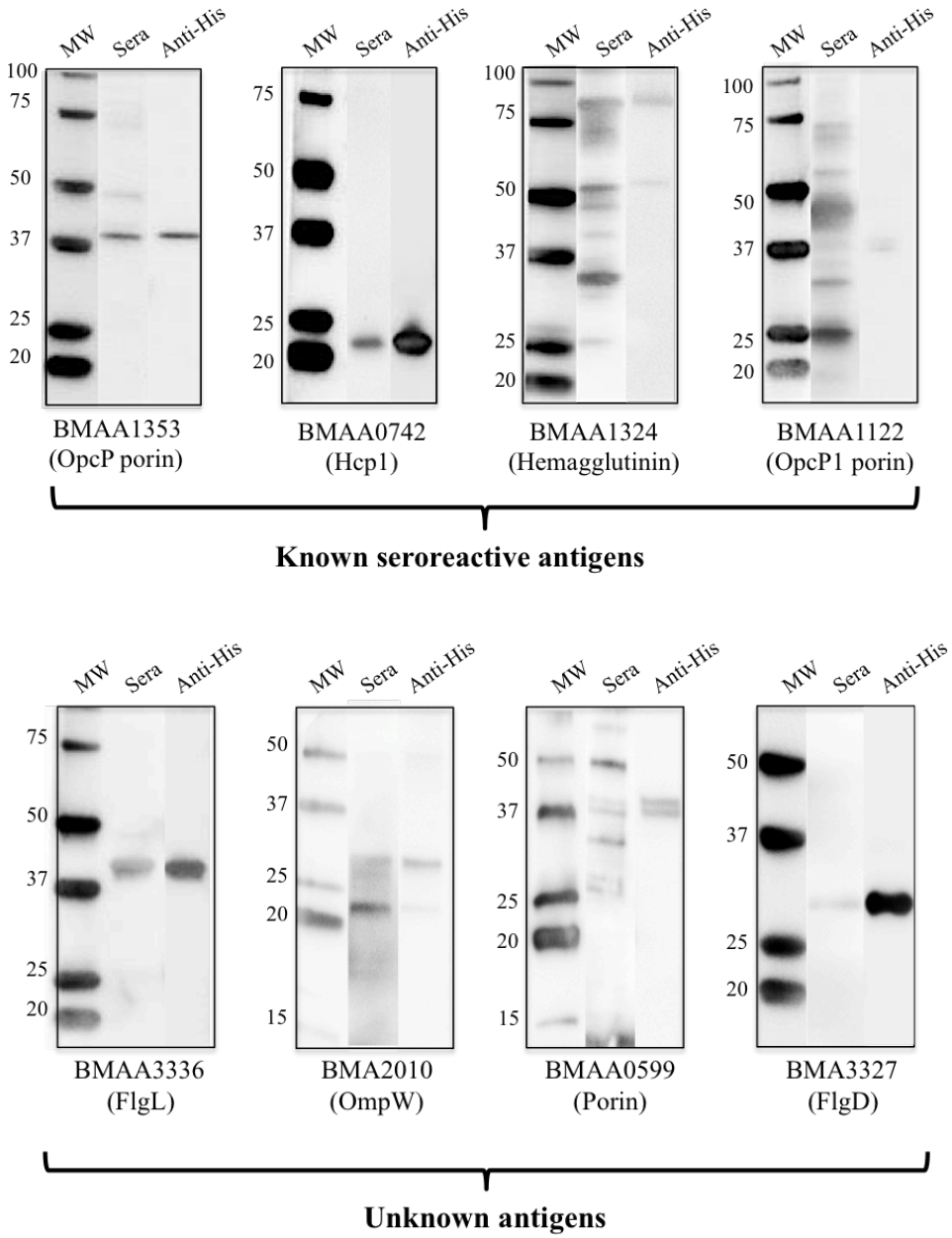


Figure 2. Representative western blots demonstrating seroreactivity of known and novel antigens with convalescent human melioiodosis sera. *MW = Molecular weight ladder (kDa).

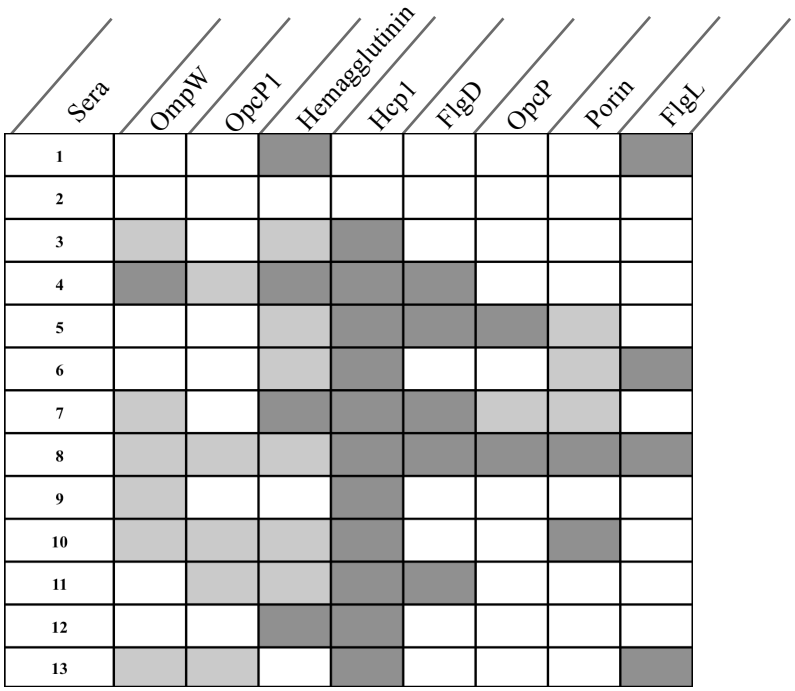


Figure 3. Proteins exhibit varying reactivity with different convalescent human melioidosis sera. White = no reactivity, light grey = faint band, dark grey = strong band.

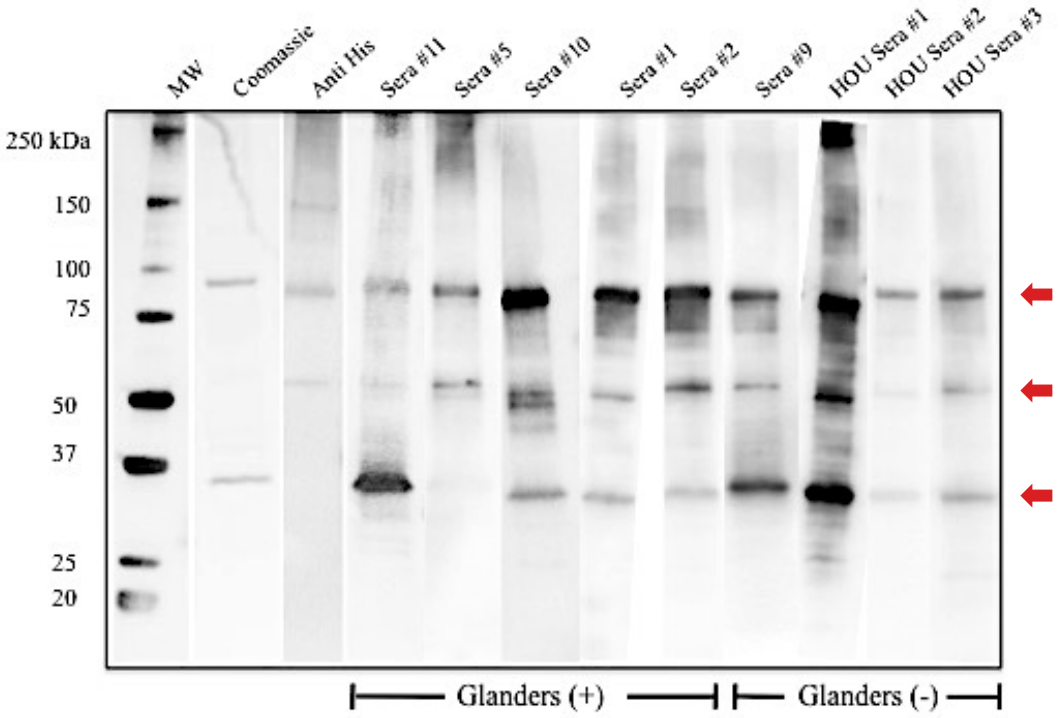


Figure 4. Recombinant hemagglutinin protein reacts strongly with all equine sera tested, including convalescent equine glanders sera (Sera #s 11, 5, 10, 1 and 2) and seronegative equine sera (Sera #9, and HOU Sera #s 1-3). Arrows indicate location of hemagglutinin protein. *MW = molecular weight ladder.

DISCUSSION

This study represents one of the first attempts to use reverse vaccinology methods to identify whole protein antigens conserved between *B. pseudomallei* and *B. mallei.* Only proteins exhibiting ≥ 98% sequence identity between *B. pseudomallei* and *B. mallei* were included in the informatics analyses. Because *B. mallei* is an obligate mammalian pathogen, selection of conserved antigens between these two species allowed the exclusion of genes involved only in environmental adaptation. Importantly, this conservation between *B. pseudomallei* and *B. mallei* also increases the probability of selecting cross-protective antigens, a phenomenon that has been demonstrated with other *Burkholderia* antigens when tested in mice (Whitlock et al., 2010). Importantly, this cross-reactivity has also been demonstrated in humans, as convalescent human melioidosis sera reacts with *B. mallei* antigens via Indirect Hemagglutinin Assay (IHA) (Tiyawisutsri et al., 2005). Taken together, these findings suggest that cross-protection can be achieved by selection of conserved antigens.

Many of the criteria for candidate selection (e.g., subcellular localization, transmembrane domains, MHC epitopes) were based on previously successful reverse vaccinology approaches against bacterial pathogens (Garcia-Angulo et al., 2014; Pizza et al., 2000; Singh et al., 2016). From the *B. mallei* proteome (>4500 proteins), 160 outer membrane and secreted proteins were identified. The selection of outer membrane and secreted proteins has been widely demonstrated in numerous reverse vaccinology studies (Moriel et al., 2010; Pizza et al., 2000; Stranger-Jones et al., 2006), as these antigens are more likely to be exposed to the host immune system. These outer membrane and secreted proteins were first down-selected based on desired physiochemical

characteristics, including: ≥ 98% conservation between *B. pseudomallei* and *B. mallei*, non-similarity with host proteins, ≤ 1 transmembrane domain, and stability. First, proteins exhibiting similarity to human or mouse proteins were eliminated. Next, proteins possessing >1 transmembrane domain were eliminated from consideration, as other studies have shown that the expression and purification of these highly hydrophobic proteins is difficult and oftentimes unsuccessful (Baker, 2010; Pizza et al., 2000). Using *in silico* screening, we also eliminated proteins with high instability index. Altogether, these analyses allowed the exclusion of 55 proteins on the basis of >1 transmembrane domain, homology with host proteins, or predicted instability.

To determine potential immunogenicity, the remaining 104 protein candidates were evaluated for desired vaccine characteristics, including adhesive properties, antigenicity, and MHC epitopes. First, proteins were evaluated for their predicted adhesive properties, as adhesins are common targets for bacterial vaccine development (Wizemann et al., 1999). Next, the proteins were screened for putative antigenicity using VaxiJen – a program designed to predict antigenicity based on similarity to other known antigens(Doytchinova and Flower, 2007). Finally, these antigens were evaluated for the number and affinity of MHCI and MHCII epitopes. These proteins were then ranked against each other on the basis of adhesive properties, antigenicity, and MHC epitopes. Through these rankings, it became evident that proteins tended to rank higher in one MHC class than the other. For this reason, we chose to rank MHCI and MHCII predictions separately.

Although *B. pseudomallei* and *B. mallei* are intracellular pathogens, the role of MHCI in pathogenesis and immunity remains indeterminate and often controversial. In

contrast, the importance of MHCII is better understood. *B. pseudomallei* is known to infect professional antigen presenting cells (APCs), including macrophages and dendritic cells (Pruksachartvuthi et al., 1990; Williams et al., 2014). Infection of dendritic cells *ex vivo* has been shown to result in upregulation of MHCII expression (Williams et al., 2014). In humans, polymorphisms in the HLA Class II alleles have been associated with severe melioidosis (Dharakul et al., 1998). Additionally, $CD4^+$ T-cells have been shown to be important for vaccine-induced protection in mice, particularly during later stages of infection (Haque et al., 2006a; Haque et al., 2006b). Together, these studies suggest an important role for MHCII in the immune response to *B. pseudomallei* and provide strong rationale for the prioritization of MHCII epitopes in this study. Because Hcp1, a well-characterized *Burkholderia* antigen, ranked 13th place for MHCII predictions, proteins ranking higher than Hcp1 were selected for further validation and immunogenicity studies.

Of the top 10 candidates, 7 novel proteins and Hcp1 were successfully cloned into a pET30a(+) expression vector (Table 5). Upon induction with IPTG, all proteins – with the exception of FlgD – were highly expressed as inclusion bodies (Figure 1B). This high expression indicates that these proteins are non-toxic to bacteria, an important consideration for scalability. In contrast, FlgD was poorly expressed despite regular bacterial growth. The limited expression of this protein might be the result of protein degradation, or may result from an unusual RNA structure that interferes with ribosomal binding. The expression of inclusion bodies can be very challenging; however, this study represents a novel method for the isolation, purification and refolding of inclusion bodies that results in highly pure, soluble protein (Figure 1A-B).

Of the seven proteins identified via informatic analyses, three proteins – FlgD, FlgL and a porin – remain novel antigens, and to our knowledge have not yet been characterized *in vitro* or *in vivo*. However, some characterization has been performed on the remaining four proteins (porin OpcP, OmpW, OpcP1 and hemagglutinin). Two of these proteins, porin OpcP and OmpW, have been shown to comprise 4.63% and 5.88% of *B. pseudomallei* total outer membrane proteins (OMP) under *in vitro* growth conditions, respectively. Importantly, porin OpcP also comprises 2.5% of *B. mallei* OMPs under these same conditions (Schell et al., 2011). This study also demonstrated that supplementation of growth media with amino acids resulted in increased expression of the porin OpcP to 11% of *B. pseudomallei* OMPs, suggesting a possible role in amino acid transport (Schell et al., 2011). Importantly, the porins OpcP and OpcP1, together with hemagglutinin, have previously been shown to react with convalescent human melioidosis sera (Felgner et al., 2009; Harding et al., 2007; Suwannasaen et al., 2011; Tiyawisutsri et al., 2007); additionally, the hemagglutinin protein also demonstrated reactivity with experimental equine glanders sera (Tiyawisutsri et al., 2007). This protein has also been shown to stimulate IFNγ production from whole blood isolated from seropositive donors (Campos et al., 2013). *In vitro*, this hemagglutinin has been shown to play a role in *B. pseudomallei* adhesion, internalization and plaque formation in A549 cells (Campos et al., 2013), as well as intracellular survival in J774.2 macrophage-like cells (Lazar Adler et al., 2015). However, the exact role of this protein in *B. pseudomallei* virulence remains controversial, with one study demonstrating a 40-fold decrease in mean lethal dose (MLD) in a mutant strain (Lazar Adler et al., 2015), while another showed no difference in bacterial colonization compared to wild type (Campos et al., 2013).

However, this discrepancy may be the result of differences in parental bacterial strains, challenge dosages and/or routes of inoculation.

When screened against experimental murine and convalescent human melioidosis sera, all proteins exhibited varying seroreactivity (Figure 2-3). These results demonstrate that these proteins are expressed during infection, and are immunogenic to the mammalian host. The wide variability in reactivity may be the result of numerous factors, including differences in donor HLA type and stage of infection (e.g., acute vs chronic). It is also important to note that the purity of the protein appeared to affect banding patterns, as more highly purified proteins demonstrated stronger, more distinct bands compared to less pure preparations. Future work will focus on addressing these shortcomings.

While these results are promising, it is also important to note some significant limitations to this study. First, the evaluation of seroreactivity is only a measurement of the humoral (IgG) immune response, and is not indicative of other antibody subclasses or cellular responses. Additionally, because proteins were denatured prior to SDS-PAGE, antibodies generated against conformational epitopes will not be detected.

When screened against convalescent human glanders sera, none of the proteins exhibited reactivity. However, only a single (n=1) sample was used and more samples are needed in order to make a definitive conclusion regarding the expression and immunogenicity of these antigens during human *B. mallei* infection. When proteins were evaluated for seroreactivity against convalescent equine glanders sera (n=5), only the recombinant hemaggluutinin protein exhibited reactivity. Interestingly, this hemagglutinin was shown to react with all equine samples tested, suggesting that homologs of this protein are expressed in healthy equines (Figure 3).

Taken together, this first part of my study represents a simplified reverse vaccinology approach to identify whole protein antigens conserved between *B. pseudomallei* and *B. mallei*. The use of reverse vaccinology allowed the identification and ranking of putative vaccine antigens based on predicted subcellular localization, antigenicity, adhesive properties and affinity for MHC molecules. By evaluating seroreactivity, we confirmed that these antigens are expressed during infection and are recognized by the human immune system. Importantly, these analyses yielded 3 novel proteins that have not yet been characterized *in vitro* or *in vivo*. To the best of my knowledge, none of these seven proteins have been evaluated in a vaccine formulation.

Chapter 8 Evaluation of the immunogenicity and protective capacity of protein candidates *in vivo*

INTRODUCTION

As described in the previous chapter, *in silico* analyses allowed the identification of novel, seroreactive antigens. In order to assess the immunogenicity and vaccine potential *in vivo*, these novel *Burkholderia* antigens were administered to mice via different vaccine formulations.

As an initial formulation screen, three of these candidates were selected for incorporation into a DNA vaccine. Because DNA vaccines are inexpensive and easy to synthesize, they allow for a rapid screen of immunogenic candidates. Administration of a DNA vaccine has the potential to stimulate both humoral and cellular immunity. Upon injection, plasmid DNA is taken up by APCs and is translocated to the cell nucleus, where it undergoes transcription to produce foreign antigens (Ingolotti et al., 2010). In animal models, DNA vaccines have been shown to generate robust immune responses against several bacterial pathogens, including *M. tuberculosis, Helicobacter pylori* and *Bacillus anthracis* (Reviewed in (Ingolotti et al., 2010)). Previously, a DNA vaccine encoding the *Burkholderia* antigen FliC provided up to 80% survival 14 days post-challenge with 10^5 CFU of a heterogenous mixture of *B. pseudomallei* strains (Chen et al., 2006a; Chen et al., 2006b). These results suggest that DNA vaccination might be a useful tool for assessing antigen immunogenicity *in vivo*.

Many studies have evaluated the ability of protein immunization to protect against *B. pseudomallei* challenge *in vivo* (Table 1). The most successful protection has been achieved in BALB/c mice receiving a prime and two boosts of recombinant protein prior

to intraperitoneal (i.p.) challenge with *B. pseudomallei*. Specifically, LolC formulated with MPL+TDM adjuvant was shown to provide 80% protection at 42 days post i.p. challenge with $4x10^4$ CFU *B. pseudomallei* K96243 (Harland et al., 2007). Additionally, when administered with Sigma Adjuvant System, Hcp2 (associated with *B. pseudomallei* T6SS cluster 2) provided 80% protection 42 days following i.p. challenge with $5x10^4$ CFU *B. pseudomallei* K96243 (Burtnick et al., 2011). Interestingly, only one protein vaccination study has evaluated the ability to protect against respiratory infection. Whitlock *et al* demonstrated that i.n. vaccination with recombinant BopA affords 60% survival at 55 days post-challenge with 2 LD_{50} of *B. pseudomallei* 1026b (Whitlock et al., 2010). Importantly, this study was the first to demonstrate that cross-protection can be achieved against *B. mallei*, as immunization with BopA also provided 100% protection against 2 LD_{50} *B. mallei* ATCC 23344. These studies demonstrate that protein immunization is unlikely to provide full protection against lethal challenge; however, these studies allow assessment of the immunological potential of recombinant proteins *in vivo*.

In this study, animals were immunized with various vaccine formulations in order to assess the humoral immune response to these novel proteins. ELISAs were used to determine protein-specific antibody titers and antibody functionality was assessed *in vitro* through opsonophagocytosis and adhesion assays. In order to evaluate the protective capacity of these proteins, immunized animals were challenged i.n. with *B. pseudomallei* 3 weeks following last boost.

RESULTS

Cloning and expression of DNA vaccine

Three high-ranking proteins (OmpW, porin OpcP1 and hemagglutinin) were selected for incorporation into a pVAX-1 vector. This vector contains the CMV eukaryotic promoter and bovine growth hormone (BGH) polyadenylation signal. Primers were designed to flank the entire ORF and incorporate a Kozak sequence for enhanced eukaryotic transcription. Prior to immunization studies, gene expression was confirmed *in vitro* via RNA isolation from pVAX-1 transfected HeLa cells. Transfection efficacy was confirmed via GFP-expressing eukaryotic vector pMAX (pMAX-GFP) (Figure 5A), a vector commonly used as a transfection control. PCR amplification of cDNA indicated that protein-encoding pVAX-1 plasmids were transcribed in mammalian cells (Figure 5B).

DNA vaccination studies

Six-to-eight week old BALB/c mice were vaccinated i.n. at two week intervals with a prime and two boosts of 60 μg plasmid DNA. For the prime immunization, animals received 1 μg Cholera toxin subunit B adjuvant. Sera collected at two weeks post-vaccination revealed increased total IgG antibody titers in immunized groups (Figure 6A). However, immunization did not provide protection against i.n. challenge with 2 x LD_{50} *B. pseudomallei* K96243 (Figure 6B).

Protein immunization studies

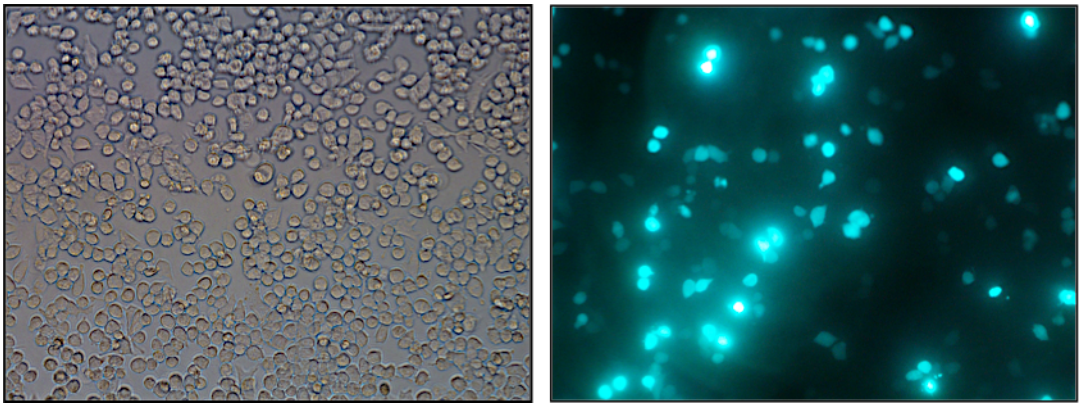
In order to fully assess immunogenicity of recombinant proteins, a series of vaccination studies were performed using two different routes (i.p. vs. s.c.), concentrations (10 μg vs. 20 μg) and adjuvants (Alhydrogel vs. CpG). All studies used the same experimental regimen, involving a prime and two boosts of recombinant protein at two week intervals, followed by serum collection and lethal challenge at 2 and 3 weeks post-boost,

respectively. Protein immunization, irrespective of route, concentration or adjuvant, generated high protein-specific antibody titers (Figure 7B, D and Figure 8B). However, despite these high antibody titers, protein immunization alone did not provide protection against lethal challenge. Notably, changes to route, concentration and adjuvant did not appear to have any effect on vaccine efficacy.

Opsonophagocytosis and adhesion assays

In order to further evaluate the humoral immune response, mice receiving a prime and 2 boosts of recombinant protein and CpG were bled retro-orbitally 2 weeks following the last boost. Opsonization of *B. pseudomallei* with 10% heat-inactivated immune sera did not affect uptake by RAW 264.7 cells (Figure 9A) or adhesion to HeLa epithelial cells (Figure 9B).

A



B

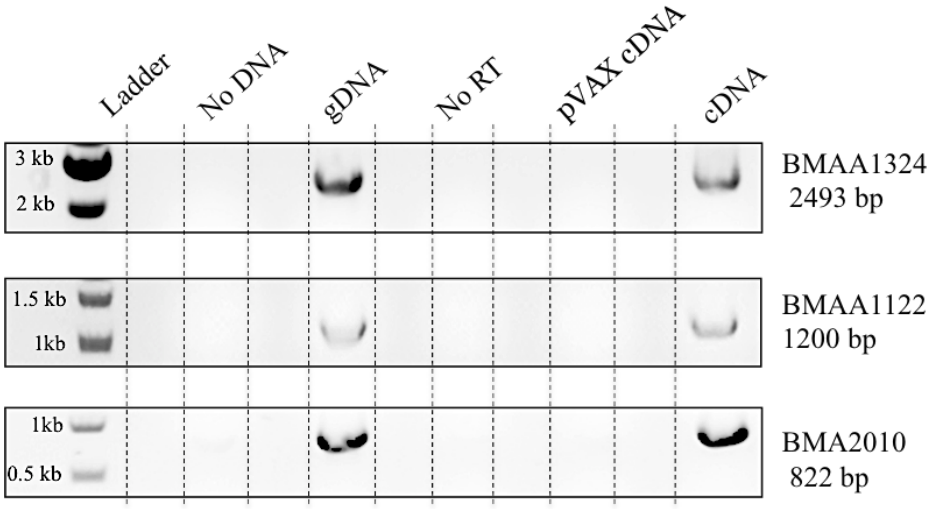


Figure 5. Transfection of HeLa cells confirms transcriptional activity of pVAX-1 encoding proteins of interest. A) Transfection efficacy was confirmed by measuring fluorescence of cells transfected with pmax-gfp. B) RNA was extracted 24 hrs post-transfection and used to synthesize cDNA. PCR amplification of cDNA using gene specific primers confirms transcriptional activity of pVAX plasmids.

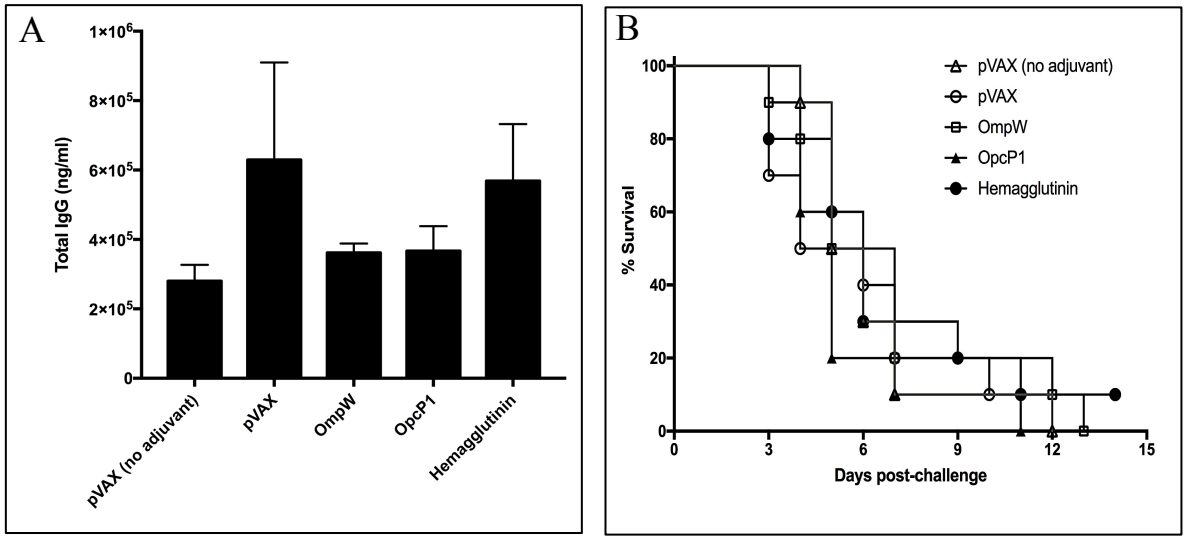


Figure 6: DNA vaccination increases serum IgG but does not afford protection against lethal *B. pseudomallei* challenge. Mice received a prime and 2 boosts of 60 µg pVAX-1 DNA, administered i.n. at 2 week intervals A) At 2 weeks post-boost, vaccinated animals demonstrated increased total serum IgG. B) No significant differences were observed between vaccinated and control groups upon challenge with 2 LD_{50} *B. pseudomallei* K96243.

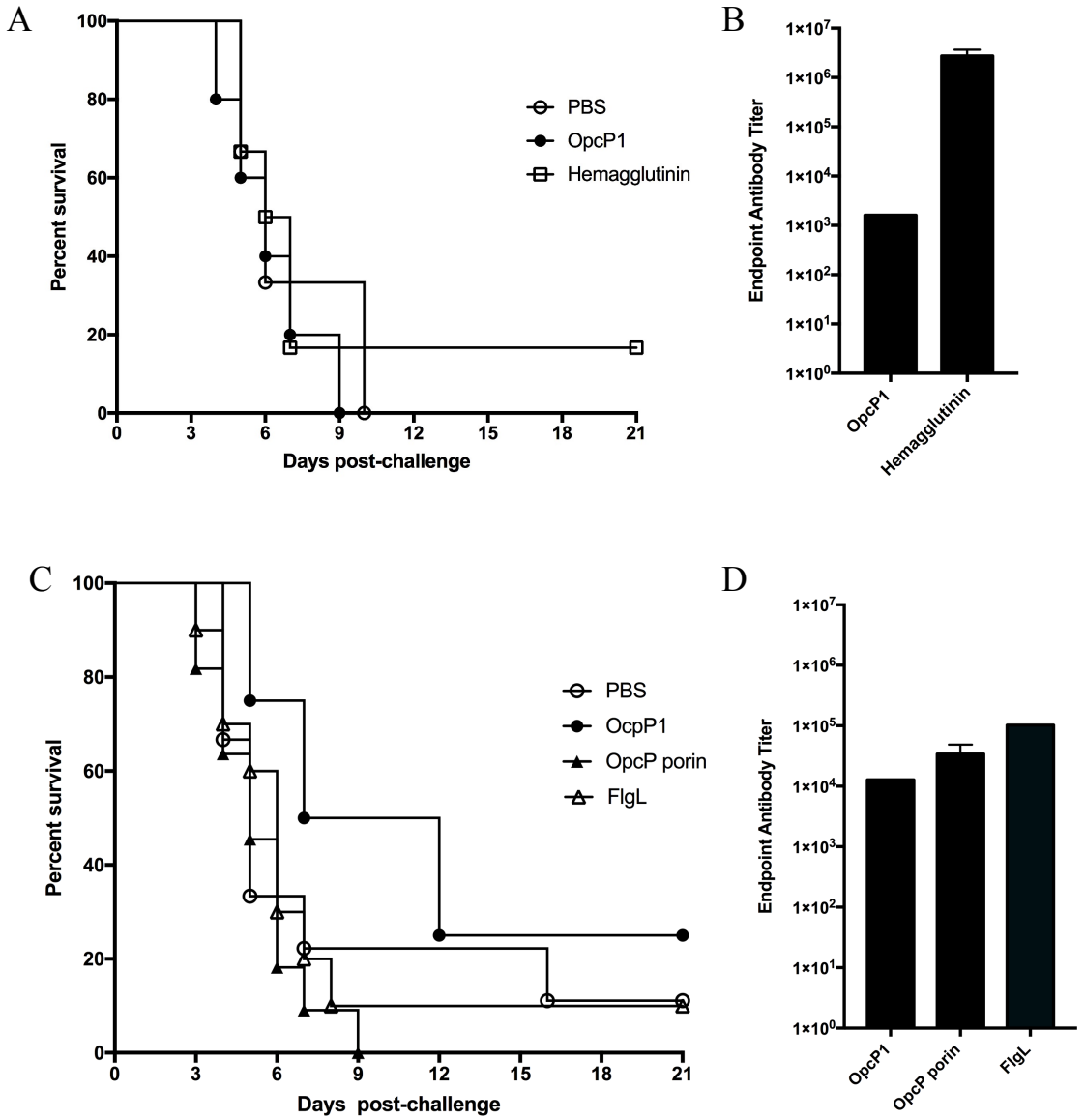


Figure 7. Intraperitoneal immunization with recombinant protein stimulates production of protein-specific antibodies, but does not afford protection against lethal *B. pseudomallei* challenge. Mice received a prime and two boosts of recombinant protein co-formulated with Alhydrogel® adjuvant (200 µg) prior to lethal challenge with 3 x LD_{50} *B. pseudomallei* K96243. A) Survival and B) Protein-specific antibody titers following a prime and two boosts immunization with 10 µg protein. C) Survival and D) Protein-specific antibody titers following a prime and two boosts immunization with 20 µg protein.

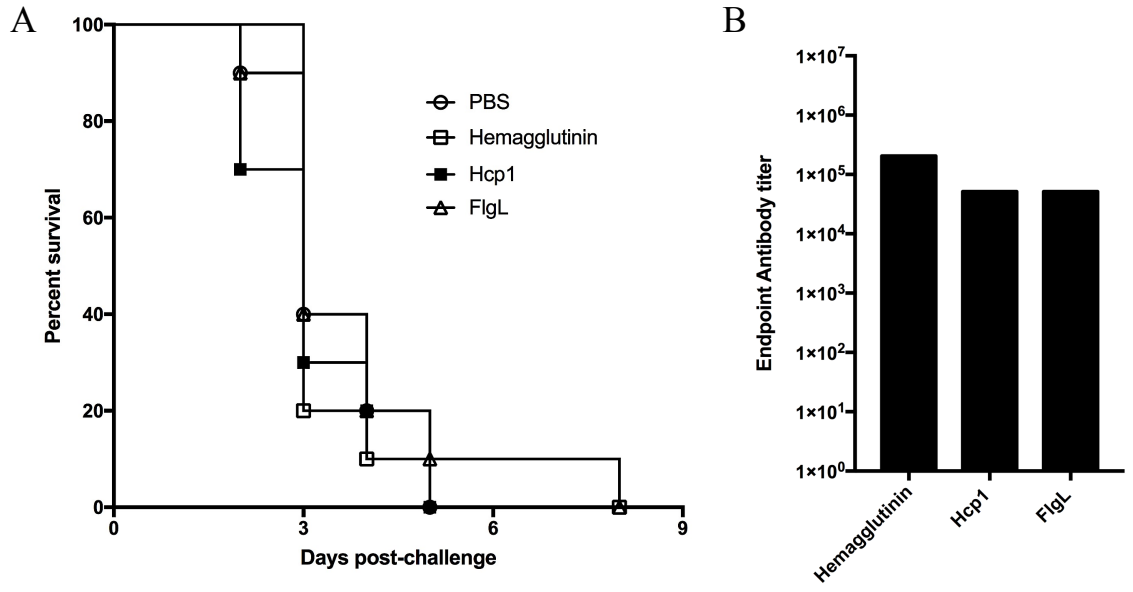


Figure 8: Subcutaneous immunization with recombinant protein stimulates production of protein-specific antibodies, but does not afford protection against lethal *B. pseudomallei* challenge. Mice received a prime and two boosts of recombinant protein (10 µg) co-formulated with CpG adjuvant (10 µg) prior to lethal challenge with 3 x LD_{50} *B. pseudomallei* K96243 (A) Survival post-challenge and B) Protein-specific serum antibody titers after completion of vaccine regimen.

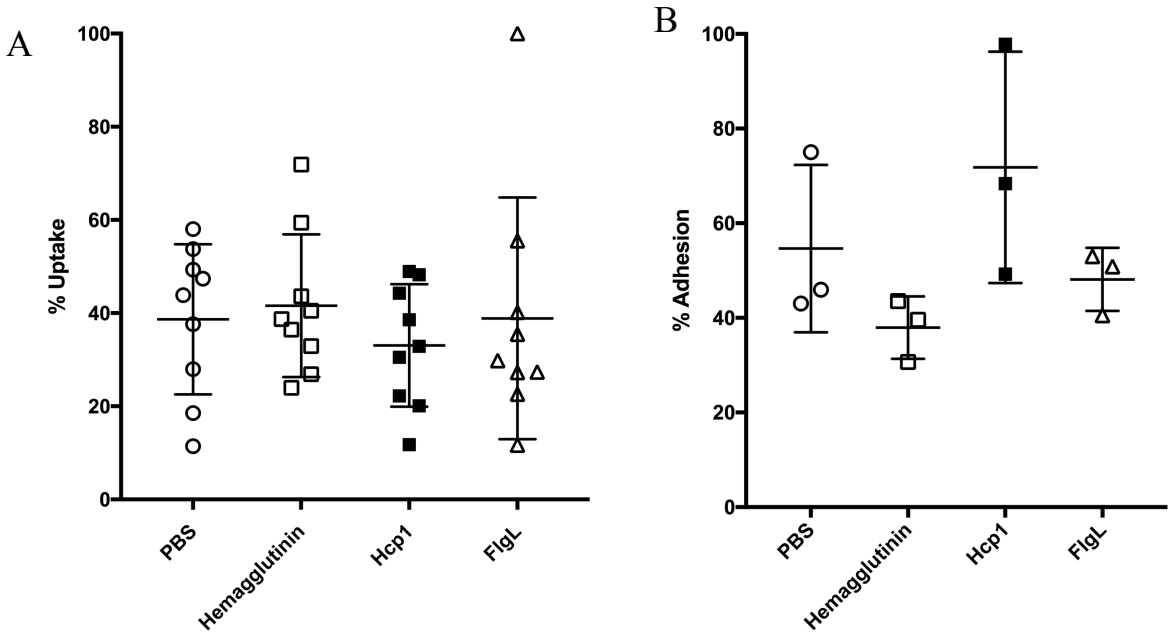


Figure 9: Immune sera does not facilitate bacterial uptake by macrophages or reduce adherence to epithelial cells. Opsonophagocytosis and adhesion assays were performed by incubating $5x10^5$ CFU *B. pseudomallei* K96243 with 10% heat-inactivated immune sera prior to infection of cell monolayer (MOI = 1). A) Bacterial uptake by RAW 264.7 macrophages. Two independent experiments are shown, and individual data points are plotted together with mean ± S.E.M. B) Bacterial adhesion to HeLa epithelial cells. One independent experiment is shown, and individual data points are plotted together with mean ± S.E.M.

DISCUSSION

This study represents an initial attempt to evaluate these novel *Burkholderia* proteins in a vaccine formulation. Administration of pVAX-1 DNA vaccine to BALB/c mice was shown to increase total serum IgG; however, this vaccine did not provide protection against lethal *B. pseudomallei* challenge. This may reflect poor cellular uptake, a problem commonly encountered with DNA vaccines (Tregoning and Kinnear, 2014). Previous studies have shown that upon entrance into the host cell cytoplasm, only 0.1% of plasmid DNA actually enters the nucleus (Capecchi, 1980). In addition, fundamental differences (e.g., rare codons and post-translational modifications) between prokaryotic and eukaryotic transcription and translation may also affect immunogenicity *in vivo* (Ingolotti et al., 2010; Strugnell et al., 1997).

In order to bypass the potential challenges associated with expressing bacterial proteins in eukaryotic host cells, mice were administered a vaccine formulation containing recombinant protein and adjuvant. First, groups of 6-8 week old female BALB/c mice (n=10) were administered a prime and two boosts (i.p) of Hemagglutinin or OcpP1 protein (10 μg) together with 200 μg Alhydrogel® adjuvant. At two weeks post boost, serology revealed high protein-specific IgG titers. However, immunization was not sufficient to protect animals from lethal respiratory challenge with 3 LD_{50} *B. pseudomallei* K96243.

In order to evaluate whether increased protein concentration could improve efficacy, animals were immunized i.p. with a prime and two boost of recombinant protein (20 μg), together with 200 μg Alhydrogel® adjuvant. Interestingly, vaccine formulations containing 20 μg Hemagglutinin or Hcp1 were shown to exhibit toxicity in animals, and

were thus excluded from the study. The remaining animals (immunized with FlgL, OpcP porin or OpcP1) were bled two weeks post-boost for serum collection prior to challenge with 2.5 LD_{50} *B. pseudomallei* K96243. Increasing protein concentration did not appear to have any significant effect on vaccine immunogenicity or protection *in vivo*, as serum titers remained similar but animals were not protected against lethal challenge (Figure 7). Furthermore, because increased protein concentration (20 μg) was poorly tolerated by immunized animals, all further vaccination studies were performed with 10 μg protein.

In order to evaluate whether alternative routes of administration or adjuvants could affect immunogenicity *in vivo*, we immunized animals s.c. with a prime and two boosts of 10 μg recombinant proteins co-formulated with 10 μg CpG adjuvant. As demonstrated in the other protein vaccination studies, immunization of mice resulted in high protein-specific antibody titers, but did not protect against lethal challenge (Figure 8).

Many conclusions can be extrapolated from these results. First, recombinant proteins (irrespective of concentration, route or adjuvant) are immunogenic in mice, confirming *in silico* predictions. However, protein alone appears to be insufficient to protect against lethal respiratory infection. Many vaccine studies have demonstrated that protein immunization can provide partial protection against i.p. challenge with *B. pseudomallei* (Table 1). However, because respiratory infection with *B. pseudomallei* is considered the most rapid and severe route, these findings suggest that a more robust, comprehensive immune response will be needed for protection.

While the immunogenicity of these proteins is evident, their ability to provide protection is entirely unknown. Several studies have demonstrated that immunogenic proteins are not always protective (Gigliotti et al., 1998; Ryder et al., 2010). For example,

Burkholderia antigen OppA reacted strongly against human sera and induced antibody and T-cell responses in mice, but only provided minimal protection against i.p. challenge with *B. pseudomallei* (Hara et al., 2009; Harland et al., 2007; Suwannasaen et al., 2011). Similarly, Druar *et al* demonstrated that immunization with seroreactive Bip proteins afforded no protection against lethal challenge (Druar et al., 2008). While it is possible that these novel antigens are not protective, this study also failed to generate protection with Hcp1, a known protective *Burkholderia* antigen. This finding suggests that further optimization of this vaccine platform is warranted in order to elucidate the full protective capacity of these proteins.

Using heat-inactivated, immune murine sera, we performed *in vitro* assays to evaluate the ability of serum antibodies to facilitate bacterial uptake by macrophages or reduce adherence to epithelial cells. Previous studies have demonstrated that immune sera can facilitate uptake of *B. mallei* by RAW 264.7 macrophages (Burtnick et al., 2012). Additionally, serum opsonization has been shown to increase uptake of *B. pseudomallei* by polymophonuclear cells (PMNs) (Ho et al., 1997; Nithichanon et al., 2015; Su et al., 2010). However, in the current study, opsonization of bacteria with 10% immune sera did not increase macrophage uptake compared to naïve sera (Figure 9A). While these results were unexpected, it may indicate that essential epitopes are hidden on the bacterial surface, resulting in diminished antibody functionality. Alternatively, this result could reflect the importance of particular antibody subtypes. In mice, IgG2a demonstrates high affinity and promiscuity in binding to multiple Fc receptors and plays a major role in macrophage effector functions, including complement fixation and opsonization (Bruhns, 2012; Buchanan et al., 1998; Nimmerjahn et al., 2005). Importantly, high ratios of

IgG2a:IgG1 are indicative of a protective Th1 response and have been correlated with protection against *Burkholderia* in several vaccination studies (Gregory et al., 2015; Hatcher et al., 2016; Scott et al., 2014b; Silva et al., 2013). Further studies will focus on elucidating antibody functionality and evaluating the importance of antibody subtypes in facilitating the uptake of *B. pseudomallei* by macrophages.

The ability of immune sera to reduce the adherence of *B. pseudomallei* to HeLa epithelial cells was also assessed. Previous studies have shown that *B. pseudomallei* can adhere to A549 lung epithelial cells *in vitro*; however, few *Burkholderia* adhesins have been identified to date (Brown, 2002). Like many other gram-negative bacteria, *B. pseudomallei* has been shown to utilize the Type IV pilus to adhere to the surface of A549 and other respiratory cell lines *in vitro* (Essex-Lopresti et al., 2005). Other *B. pseudomallei* adhesins include: flagellum, BoaA and BoaB proteins, and several trimeric autotransporters, including the hemagglutinin used in this study (Balder et al., 2010; Campos et al., 2013; Inglis et al., 2003; Lafontaine et al., 2014). In order to evaluate whether immune sera could inhibit bacterial adhesion, *B. pseudomallei* was incubated with 10% heat-inactivated sera prior to infection of HeLa epithelial cells (MOI =1). At three hrs post-infection, bacteria incubated with immune sera did not display reduced adherence compared to naïve sera (Figure 9B). This finding was unexpected, as both hemmaglutinin and flagella have been shown to mediate *B. pseudomallei* adhesion *in vitro* (Campos et al., 2013; Inglis et al., 2003). These dissimilar findings may reflect differences in experimental conditions or cell lines. Because the adhesive capacity of *B. pseudomallei* remains poorly understood, the importance of adhesion in *B. pseudomallei*

pathogenesis *in vivo* is unclear and further studies are needed to elucidate the significance of these findings.

Altogether, this study demonstrates that these novel proteins are immunogenic in mice but are unable to provide protection against respiratory *B. pseudomallei* challenge when administered alone. Together, these findings provide rationale for incorporation of these antigens into alternative vaccine platforms in order to achieve increased efficacy.

Chapter 9 Optimization of a nano-glycoconjugate vaccine against *Burkholderia*

INTRODUCTION

Perhaps the most successful *Burkholderia* antigens identified to date are LPS and CPS. Anti-polysaccharide antibodies have been associated with protection against *B. pseudomallei* in both humans and animals (Charuchaimontri et al., 1999; Ho et al., 1997; Silva and Dow, 2013; Titball et al., 2017). Additionally, monoclonal antibodies generated against LPS and CPS have been able to provide up to 100% protection in murine models of melioidosis (AuCoin et al., 2012; Jones et al., 2002; Zhang et al., 2011). These findings strongly support the incorporation of these polysaccharides into multivalent vaccines against *Burkholderia.* However, polysaccharides are T-cell independent antigens; therefore, generating robust anti-polysaccharide immune memory requires conjugation of polysaccharide to a carrier proteins. Importantly, glycoconjugate vaccines have been shown to increase vaccine immunogenicity and provide enhanced protection against *Burkholderia* compared immunization with polysaccharide alone (Burtnick et al., 2012; Gregory et al., 2015; Scott et al., 2014a; Scott et al., 2014b; Torres et al., 2014).

Previous studies by Gregory *et al* and Torres *et al* demonstrated that AuNPs can serve as novel carriers for glycoconjugate vaccines. AuNPs remain an attractive vaccine platform as they are non-immunogenic, can be modified to carry a variety of molecules, and have the potential to stabilize antigens in order to achieve longer immune exposure. In these studies, administration of AuNP-glycoconjugate vaccines resulted in increased anti-LPS antibodies, decreased splenic colonization and increased survival following lethal respiratory challenge with *B. mallei* (Gregory et al., 2015; Torres et al., 2014).

While these findings are promising, this vaccination strategy was unable to induce sterilizing immunity.

In an effort to increase the immunogenicity of this vaccine platform, we optimized a method for incorporating our immunogenic proteins into an AuNP-glcoconjugate platform. This platform was administered to animals and evaluated for its ability to induce a robust humoral immune response and protect animals from challenge.

RESULTS

Lipopolysaccharide synthesis

LPS was purified from *B. thailandensis* E264 via a modified hot-phenol extraction as previously described (Burtnick et al., 2012). Approximately 15 mg of purified LPS was obtained from 2.5 L of bacterial culture. Polysaccharide purity was assessed via silver stained 12% SDS-PAGE (Figure 14B). Silver staining allowed visualization of the typical O-antigen banding pattern (35-75 kDa) and the lack of additional bands indicated that the sample was not contaminated by protein or other bacterial products. When transferred to PVDF membrane and probed with 1 µg/ml *B. pseudomallei* O-antigen mAb C5A (generously provided by David AuCoin) via western blot, a distinctive smear was visualized between 35-75 kDa, confirming LPS identity (Figure 14).

Gold nanoparticle synthesis

Spherical, 15 nm gold nanoparticles were synthesized according to the Turkevich method (Turkevich J, 1951). Upon addition of 90 mM sodium citrate ($Na_3C_6H_5O_7$) to 1 mM gold chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), the solution immediately underwent several color changes, including pale yellow, clear, black, and burgundy. These colors are

representative of the oxidative states of gold (Figure 10A). The presence of sodium citrate functions in stabilizing the gold particles, as electrostatic repulsion between the particles prevents aggregation. This stability was easily assessed by the color of the solution, as unstable particles would aggregate and solution would turn from burgundy to blue. Following synthesis, the size and shape of the particles were confirmed via TEM (Figure 10B).

Optimization of AuNP-glycoconjugate platform

Gold nanoparticle glycoconjugates were synthesized as described previously (Gregory et al., 2015), with modifications. First, gold nanoparticles were coated with 0.1 mM of the thiol-containing ligand 16-MHDA. Successful coating was confirmed by measuring surface plasmon resonance via UV/Vis (Figure 11). Naked AuNPs generated a distinctive lambda max (λ_{max}) at 520 nm, as expected. Upon the addition of 16-MHDA, a red shift to ~527 nm was indicative of increased particle diameter. To covalently attach protein to the 16-MHDA-modified AuNPs, a carbodiimide coupling approach was first attempted, as described by Gregory *et al.* This approach utilizes the coupling reagents EDC and NHS to covalently link the carboxylic acid residue of the linker (16-MHDA) with the primary amine of the protein. These reagents function at an optimal pH of 4.7-6; therefore, conjugation reactions were first performed in 50 mM MES buffer at pH 5.5 (Gregory et al., 2015). However, attempting to conjugate protein via this method resulted in irreversible aggregation of AuNPs, irrespective of the concentration or identity of protein (Figure 12B).

In order to reduce aggregation, protein conjugation was performed in a 96-well plate (Figure 12A). This method allowed the optimization of a wide range of parameters,

including: conjugation buffer, pH, surfactant, protein concentration, BSA stabilization, conjugation time, and activating reagent. The results of these assays are summarized in Table 6. Parameter optimization was assessed visually by reduction in aggregation; additionally, stable AuNPs did not undergo aggregation in the presence of 10% NaCl. Conjugation efficacy was first assessed via UV/Vis; however, only a minor shift in λ_{max} was observed upon addition of protein (Figure 11). In order to confirm efficacy of conjugation, protein was cleaved from the surface of the AuNP using 11-mercapto-1-undecanol. Samples were denatured and run on 4-20% SDS-PAGE. The minimum concentration of protein at which band density did not increase was considered the saturation point. Additionally, this was also the lowest concentration of protein required to prevent color change from burgundy to blue upon addition of 10% NaCl (Figure 12A). Most proteins were shown to saturate the AuNPs at approximately 20 µg/ml (Figure 13). However, three proteins [OmpW, OpcP1 and porin (BMA0599)] demonstrated very poor conjugation efficacy (Figure 13).

Upon optimization of the parameters for protein conjugation, we selected 3 *Burkholderia* antigens (Hemagglutinin, Hcp1 and FlgL) and BSA to continue into vaccination studies. In order to conjugate LPS to protein-conjugated AuNPs, we utilized a modified thiol-malemide coupling approach, as described by Gregory *et al*. To maintain consistency between vaccination groups, all proteins were conjugated to AuNPs at concentrations of 20 µg/ml; additionally, LPS conjugations were also performed at concentrations of 20 µg/ml. Because solutions were filtered (3kDa MWCO) during synthesis, any unconjugated molecules remained in solution. However, previous studies have shown that unconjugated soluble protein can enhance the antibody response to gold

nanoparticle vaccines (Tao and Gill, 2015; Tao et al., 2017). Confirmation of LPS conjugation was confirmed, indirectly, via SDS-PAGE (Figure 15), by visualization of increased AuNP smearing (~250kDa) and lack of unconjugated protein in the wells.

In vivo vaccination studies

To evaluate the immunogenicity of this optimized AuNP-glycoconjugate platform *in vivo,* 6-8 week old female BALB/c mice were immunized s.c. with a prime and two boosts of AuNPs containing 10 µg protein and 10 µg LPS. Vaccine groups consisted of AuNP-BSA-LPS, AuNP-Hemagglutinin-LPS, AuNP-Hcp1-LPS, and AuNP-FlgL-LPS. Additionally, one group (AuNP-Combo-LPS) received equal parts of Hemagglutinin, Hcp1 and FlgL proteins. Immunizations were formulated with 30 µg VacciGrade™ Poly (I:C) and 500µg Alhydrogel® adjuvants and were administered bi-weekly.

Antibody titers

Sera were collected from immunized animals at 2 weeks following the second boost. Indirect ELISAs were utilized to determine protein- and LPS-specific serum IgG titers (Figure 16). Endpoint titers were determined to be the $OD_{450\text{-}570}$ value equivalent to twice the standard deviation of naïve sera. All immunized groups, with the exception of FlgL, generated high protein-specific antibody titers. While FlgL antibody titers were 1:50, other groups ranged from 1:2,600 to 1:25,600 (Figure 16A). Interestingly, despite immunization with lower concentrations of individual proteins (e.g., 3.33 µg of Hemagglutinin, FlgL and Hcp1 for total concentration of 10 µg), AuNP-Combo-LPS generated equivalent protein-specific antibody titers. Immunization with AuNP-

glycoconjugates also generated high anti-LPS serum antibody titers ranging from 1:3200 (AuNP-Hcp1-LPS) to 1:25600 (AuNP-Combo-LPS) (Figure 16B).

Serum opsonophagocytosis assays

In order to characterize the humoral immune response and evaluate antibody functionality, opsonization assays were performed with immune sera. Sera taken from chronically infected mice (n=5) were used as a positive control. Opsonization of bacteria with 10% heat-inactivated, pooled (n=10) immune sera resulted in significant increase in macrophage uptake compared to naïve sera (Figure 17). Interestingly, sera taken from the combination vaccine appeared to be less effective at facilitating uptake compared to AuNP-FlgL-LPS or AuNP-Hcp1-LPS alone, despite similar protein- and LPS-specific IgG titers.

Adhesion and invasion assays

For adhesion and invasion assays, *B. pseudomallei* were incubated in 10% heat-inactivated, pooled (n=10) sera prior to infection of LA-4 cells (MOI=1). At 3 hrs post-infection, no differences in adhesion (Figure 18A) were seen between naïve and immune groups. Additionally, after extracellular killing with 250 µg/ml Km, no differences in bacterial invasion were observed between naïve and immune groups (Figure 18B). Interestingly, sera taken from chronically infected mice (anti-*Bpm*) had no effect on adhesion or invasion.

Survival studies

Three weeks post-boost, animals were challenged i.n. with 3.7 LD_{50} *B. pseudomallei* or 4.4 LD_{50} *B. mallei lux*. Upon challenge, all animals challenged with *B. mallei* quickly

succumbed to infection within 4 days post-challenge. Similarly, only one animal (9/10) from the AuNP-Hcp1-LPS and AuNP-Hemagglutinin-LPS groups survived *B. pseudomallei* challenge.

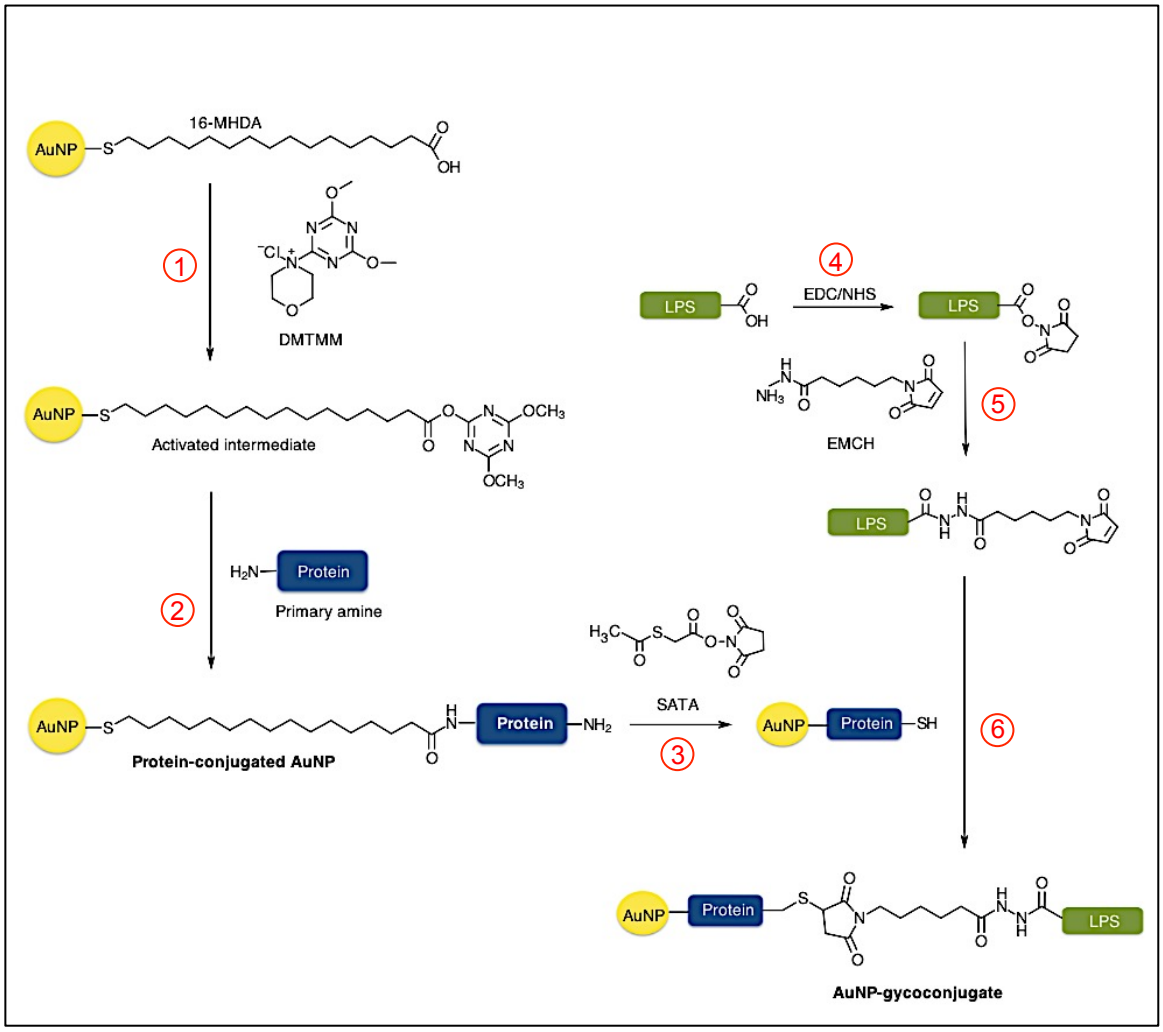
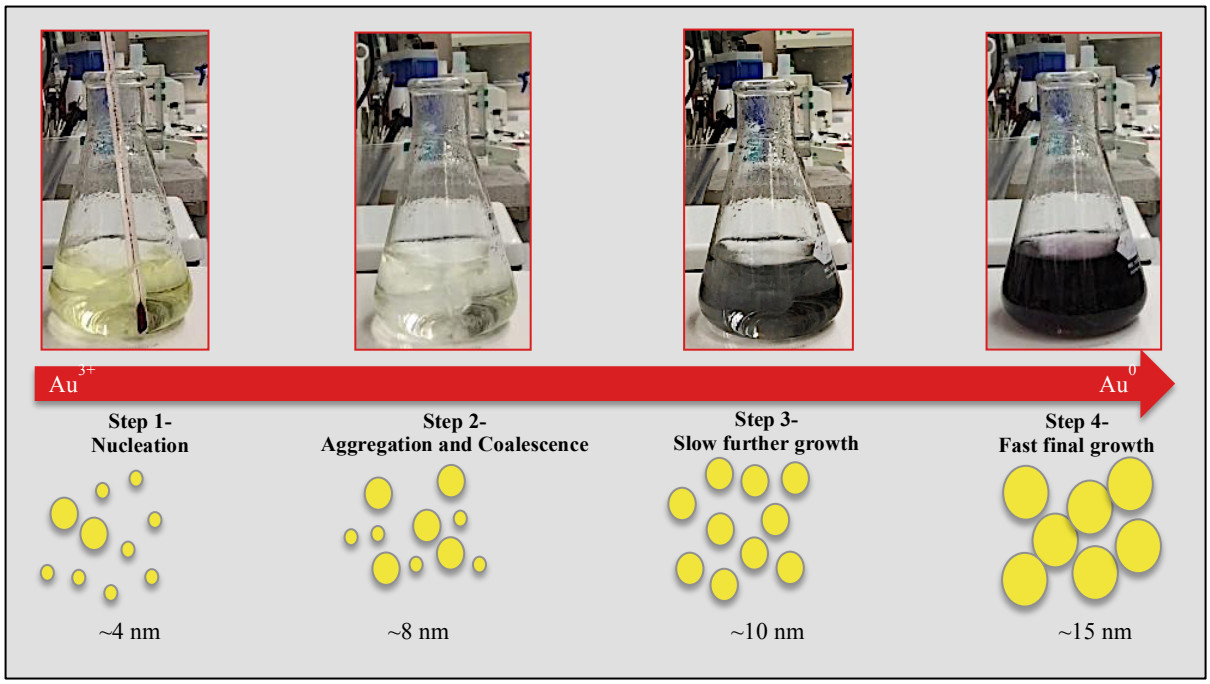


Illustration 2: Six steps in the synthesis of AuNP-glycoconjugates, adapted from source (Gregory, 2013): 1) Modification of the carboxylic acid residue on 16-MHDA to produce an activated intermediate 2) Addition of protein of interest, 3) Use of SATA to convert amine to thiol group, meanwhile, 4) Use of EDC/NHS to activate carboxylic acid residue on LPS molecule, 5) Addition of EMCH to facilitate crosslinking and 6) Reaction between modified LPS and thiol group on protein to form stable gold-nanoparticle glycoconjugate.

A



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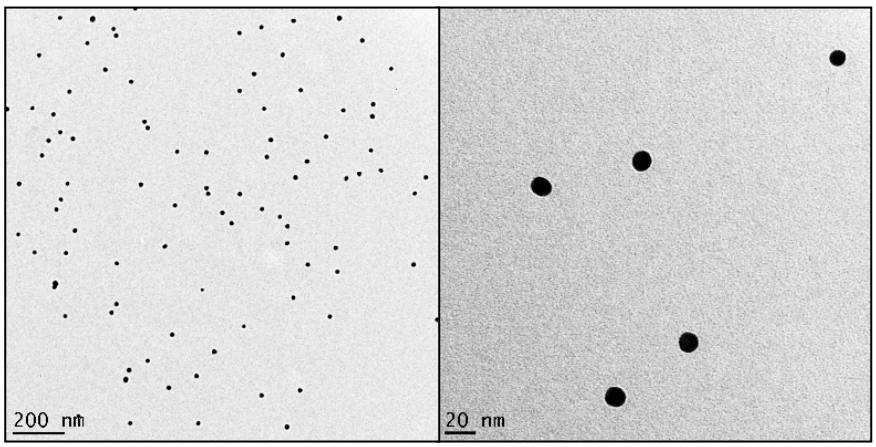


Figure 10. Synthesis of citrate-stabilized gold nanoparticles. A) Figure representing color changes and corresponding oxidative states during AuNP synthesis. Figure adapted from source (Wang et al., 2014). B) Confirmation of size and shape via Transmission Electron Microscopy.

Table 6. Summary of conjugation parameters and effect on AuNP aggregation

Parameter(s)	Concentration/range	Results
BSA stabilization	0.1, 0.5 and 1% (w/v)	---
Triton-X™100	0.05, 0.1 or 0.5% (v/v)	*
Tween-20®	0.05, 0.1 or 0.5% (v/v)	**
Protein concentration	0 µg – 50 µg/ml	**
Conjugation time	1.5 hrs – 18 hrs	**
Conjugation Buffer: PBS 0.1M Borate 50mM MES Tris-HCl	 pH 6.5, 7.5, 8.5 pH 7.5 and 8.5 pH 4.5 and 5.5 pH 7.5 and 8.5	***
Activators: EDC/NHS DMTMM	 0.15 mM/0.6 mM 0.01 mM, 0.1 mM and 0.4 mM	****

Ability to reduce AuNP aggregation, ranging from minimal effect (*) to major effect (****)

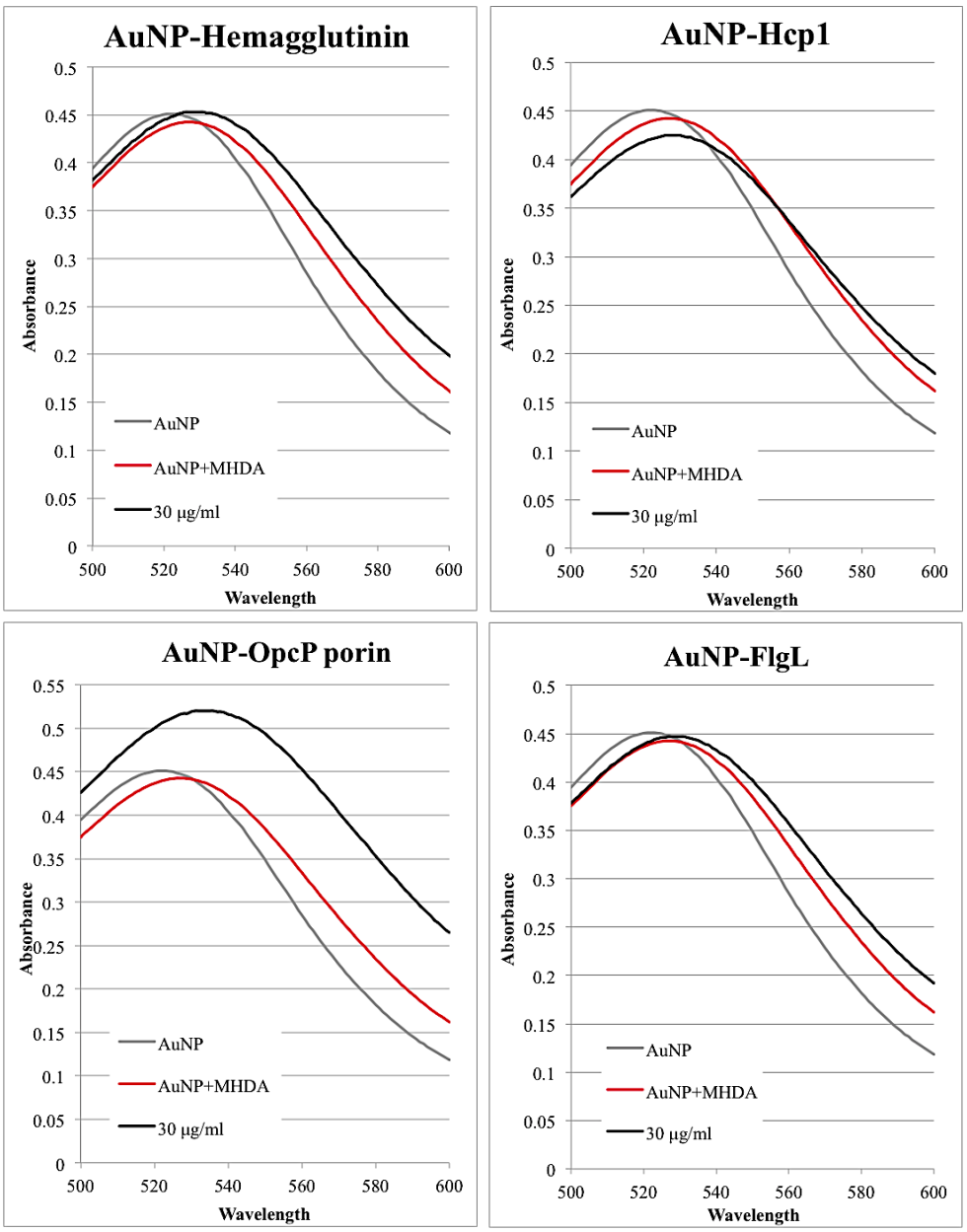


Figure 11: UV/Vis spectroscopy of protein-conjugated AuNPs demonstrates a red shift in plasmon resonance of AuNPs upon addition of MHDA and protein (30 µg/ml).

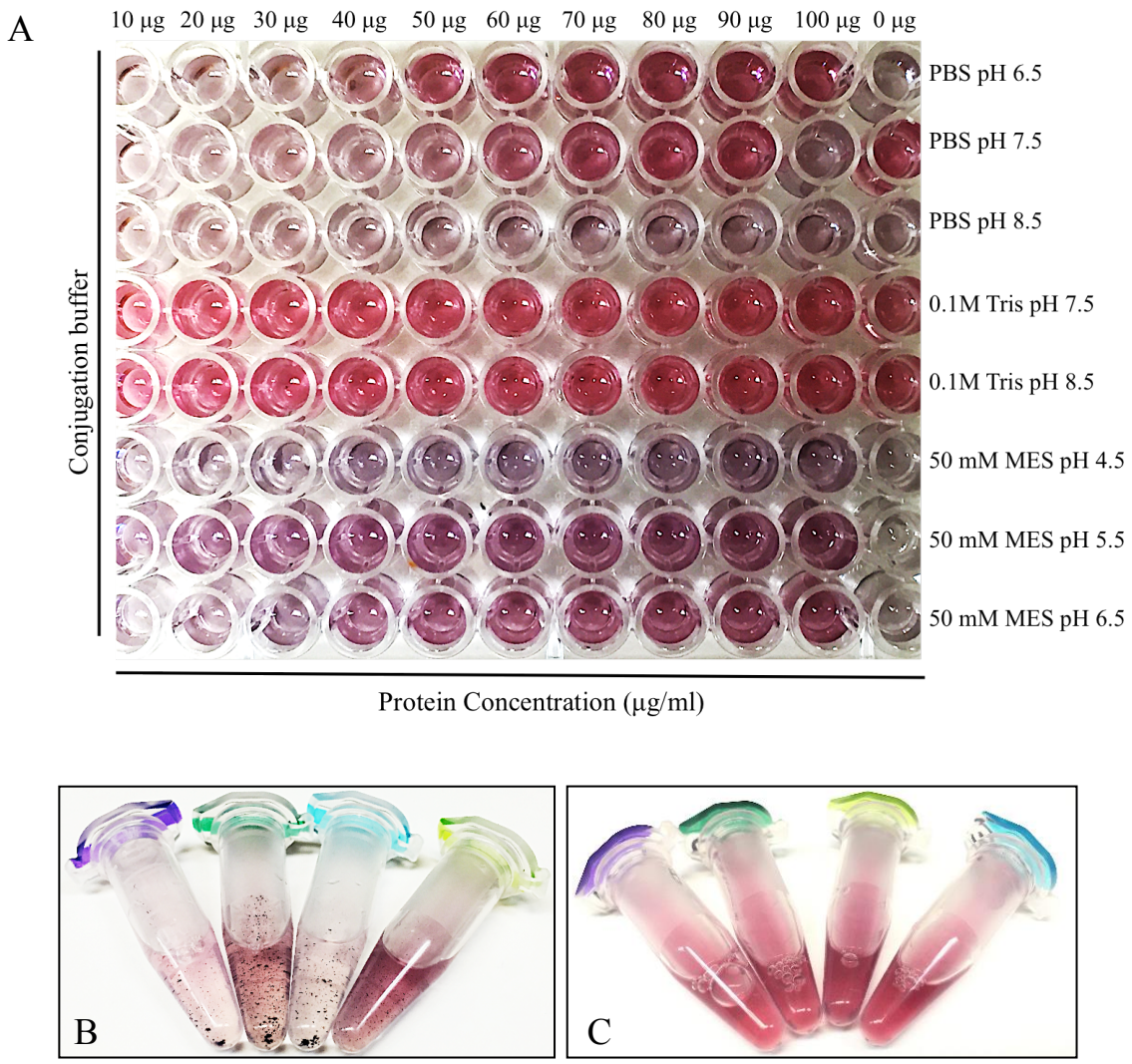


Figure 12: Optimization of parameters for protein conjugation. A) Representative 96-well plate format used for optimization of various parameters. Unstable solutions are indicated by a color change from red (non-aggregated) to blue (aggregated) in the presence of high salt. B) Aggregation of protein-conjugated AuNPs before optimization. C) Aggregation of protein-conjugated AuNPs is eliminated after optimization.

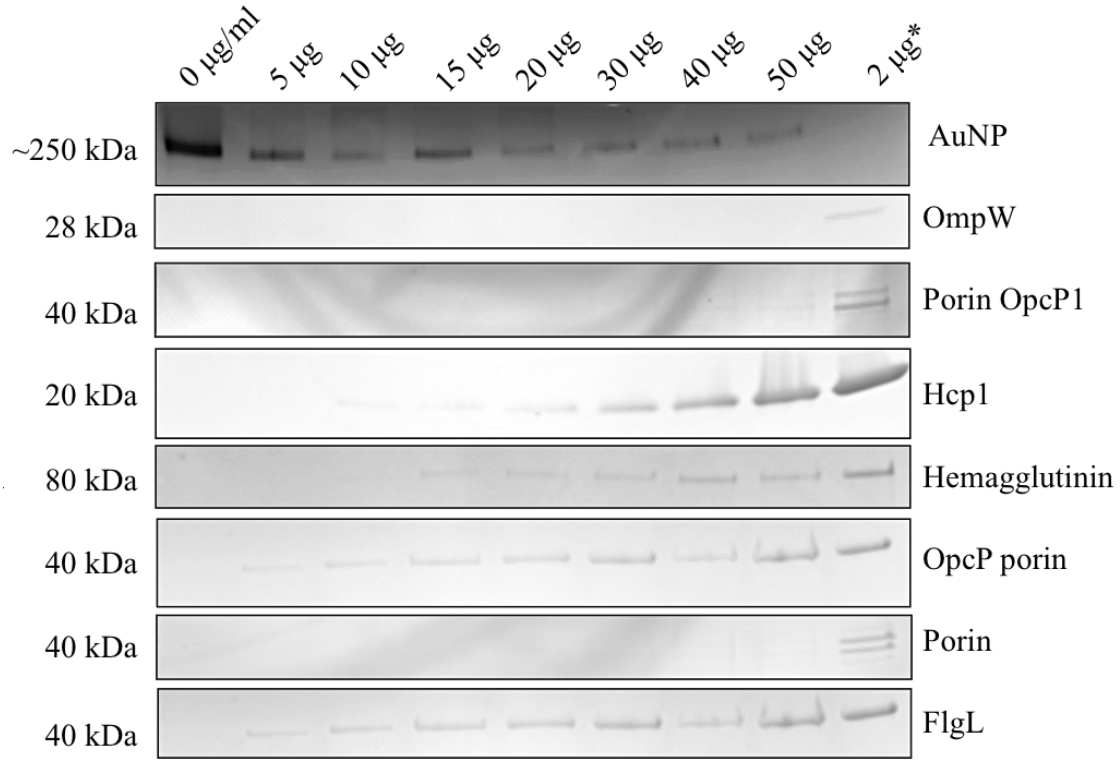


Figure 13: SDS-PAGE of protein-conjugated AuNPs indicates that conjugation efficacy is dependent on the protein. Additionally, concentration of protein (µg/ml) needed for AuNP saturation is visualized as the point at which protein concentration does not increase (~20 µg/ml for most proteins). *Purified, unconjugated protein for comparison

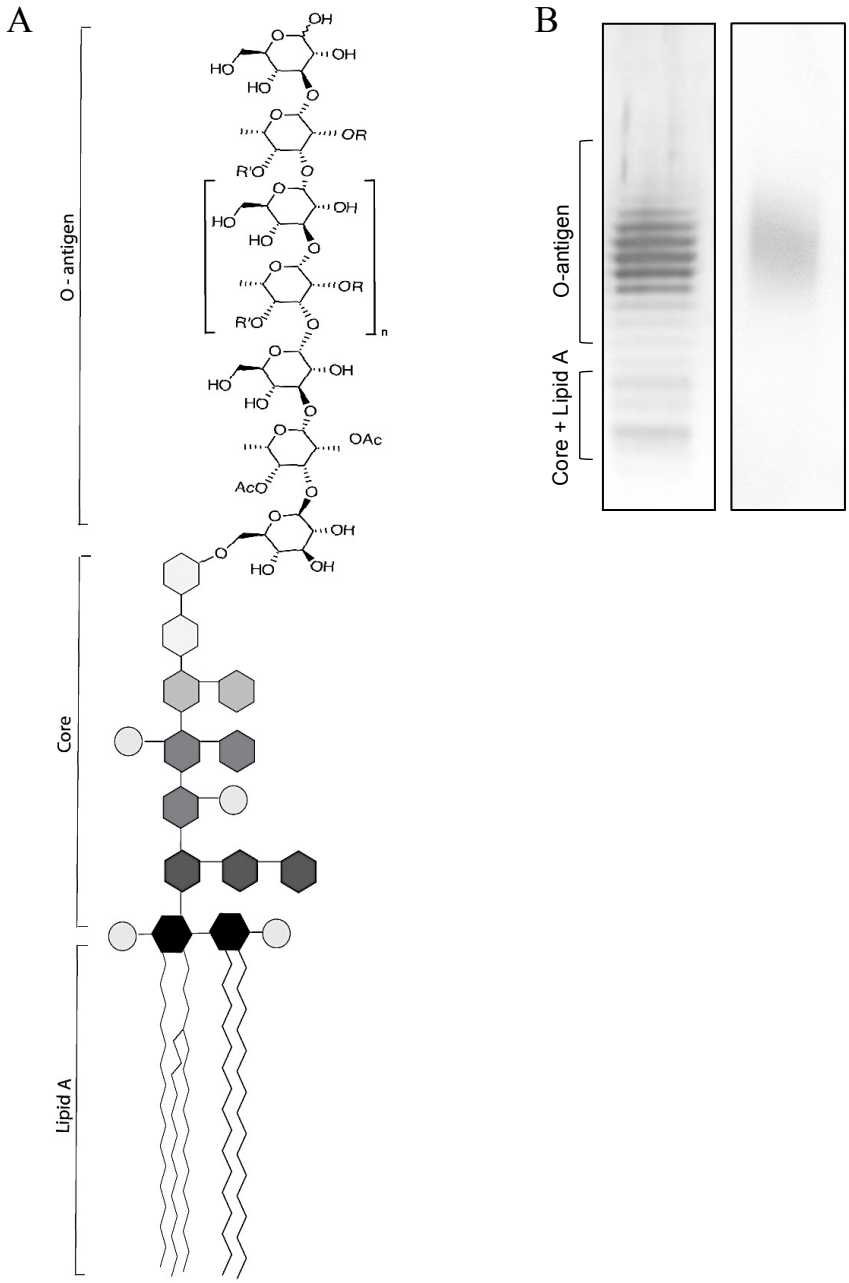


Figure 14: Purification of *B. thailandensis* E264 lipopolysaccharide. A) Simplified skeletal structure of *B. thailandensis* LPS, adapted from source (Gregory, 2013) R = OMe, R' = OAc. B) Left: 12% SDS-PAGE containing 1 µg LPS and stained with Silver Stain. Right: Western blot of 1 µg LPS probed with cross-reactive *B. pseudomallei* murine mAb C5A and HRP-conjugated goat-anti-mouse.

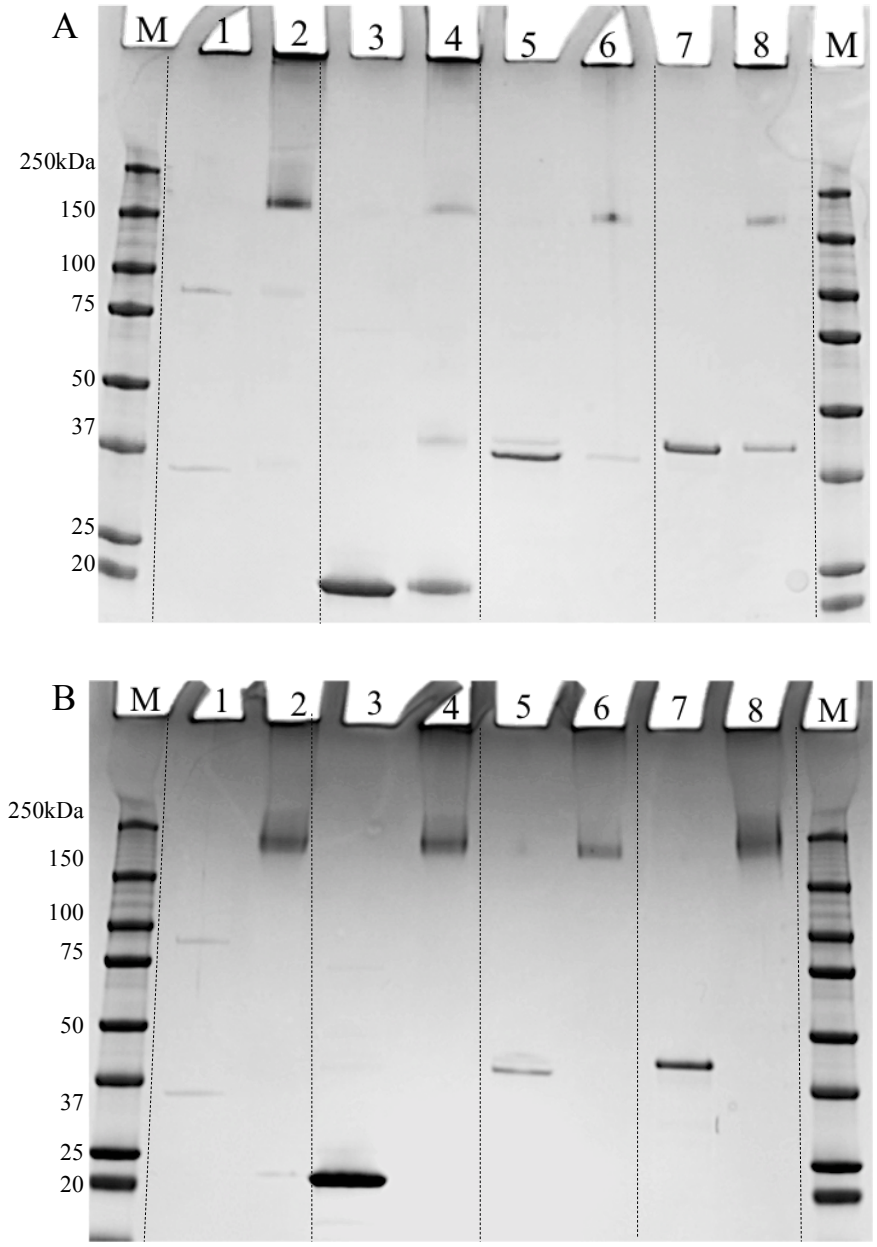


Figure 15. SDS-PAGE (4-20%) of AuNP-Protein and AuNP-Protein-LPS after cleavage with 11-mercapto-1-undecanol. A) AuNP-protein conjugates, B) AuNP-protein-LPS conjugates. Lanes (from L to R): M- Molecular weight ladder, 1- 2 µg Hemagglutinin, 2- AuNP-Hemagglutinin conjugate, 3- 2 µg Hcp1, 4- AuNP-Hcp1 conjugate, 5- 2 µg OpcP porin, 6- AuNP-OcpP porin conjugate, 7- 2 µg FlgL, and 8- AuNP-FlgL conjugate

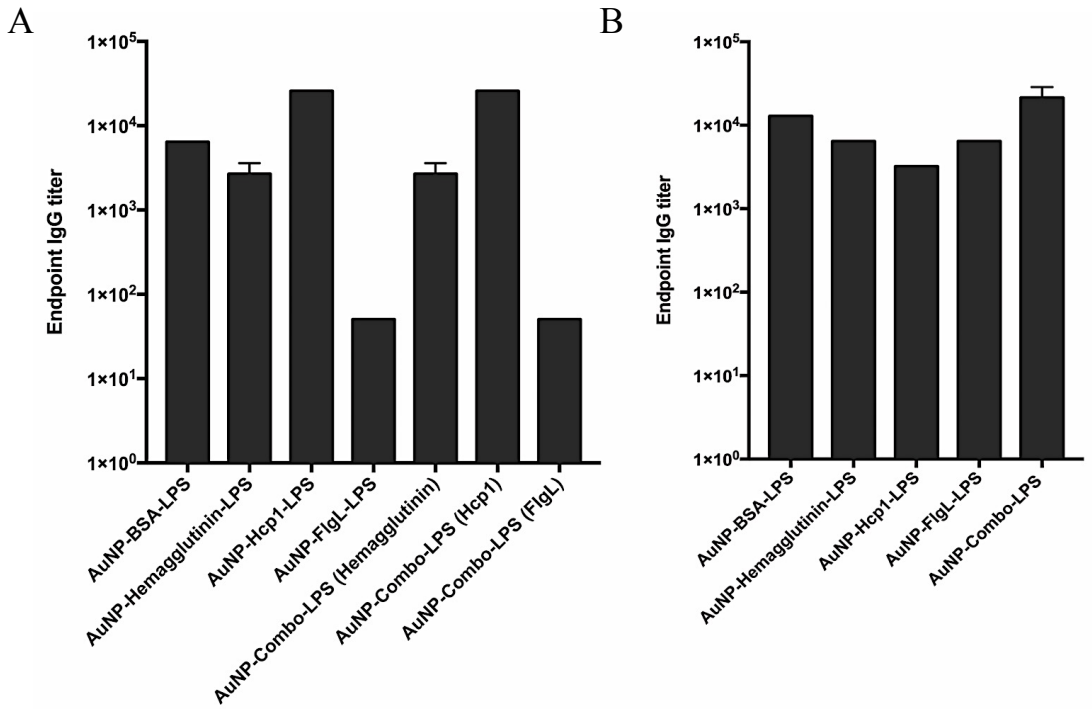


Figure 16. Antibody responses following nano-glycoconjugate vaccination. Serum was collected from vaccinated animals at 2 weeks post-boost and pooled (n=10). Protein- or LPS-specific IgG titers were assessed via indirect ELISA, with endpoint titers determined to be twice the standard deviation of naïve sera. Bars represent the mean of three replicates. (A) Protein-specific IgG responses across vaccinated groups. Sera taken from the AuNP-Combo-LPS group was assessed against each individual protein (Hemagglutinin, Hcp1 and FlgL, shown in parentheses). (B) LPS-specific IgG responses.

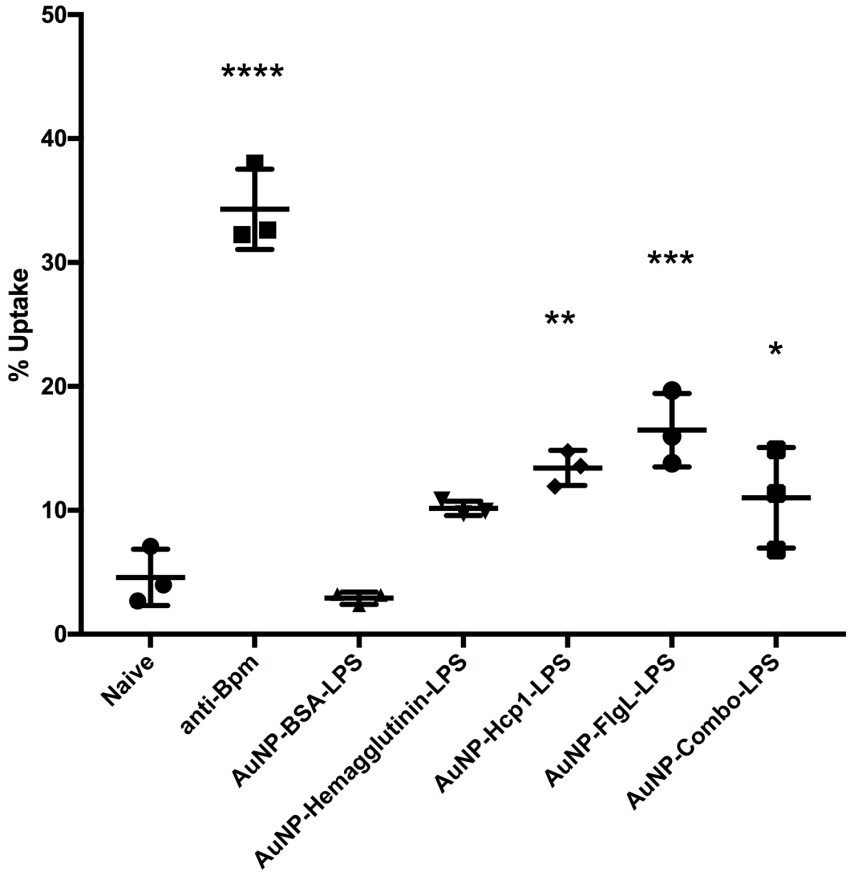


Figure 17. Opsonization of *B. pseudomallei* K96243 with immune sera resulted in increased bacterial uptake by RAW 264.7 murine macrophages. Opsonophagocytosis assays were performed by incubating $5x10^5$ CFU *B. pseudomallei* K96243 with 10% heat-inactivated immune sera prior to infection of RAW 264.7 macrophages (MOI = 1). Figure is representative of two independent experiments. Individual data points are plotted, together with mean ± S.E.M. Statistical analyses were performed using a standard one-way ANOVA. Levels of significance (compared to naïve sera): *$p < 0.05$, **$p< 0.005$, ***$p<0.0005$, ****$p=0.0001$.

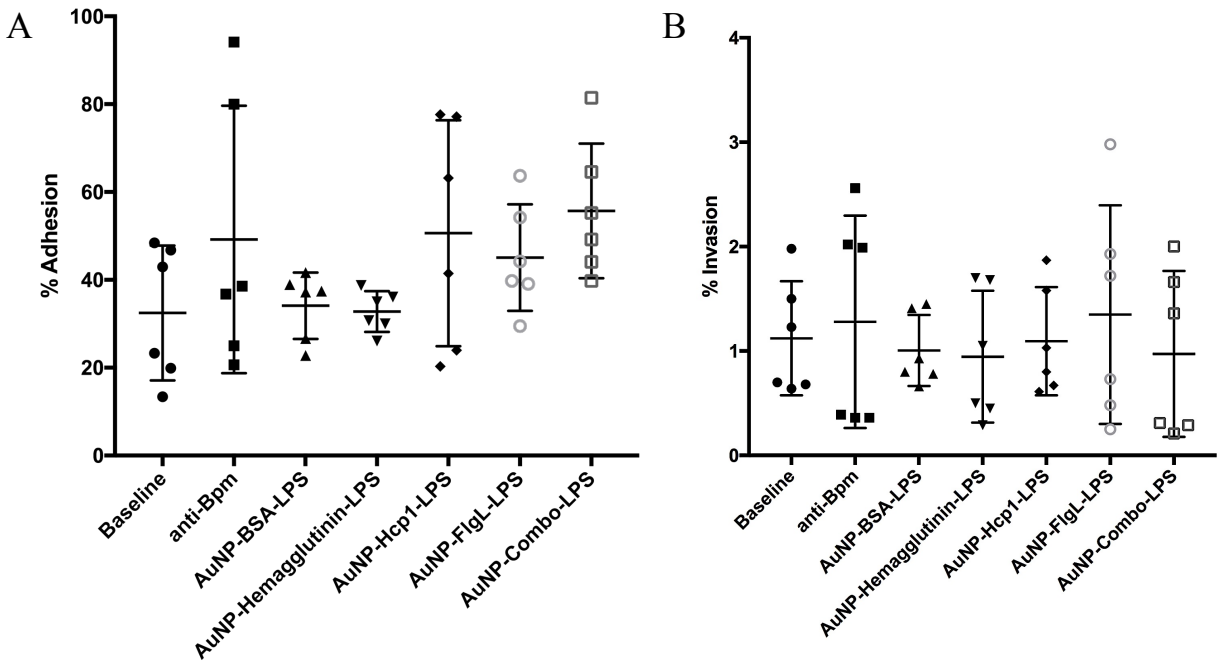
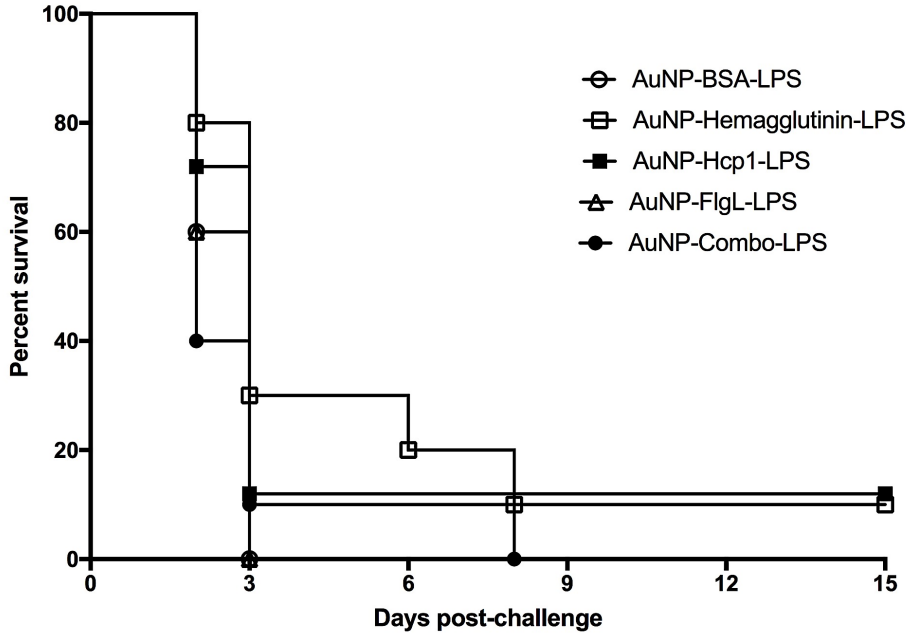


Figure 18. Opsonization of *B. pseudomallei* K96243 with immune sera did not affect the ability of *B. pseudomallei* K96243 to adhere or invade LA-4 murine lung epithelial cells. Adhesion and invasion assays were performed by incubating $5x10^5$ CFU *B. pseudomallei* K96243 with 10% heat-inactivated immune sera prior to infection of LA-4 cells (MOI = 1). Two independent experiments are shown, and individual data points are plotted together with mean ± S.E.M. A) Adhesion of LA-4 cells by *B. pseudomallei* and B) Invasion of LA-4 cells by *B. pseudomallei* following extracellular killing with 250 µg/ml kanamycin for 1 hour.

A



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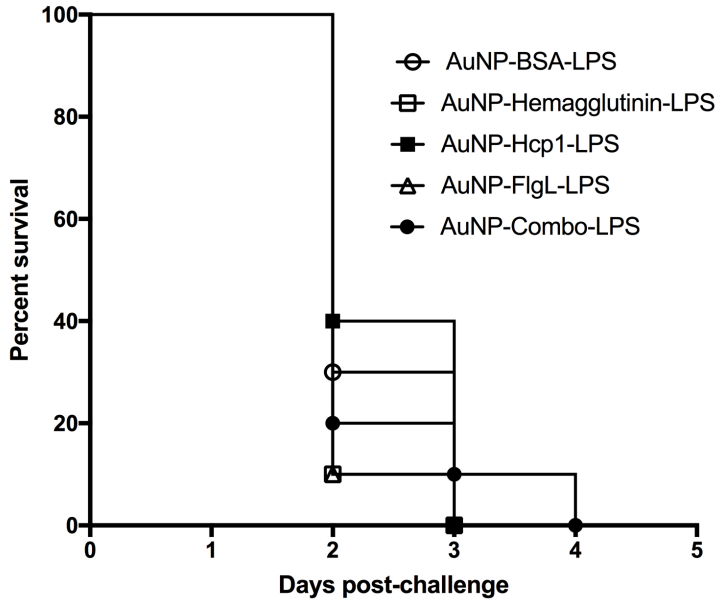

Figure 19: AuNP-glycoconjugate immunization does not protect BALB/c mice against lethal challenge. Animals received a prime and two boosts of AuNP-glycoconjugate vaccine at two week intervals. A) Survival following challenge with 3.7 LD_{50} *B. pseudomallei* K96243 and B) Survival following challenge with 4.4 LD_{50} *B. mallei lux.*

DISCUSSION

The most efficacious *Burkholderia* subunit vaccines to date contain high antigenic polysaccharides (CPS or LPS), and anti-polysaccharide antibodies are associated with protection against *Burkholderia* in both animals and humans (Charuchaimontri et al., 1999; Ho et al., 1997; Silva and Dow, 2013; Titball et al., 2017). However, generating robust anti-polysaccharide responses is challenging, as polysaccharides are T-cell independent antigens. Because polysaccharide molecules contain highly repetitive sugar residues, they are capable of crosslinking BCRs and inducing antibody production (Vinuesa and Chang, 2013). However, because they do not engage T-cells, these antibodies are often short-lived, and do not undergo the isotype switching and affinity maturation needed for immunological memory (Mond et al., 1995). It has been shown that conjugation of bacterial polysaccharides to carrier proteins can elicit T-cell help, thereby generating memory responses (Avci et al., 2011).

Previous studies have shown that glycoconjugate vaccines containing antigenic LPS or CPS can elicit a protective immune response against *Burkholderia* (Burtnick et al., 2012; Gregory et al., 2015; Nelson et al., 2004; Scott et al., 2014a; Scott et al., 2014b; Torres et al., 2014). Because subunit vaccines are often quickly degraded, achieving sufficient immune exposure to mount a robust response remains a significant challenge. AuNPs have the potential to enhance the immunogenicity of glycoconjugate vaccines by providing increased stability *in vivo*. Our lab has previously demonstrated that AuNP-glycoconjugates can provide significant protection from lethal *B. mallei* challenge (Gregory et al., 2015; Torres et al., 2014). However, surviving animals remained colonized by *B. mallei*, indicating that a more robust immune response is needed.

In an effort to increase the immunogenicity of this AuNP-glycoconjugate platform, we optimized an approach to incorporate our novel antigens into this platform. Upon vaccination with nano-glycoconjugates, mice generated high LPS-specific IgG titers ranging from 1:3200 to 1:25600. Because all mice received equivalent LPS (10 μg) immunizations, this wide range in anti-LPS endpoint titers could be indicative of protein immunogenicity (e.g., increased processing and presentation on MHC), or may reflect differential proportions of unconjugated LPS. Importantly, these titers are evident of increased optimization of this nano-glycoconjugate platform, as previous studies generated endpoint titers of up to 1:100 (Gregory et al., 2015). It is important to note that differences in nano-glycoconjugate construction (e.g., incorporation of novel proteins) and vaccination strategies (e.g., LPS concentration, route of immunization, and adjuvant) may also account for this improved immunogenicity.

Opsonization of *B. pseudomallei* with immune sera was shown to facilitate uptake by murine macrophages (Figure 17). The increase in bacterial uptake by macrophages seems to be at least partly dependent on protein-specific antibodies, as AuNP-BSA-LPS immune sera did not increase uptake despite high anti-LPS endpoint titers (1:12,800). However, because endpoint titers were determined based on total serum IgG, these results may also reflect the importance of other immunoglobulin subtypes in bacterial uptake. Future studies will aim to elucidate the importance of protein- and LPS-specific antibody subtypes on *B. pseudomallei* uptake by macrophages. Interestingly, sera from the combination group also appeared to be less effective at facilitating bacterial uptake than AuNP-FlgL-LPS or AuNP-Hcp1-LPS alone. This may suggest steric hindrance between antibodies, as protein-specific titers remain similar between groups. While it is currently

thought that incorporation of multiple antigens into a single vaccine formulation may increase immunogenicity and protection against *Burkholderia* (Titball et al., 2017); these findings expose the challenges and complexity of selecting the right antigens to achieve a synergistic response.

As demonstrated with protein immunization alone, incubation of bacteria with immune sera from AuNP-glycoconjugate immunized mice did not prevent or reduce adhesion or invasion of LA-4 murine lung epithelial cells. Surprisingly, incubation with anti-*B. pseudomallei* sera also had no effect on adhesion or invasion of this pathogen. While unexpected, this finding suggests that the bacteria may exhibit different phenotypes *in vitro* vs. *in vivo*. *B. pseudomallei* has been shown to exhibit significantly increased adherence to epithelial cells at a low temperature (30°C), suggesting that environmental factors may influence differences in *B. pseudomallei* adherence (Brown, 2002).

One limitation of this study is that it only evaluates the humoral immune response. Previous studies have shown that antibodies are essential for protection against *Burkholderia*, as B-cell depletion results in exacerbated disease and decreased protection following vaccination (Silva et al., 2013; Whitlock et al., 2008). Therefore, incorporation of antibody-inducing proteins is necessary when designing a subunit vaccine against *Burkholderia*. Nevertheless, it is currently understood that a balanced cellular and humoral immune response will likely be required for vaccine-induced protective immunity against *B. pseudomallei* and *B. mallei* (Choh et al., 2013; Hatcher et al., 2016; Mott et al., 2015; Silva and Dow, 2013). Therefore, further studies will focus on

elucidating the cellular responses evoked by AuNP-glycoconjugate immunization and the importance of these responses for vaccine-induced protection against *Burkholderia* .

Despite the increased antibody titers and macrophage uptake *in vitro*, vaccination of animals did not provide protection against lethal challenge with 3.7 LD_{50} *B. pseudomallei* or 4.4 LD_{50} *B. mallei*. The lack of protection against *B. mallei* was unexpected, as previous studies had demonstrated that AuNP-glycoconjugate vaccines were protective in a murine and NHP model of inhalational glanders (Gregory et al., 2015; Torres et al., 2014). Interestingly, AuNP-glycoconjugate vaccines containing Hcp1 – a known protective antigen – were also unable to protect. It is possible that the purification or conjugation process may have altered essential protective epitopes within the protein or polysaccharide antigens. To assess this possibility, further studies involving antibody-binding assays and alternative purification/conjugation approaches are needed. Together, the lack of protection afforded by AuNP-glycoconjugate immunization indicates that sufficient immunity was not achieved and further optimization of this platform is required.

It is important to note that significant dissimilarities between the present study and the study by Gregory *et al* make direct comparison difficult. In the present study, AuNP-glycoconjugate synthesis required extensive optimization, including the use of novel proteins, alternative conjugation buffers, different pH values, Tween-20®, and DMTMM activation. It is possible these differences may have negatively affected immunogenicity.

Of all the antigens tested, only Hcp1 is known to be expressed by *B. mallei in vivo* (Burtnick and Brett, 2013; Hatcher et al., 2016). Convalescent equine glanders sera did

not react with any of the recombinant antigens tested, with the exception of the hemagglutinin protein. However, this hemagglutinin was also reactive with seronegative equine sera (Figure 4). Therefore, it is possible that these antigens are expressed during *B. pseudomallei* infection, but not during *B. mallei* infection. Because naturally occurring *B. mallei* infections are rare and seropositive sera are hard to obtain, further studies will need to focus on evaluating the expression and functionality of these antigens in experimental models of infection.

One important difference between this study and the one performed by Gregory *et al* was the route of vaccination. AuNP-glycoconjugate immunizations were performed s.c., as i.n. immunization was poorly tolerated by animals. It is possible that administration of AuNP-glycoconjugate vaccines through i.n. route may have induced a mucosal immune response important for protection. However, the importance of mucosal immunity for protection against respiratory *Burkholderia* infection remains uncertain, as few groups have evaluated mucosal immunity to *B. pseudomallei* or *B. mallei.* While IgA has been shown to be elevated in melioidosis patients, its role in immunity remains unknown (Chenthamarakshan et al., 2001). Interestingly, work by Nieves *et al* demonstrated that s.c. immunization with OMVs provided better protection against respiratory *B. pseudomallei* infection than i.n. immunization, despite decreased IgA responses (Nieves et al., 2011). However, the exact role of IgA in the protective immune response to either *B. pseudomallei* or *B. mallei* infection remains entirely unknown.

The present study also incorporated Poly (I:C) into vaccine formulations. This TLR3 agonist not only enhances innate immune signaling, but has also been shown to induce DC maturation (Verdijk et al., 1999), and promote antigen-specific antibody

responses (Ma and Ross, 2009; Rookhuizen and DeFranco, 2014). It is possible that the administration of Poly (I:C) may have altered the immune profile to vaccination, thus affecting the ability to provide protection.

Finally, differences in *B. mallei* challenge strains may have accounted for the differences in protection observed between these two studies. Although bioluminescent *B. mallei lux* (CSM001) was generated from the *B. mallei* ATCC 23344 parent strain, the LD_{50} of these two strains differs 100-fold, with *B. mallei lux* being significantly more virulent *in vivo* (Massey et al., 2011). While similar LD_{50} were administered between studies, it is possible that differences in strain virulence could have affected the outcome.

The lack of protection afforded by AuNP-glycoconjugate immunization indicates that additional optimization is needed. Many parameters can be altered to enhance immunogenicity, including route of administration, dose concentration and adjuvantation. Further studies will focus on optimizing these parameters in order to fully elucidate the protective capacity of this AuNP-glycoconjugate platform.

Conclusions

Taken together, this study represents a novel approach to rational vaccine design that can be applied to other pathogens. First, exploitation of the *B. mallei* proteome led to the identification of 160 outer membrane and secreted proteins. Many of these proteins were predicted to be "antigenic" based on similarity with known antigens, and had high numbers and affinities of MHCI and MHCII epitopes. The top 7 proteins were expressed *in vitro* and recombinant proteins were shown to react with convalescent human melioidosis sera. This seroreactivity validates our informatics predictions, confirming that these antigens are expressed during human infection and are recognized by the human immune response. When tested against convalescent equine glanders sera, only the hemagglutinin protein exhibited significant seroreactivity. However, limited number (n=5) and volume of sera samples prevented extensive testing of seropositive sera. Interestingly, this hemmaglutinin protein was strongly reactive with healthy (seronegative) equine sera, suggesting that this protein might be expressed by environmental *Burkholderia* or other bacterial strains to which the animal may have become exposed.

When administered to mice, recombinant proteins generated high IgG antibody titers, irrespective of the route, adjuvant or concentration administered. However, immunized mice did not survive lethal challenge, indicating that these antibody titers are not sufficient for protection against respiratory challenge.

In order to evaluate these proteins in a multivalent platform, we optimized a method for the synthesis of AuNP-glycoconjugates. Assessment of humoral immune response suggest superior immunogenicity of this platform over previously tested AuNP-

glycoconjugates, as indicated by increased anti-LPS titers. However, despite this increased immunogenicity, no protection was afforded by AuNP-glycoconjugate vaccination. Future studies will evaluate cellular immune response and the importance of antibody subtypes in mediating protection against *Burkholderia.*

Subunit vaccines often require extensive optimization of numerous parameters (e.g., route, dose and adjuvant) in order to achieve robust immunity and protection. Future studies will focus on optimizinig these parameters in order to fully evaluate the immunogenicity of AuNP-glycconjugate vaccines against *Burkholderia.* Together, this study identified novel, immunogenic antigens and has optimized the synthesis of AuNP-glycoconjugate vaccines, providing the foundation for continued optimization of these subunit vaccines in pre-clinical models of infection.

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Publications

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