Use of Reverse Vaccinology in the Design and Construction of Nanoglycoconjugate Vaccines against *Burkholderia pseudomallei*

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**ABSTRACT** *Burkholderia pseudomallei* is a Gram-negative, facultative intracellular pathogen that causes the disease melioidosis in humans and other mammals. Respiratory infection with *B. pseudomallei* leads to a fulminant and often fatal disease. It has previously been shown that glycoconjugate vaccines can provide significant protection against lethal challenge; however, the limited number of known *Burkholderia* antigens has slowed progress toward vaccine development. The objective of this study was to identify novel antigens and evaluate their protective capacity when incorporated into a nanoglycoconjugate vaccine platform. First, an *in silico* approach to identify antigens with strong predicted immunogenicity was developed. Protein candidates were screened and ranked according to predicted subcellular localization, transmembrane domains, adhesive properties, and ability to interact with major histocompatibility complex (MHC) class I and class II. From these *in silico* predictions, we identified seven “high priority” proteins that demonstrated seroreactivity with anti-*B. pseudomallei* murine sera and convalescent human melioidosis sera, providing validation of our methods. Two novel proteins, together with Hcp1, were linked to lipopolysaccharide (LPS) and incorporated with the surface of a gold nanoparticle (AuNP). Animals receiving AuNP glycoconjugate vaccines generated high protein- and polysaccharide-specific antibody titers. Importantly, immunized animals receiving the AuNP-FlgL-LPS alone or as a combination demonstrated up to 100% survival and reduced lung colonization following a lethal challenge with *B. pseudomallei*. Together, this study provides a rational approach to vaccine design that can be adapted for other complex pathogens and provides a rationale for further preclinical testing of AuNP glycoconjugate in animal models of infection.

**KEYWORDS** *Burkholderia pseudomallei*, melioidosis, nanoglycoconjugate, nanovaccine, reverse vaccinology

*Burkholderia pseudomallei* is a Gram-negative aerobic bacterium common to tropical and subtropical climates worldwide. This saprophytic bacterium can survive in soil and water and, upon transmission to humans or other susceptible mammals, cause the disease melioidosis. Human disease can present with a wide variety of clinical manifestations, including cutaneous and soft tissue abscesses, lymphadenopathy, and sepsis (1, 2). Clinical symptoms have been correlated with the route of infection; percutaneous infection often results in a purulent lesion at the site of inoculation, whereas respiratory
infection causes a more rapid fulminant disease that often includes pneumonia, fever, bacteremia, and sepsis (1, 3, 4). Melioidosis treatments are limited, as *B. pseudomallei* is inherently resistant to major antibiotic classes, including many β-lactams, aminoglycosides, and macrolides (5, 6). Additionally, there are currently no vaccines available, either licensed or in clinical trials. High morbidity and mortality among respiratory cases, together with the limited treatment options, have resulted in the categorization of *B. pseudomallei* as a tier 1 select agent. The public health threat associated with *B. pseudomallei* emphasizes the need for an effective vaccine that can be administered to at-risk military personnel and aid workers, together with populations living in areas where it is endemic.

For the past 2 decades, many groups have attempted to develop a vaccine against *B. pseudomallei* through various approaches, including live-attenuated, whole-cell killed/irradiated, and subunit vaccines (reviewed in references 7 and 8). While these experimental vaccines have been shown to provide protection against a lethal challenge, sterilizing immunity remains elusive. Subunit vaccines remain an attractive alternative to live-attenuated vaccines, because they can be administered to a more diverse population without the concern for dormancy or reversion to the wild type. Subunit vaccines also do not require the use of biosafety level 3 (BSL3) facilities, an important consideration for cost-effectiveness and personnel safety. The most common subunit vaccines today, both licensed and experimental, contain immunogenic proteins. These proteins are known to stimulate a T-cell-dependent immune response, resulting in T-cell and B-cell memory, along with antibody affinity and isotype switching (9, 10). In fact, previous studies have shown that proteins alone can provide significant, albeit incomplete, protection against *Burkholderia* challenge (11–14). However, more recent studies have shown that when immunogenic proteins are incorporated in a glycoconjugate vaccine in conjunction with the highly antigenic *Burkholderia* lipopolysaccharide (LPS) or capsular polysaccharide (CPS), a more robust immune response is generated (15–18). Our lab has previously demonstrated that the incorporation of a glycoconjugate with the surface of a gold nanoparticle can enhance the immune response and increase protection in a murine model of inhalational glanders, a disease caused by the closely related host-adapted pathogen *B. mallei* (17). Additionally, these nanoglycoconjugates were also shown to be safe and immunogenic in nonhuman primates (18). While these studies are highly promising, complete protection was not achieved, and further optimization is needed for increased efficacy. However, the current pool of well-characterized *Burkholderia* proteins is limited, impeding the progress toward vaccine optimization. To address this need, we have adapted and optimized an *in silico* methodology for selecting novel protein candidates based on their predictive subcellular localization, antigenicity, and affinity for major histocompatibility complex (MHC) class I and class II. The top 7 vaccine candidates, together with known *Burkholderia* antigen Hcp1, were selected for expression and purification. Hcp1 was chosen for comparison studies because of its well-characterized immunogenicity, reactivity with convalescent human melioidosis sera, and ability to provide protection against lethal *B. pseudomallei* challenge in a mouse model (11, 19, 20). To validate our *in silico* predictions, we confirmed the seroreactivity of these 7 novel proteins with anti-*B. pseudomallei* murine sera, as well as with human convalescent melioidosis sera. Upon confirmation of immunogenicity, we incorporated two of these novel proteins into a gold nanoparticle (AuNP) glycoconjugate vaccine platform and evaluated immunogenicity in mice. AuNP glycoconjugates were immunogenic, generating high protein- and LPS-specific IgG titers. Additionally, immunization with AuNP glycoconjugates containing the novel flagellar protein FlgL and a protein combination (FlgL, Hcp1, and hemagglutinin) demonstrated 90% and 100% survival following a lethal challenge, respectively. Surviving animals also demonstrated a significant reduction in lung colonization compared to groups receiving adjuvant alone. Together, these studies have confirmed and validated the use of *in silico* methodologies for the identification of novel vaccine candidates. The use of these methodologies abrogates the need for high-containment facilities and can easily be applied to other complex pathogens.
Importantly, by using reverse vaccinology predictions, we could identify previously unknown antigens that reacted with convalescent human melioidosis sera. In this study, we have optimized the construction of an AuNP glycoconjugate vaccine and demonstrated its ability to protect against lethal respiratory challenge with \textit{B. pseudomallei}. Together, these studies provide the rationale for continued testing of AuNP glycoconjugate vaccines in preclinical models of melioidosis.

\textbf{RESULTS}

Identification of novel \textit{Burkholderia} antigens via bio- and immunoinformatics approaches. To identify potential \textit{Burkholderia} vaccine antigens, we first selected outer membrane and secreted proteins with $>98\%$ conservation between \textit{B. pseudomallei} and the closely related host-adapted \textit{B. mallei} pathogen. \textit{B. mallei} was chosen for informatics analyses based on its smaller genome size, $99\%$ genetic identity with \textit{B. pseudomallei} (increased probability of cross-protection), and its mammalian reservoir (loss of genes involved only in environmental adaptation). From this pool, we eliminated proteins with $>1$ transmembrane domain to simplify downstream protein purification. To evaluate the potential immunogenicity, we selected proteins with high predicted antigenicity and similarity to known adhesins. Each protein was screened for the number and affinity of MHC-I and MHC-II epitopes. Finally, proteins were ranked against each other based on the predicted immunogenicity (e.g., adhesion, antigenicity, and number/affinity of MHC-I and MHC-II epitopes) (Fig. 1). Based on these characteristics, the top seven proteins were selected from this final pool and compared with the known \textit{Burkholderia} antigenic protein Hcp1 in downstream validation and immunization studies (Table 1).
Protein expression and purification. Proteins of interest were amplified with genespecific primers (see Table S3 in the supplemental material) and inserted into a pET30a(H11001) expression vector. Upon induction with IPTG (isopropyl-β-D-thiogalactopyranoside), all proteins were expressed in high yields (ranging from 20 to 60 mg per liter of bacterial culture) as inclusion bodies, except for FlgD (Fig. 2A). Despite sufficient bacterial growth, FlgD was expressed in very low yields (0.1 mg per liter culture). Following inclusion body isolation and refolding, SDS-PAGE revealed the high purity of recombinant proteins (Fig. 2B).

Validation of in silico predictions. To validate the immunogenicity of these proteins, we first examined their reactivity against sera taken from chronically infected mice. When evaluated via enzyme-linked immunosorbent assay (ELISA), all recombinant proteins exhibited reactivity at dilutions of 1:50 (data not shown), with endpoint titers determined to be twice the standard deviation from those of naive sera. Further, when transferred to a polyvinylidene difluoride (PVDF) membrane, all proteins exhibited various reactivities with convalescent human melioidosis sera (Fig. 3A and B) but did not react with seronegative human sera (data not shown).

Burkholderia proteins enhance immunogenicity of AuNP glycoconjugate platform. Our lab has previously shown that gold nanoparticle AuNP glycoconjugates can provide significant protection in murine and nonhuman primate models of inhalational glanders (17, 18). However, these vaccines were only partially protective against a lethal challenge and generated weak anti-LPS antibody titers. To improve the immunogenicity of this vaccine platform, we optimized a method for incorporating these novel proteins into a nanoglycoconjugate vaccine formulation. When incorporated into this AuNP glycoconjugate platform, all proteins exhibited various degrees of conjugation

### TABLE 1 Top seven candidates (together with known antigen Hcp1) identified via bioinformatics and immunoinformatics analyses

| Protein | Locus Placement | Seroreactivity Expression |
|---------|----------------|----------------------------|
| Hemagglutinin | BMAA1324 BPS50908 | 1 Yes Yes (22, 23) |
| FlgD | BMA3327 BPSL0272 | 4 Unknown Unknown |
| OmpW | BMA2010 BPSL2704 | 5 7 Unknown in vitro(b) (21) |
| FlgL | BMA3336 BPSL0281 | 8 21 Unknown Unknown |
| Porin OpcP1 | BMAA1122 BPS50708 | 9 3 Yes Yes (22) |
| Porin | BMAA0599 BPS50757 | 12 4 Unknown Unknown |
| Porin OpcP | BMAA1353 BPS50879 | — 1 Yes in vitro(a) (21, 25) |
| Hcp1 | BMAA0742 BPS51498 | 13 — Yes Yes (14) |

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**FIG 2 Expression of Burkholderia antigens.** His-tagged (6×) recombinant proteins were expressed as inclusion bodies in a pET30a(+) IPTG-inducible expression vector. Inclusion bodies were purified, denatured, and solubilized into a refolding buffer prior to dialysis against PBS. (A) Protein yields per liter bacterial culture. (B) SDS-PAGE of top seven candidates and Hcp1. Lane 1, OmpW; 2, OpcP1; 3, hemagglutinin; 4, Hcp1; 5, FlgD; 6, OpcP porin; 7, porin; 8, FlgL.

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efficacy as determined by SDS-PAGE (not shown). To evaluate the immune response to our optimized nanoglycoconjugate platform, two novel vaccine candidates (together with Hcp1 and bovine serum albumin [BSA]) exhibiting the highest conjugation efficacies were selected for in vivo immunization studies. The mice receiving a prime and two boosts of AuNP glycoconjugate vaccine generated high protein- and LPS-specific IgG titers (Fig. 4A and B). Importantly, immunization with AuNP glycoconjugates generated anti-LPS antibody titers ranging from 1:10^4 to 1:10^5 (Fig. 4B).

FIG 3 Validation of in silico predictions. Purified proteins were run by 4 to 20% SDS-PAGE and Western blotting was performed using human sera (diluted 1:500 to 1:1000) or anti-His antibody (diluted 1:10,000) prior to the addition of goat anti-human or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody, respectively. (A) Representative Western blots of purified protein with convalescent human melioidosis sera compared to anti-His antibody. *, known seroreactive antigens. (B) Chart representing the various reactivities of proteins with convalescent human sera. Dark shading, strong reactivity (e.g., FlgL); light shading, weak reactivity (e.g., Porin OpCp1); no shading, no reactivity.

FIG 4 Antibody responses following nanoglycoconjugate vaccination. Sera were collected from vaccinated animals at 2 weeks postboost and pooled (n = 10). Protein- or LPS-specific IgG titers were assessed via ELISA, with endpoint titers determined to be twice the standard deviation from those of naive sera. Bars represent the means from three replicates. (A) Protein-specific IgG responses across vaccinated groups. Sera taken from the AuNP-combo-LPS group were assessed against each individual protein (hemagglutinin, Hcp1, and FlgL). (B) LPS-specific IgG responses. Significant differences in LPS-specific IgG titers were determined via one-way ANOVA followed by Tukey’s post hoc test. Levels of significance (compared to adjuvant-only): **, P < 0.005; ***, P < 0.0005.
Protection studies. To evaluate the protective capacity of these novel AuNP glycoconjugate platforms, immunized animals were challenged intranasally (i.n.) with 3.4 times the 50% lethal dose (LD50) of B. pseudomallei K96243. Survival was monitored for 35 days postinfection. Animals receiving AuNP-FlgL-LPS and AuNP-combo-LPS demonstrated the highest protection, with 90% and 100% survival at 35 days postinfection, respectively (Fig. 5). All animals immunized with AuNP-BSA-LPS succumbed to infection by day 8 postinfection (Fig. 5). Groups receiving adjuvant only or AuNP-hemagglutinin-LPS demonstrated 20% survival at day 35 postinfection. In addition, both AuNP-Hcp1-LPS and LPS-only immunized groups demonstrated 10% survival at day 35 postinfection.

CFU enumeration. Bacterial burden in the lung, liver, and spleen from surviving mice was assessed by standard bacterial enumeration after tissue processing. Animals that received adjuvant alone had the highest lung bacterial colonization, whereas other groups receiving LPS alone or AuNP glycoconjugate vaccines demonstrated a significant reduction in bacterial burden (Fig. 6A). Importantly, animals receiving AuNP-FlgL-LPS or the combination (AuNP-combo-LPS) demonstrated the most significant reduction in lung colonization (P < 0.001) compared to those receiving adjuvant alone. However, no significant differences were observed in the livers or spleens of the control or AuNP glycoconjugate-immunized groups, except for the AuNP-Hcp1-LPS group (Fig. 6B and C). The surviving animal (n = 1) from this group demonstrated significantly increased splenic colonization (P < 0.0001) compared to those receiving adjuvant alone (n = 2).

DISCUSSION

In this study, we optimized a reverse vaccinology approach to identify immunogenic Burkholderia proteins using publicly available informatics programs. This simplified approach to candidate identification is widely applicable to other pathogens. We first selected proteins based on the conservation (>98% identity) between B. pseudomallei and B. mallei. Because B. mallei is an obligate mammalian pathogen, the selection of these conserved antigens allowed the exclusion of genes involved only in environmental adaptation. The top seven proteins, together with Hcp1, were selected based on their predicted subcellular localization, antigenicity, adhesion, and MHC epitopes (Fig. 1; see also Table S1 in the supplemental material). The selection of protein candidates with predicted extracellular or outer membrane localization was important to increase the chance that these antigens would encounter the host’s immune system. The high expression of recombinant protein upon IPTG induction (Fig. 2A) indicates that these proteins are nontoxic to bacteria, an important consideration for vaccine scalability. In contrast, the low yields of FlgD produced by Escherichia coli suggest that this protein
might be unstable and easily degraded or may result from an unusual secondary structure of RNA that interferes with ribosomal binding. Future studies will evaluate whether altering the codons or expressing in a protease-deficient *E. coli* strain may improve protein production of FlgD.

When screened against experimental murine and convalescent human melioidosis sera, all proteins exhibited seroreactivity. The wide range in reactivity with human sera (Fig. 3B) may be indicative of numerous factors, including differences in human HLA alleles or the infecting *B. pseudomallei* strain. Importantly, only IgG was assessed; therefore, the stage of infection may also play a role in the different seroreactivities observed. Taken together, these results indicate that these proteins are expressed during infection and are immunogenic in murine and human hosts.

To our knowledge, none of these antigens has been tested in a vaccine study; however, a few studies have examined the immunogenicity and functionality of these proteins. Of the seven proteins identified via informatics analyses, three proteins, namely, FlgD, FlgL, and a porin (BMA0599), remain entirely 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. Specifically, other groups have shown that two of these proteins, porin OpcP and OmpW, comprise 4.63% and 5.88% of *B. pseudomallei* total outer membrane proteins (OMPs) under *in vitro* conditions.

**FIG 6** Animals immunized with AuNP-glycoconjugate vaccines exhibited reduced bacterial colonization in lungs. Bacterial colonization in the lungs (A), livers (B), and spleens (C) at 35 days postchallenge with *B. pseudomallei* K96243. The plots represent individual averages and standard errors of the means from individual mice. Significant differences in colonization were individually ascertained via one-way ANOVA followed by Tukey’s *post hoc* test. Levels of significance (compared to adjuvant-only): **, *P* < 0.005; ***, *P* < 0.0005.
growth conditions, respectively (21). Interestingly, these authors also demonstrated that supplementing the growth medium with amino acids resulted in increased expression of the porin OpcP to 11% of *B. pseudomallei* OMPs, suggesting a possible role for this predicted porin in amino acid transport (21). Importantly, the porins OpcP and OpcP1, together with hemagglutinin, have previously been shown to react with convalescent human melioidosis sera (22–25), with the hemagglutinin protein also demonstrating reactivity with experimental equine glanders sera (24). This protein has also been shown to stimulate interferon gamma (IFN-γ) production from whole blood isolated from seropositive donors (26). Additionally, this hemagglutinin has been shown to play a role in *B. pseudomallei* adhesion, internalization, and plaque formation in A549 cells (26), as well as survival in J774.2 macrophage-like cells (27). However, the exact role of this protein in *B. pseudomallei* virulence remains controversial, as one study demonstrated a 40-fold decrease in mean lethal dose (MLD) in a mutant strain (27), while another showed no difference in bacterial colonization compared to the wild type (26). However, this discrepancy may be the result of differences in parent bacterial strains, challenge dosages, or routes of inoculation.

To our knowledge, this study represents the first attempt to evaluate these novel *Burkholderia* proteins in a vaccine formulation. The incorporation of highly antigenic polysaccharides into *Burkholderia* vaccine formulations is important, as antipolysaccharide antibodies are associated with protection against *Burkholderia* in both animals and humans (28–31). However, *B. pseudomallei* and *B. mallei* are BSL3 pathogens, making the isolation of antigenic components (e.g., LPS) difficult. To avoid these complications, many researchers have turned to excluded strains of *B. pseudomallei* or the avirulent *B. thailandensis* for LPS isolation (15, 20, 32). Importantly, the structure of *B. thailandensis* LPS has been shown to be nearly identical to that of *B. pseudomallei* and is able to protect against lethal *B. pseudomallei* challenge (32, 33). It has been shown that the conjugation of bacterial polysaccharides to carrier proteins can elicit T-cell help, thereby generating memory responses (34). As such, there is currently considerable interest in generating glycoconjugate vaccines against various pathogens. Our nanoglycoconjugate vaccine design represents a unique approach for screening various antigen combinations to achieve increased vaccine efficacy.

When incorporated into this nanoparticle platform, all proteins exhibited various levels of conjugation efficacy. Because this conjugation is highly dependent on the availability of primary amines (e.g., lysine residues), it is likely that the three-dimensional structure of the protein is hindering the availability of these residues. To evaluate the efficacy of these AuNP glycoconjugate vaccines *in vivo*, we selected two novel proteins (hemagglutinin and FlgL) that demonstrated high conjugation efficacy. After a prime and two boost vaccinations with nanoglycoconjugates containing immunogenic proteins (FlgL, hemagglutinin, and Hcp1) or BSA, mice generated high LPS-specific serum IgG titers ranging from 1:10^4 to 1:10^5. Interestingly, animals receiving LPS alone also generated high anti-LPS titers of 2 × 10^5. Because all immunized mice received equivalent LPS (10 μg) immunizations, the wide range in anti-LPS endpoint titers could be indicative of protein immunogenicity (e.g., increased or decreased processing and presentation on MHC) or may reflect differential proportions of unconjugated LPS. To address this question, further studies will focus on elucidating the conjugation efficacy and concentration needed to achieve the most robust anti-LPS titers.

Importantly, these anti-LPS titers are evidence of increased optimization of this nanoglycoconjugate platform, as previous studies generated endpoint titers of 1:100 (17). It is important to note that differences in nanoglycoconjugate 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.

After the final vaccination, immunized animals were challenged i.n. with 3.4 LD₅₀ of *B. pseudomallei* K96243. The AuNP-FlgL-LPS and AuNP-combo-LPS vaccination groups showed 90% and 100% protection at day 35 postinfection, respectively, whereas other groups demonstrated 20% survival (adjuvant only and AuNP-hemagglutinin-LPS), 10% survival (LPS and AuNP-Hcp1-LPS), and 0% survival (AuNP-BSA-LPS) (Fig. 5). These
results suggest that the immunogenic protein plays an important role in mediating protection. The 20% survival observed in the adjuvant-only-immunized group was likely a remnant of innate stimulation following adjuvant administration. Future studies will focus on increasing the delay between immunization and challenge to more fully evaluate the longevity of this protective immune response.

In the lungs of surviving animals, all immunized groups demonstrated a significant reduction in bacterial colonization compared to those receiving adjuvant alone (Fig. 6). However, the most significant reduction ($P < 0.001$) was observed in the AuNP-FlgL-LPS- and AuNP-combo-LPS-vaccinated groups. A similar trend was seen in the liver and spleen, with many animals receiving AuNP-FlgL-LPS or AuNP-combo-LPS and demonstrating colonizations below the limit of detection. Meanwhile, the surviving animals from the AuNP-Hcp1-LPS ($n = 1$) and AuNP-hemagglutinin-LPS ($n = 2$) groups demonstrated both spleen and liver colonizations. However, it is important to note that the limited number of surviving animals prevents further conclusions, and additional studies are needed to fully understand the protective capacity of these vaccine formulations.

The limited survival observed in the AuNP-Hcp1-LPS group was unexpected, as this antigen has previously been shown to provide protection against both $B. mallei$ and $B. pseudomallei$ in several studies (11, 14, 17). However, since these studies administered Hcp1 through alternate routes (e.g., intraperitoneal and i.n.), it is possible that the protection is route dependent. Additionally, the present study used $B. mallei$ Hcp1. While it differs from $B. pseudomallei$ K96243 Hcp1 by only 1 amino acid, it is possible that differences in the 3-dimensional structure could have affected immune recognition. Further studies are needed to test these hypotheses.

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 (8, 35). Therefore, the incorporation of antibody-inducing proteins is important when designing a subunit vaccine against $Burkholderia$. Nevertheless, it is currently understood that balanced cellular and humoral immune responses will be required for vaccine-induced protective immunity against $B. pseudomallei$ and $B. mallei$ (29, 36–38). Interestingly, in the present study, there does not appear to be a direct correlation between antibody titers (anti-LPS or antiprotein) and protection. It is important to note that this study only evaluated total IgG, and it is possible that specific antibody isotypes are mediating the protection. However, the lack of correlation between antibody responses and protection suggests that the cellular immune response is playing a significant role in protection. Further studies will focus on elucidating the correlates of protection mediated by these AuNP glycoconjugate vaccines. Together, the results of this study represent a unique approach to identifying novel vaccine antigens and provide the foundation for continued optimization of $Burkholderia$ glycoconjugate vaccines.

**MATERIALS AND METHODS**

**Bioinformatics and immunoinformatics.** To identify potential vaccine candidates, we screened for desirable physiochemical properties and predicted immunogenicity. First, we evaluated the gene conservation between $B. pseudomallei$ and the closely related host-adapted pathogen $B. mallei$. Using the $B. mallei$ proteome of $>5,000$ proteins (obtained from the Burkholderia Genome Database [39]), we used BLAST analyses to select proteins that exhibited $>98\%$ conservation between the two $Burkholderia$ species. Using the Vaxign (40) program, we eliminated any proteins exhibiting sequence homology with host (human and mouse) proteins. Next, we used pSORTb (41) to screen for outer membrane and extracellular (e.g., secreted) proteins, selecting proteins with prediction scores of $>9.5$. We used the transmembrane prediction programs TMHMM (42), Phobius (43), and HMMTOP (44) to select proteins with $\leq 1$ transmembrane domain. Following this analysis, we examined the predicted antigenicity (threshold $= 0.4$) and adhesive properties via VaxiJen (45) and Vaxign, respectively. Finally, we evaluated the predicted stability of the protein via ProtParam (46). Following these physicochemical analyses, we screened for predicted immunogenicity based on the binding to MHC-I and MHC-II molecules. To evaluate potential MHC-I and MHC-II epitopes and their corresponding affinities, we used the NetCTL 1.2 (47) and NetMHC 2.2 (48) programs, respectively. A summary of these analyses is included in Table S1 in the supplemental material. Finally, to downselect to the most ideal vaccine candidates, proteins were ranked against each other based on their adhesion probability and predicted antigenicity, as well as the
number and affinity of MHC epitopes. The top seven protein candidates (together with the known
Burkholderia antigen Hcp1) were selected for further validation studies.

Cloning and expression of Burkholderia antigens. B. mallei ATCC 23344 DNA was isolated via the
Qiagen DNAeasy blood and tissue kit, according to the manufacturer’s directions. Sequences encoding
OmpW (BMAA010 or BPSL2704), porin OpcP1 (BMAA1122 or BPSS0708), hemagglutinin (BMAA1324 or
BPSS0908), Hcp1 (BMAA0742 or BPSS1498), FlgD (BMAA3327 or BPSS0272), OpcP porin (BMAA1353 or
BPSS0879), porin (BMAA0599 or BPSS0757), and FlgL (BMAA3336 or BPSSL0281) were amplified via Phusion
polymerase (New England BioLabs) and cloned into a PET30a(+) expression vector using NdeI and Xhol
or HindIII-HF (New England BioLabs) restriction sites (see Table S2). The open reading frame (ORF) for
each protein was inserted in-frame with a 6-histidine (His) tag at the C terminus (see Table S3). Ligation,
transformation, and expression were performed according to the manufacturer’s directions (pET system;
Novagen) with some modifications. Upon confirmation of successful gene insertion via gel electropho-
resis and directional sequencing (UTMB Genomics Core), plasmids were transformed into BL21(DE3)
competent E. coli (New England BioLabs) via heat shock treatment. To induce protein expression,
overnight cultures were diluted 1:100 in 40 ml of Luria-Bertani (LB) broth, grown to an optical density at
600 nm (OD$_{600}$) of ~0.5, and induced with a 1 mM final concentration of IPTG. At 3 h postinduction,
cultures were centrifuged (4000 × g for 15 min), and the resulting bacterial pellets were frozen at −20°C.
To confirm protein expression, bacterial pellets were resuspended in cold 20 mM Tris-HCl (pH 7.5) and
sonicated with a 750-W ultrasonic processor on ice for 10 pulses (30 s on, 30 s off). The resulting material
was centrifuged (14,000 × g for 10 min) to separate soluble and insoluble fractions. Following the
addition of 2× Laemmli buffer (Bio-Rad), samples were heated at 95°C for 5 min and run on 10% gels
for SDS-PAGE (Bio-Rad). Protein bands were cut out, digested, and analyzed via matrix-assisted laser
desorption ionization–time of flight (MALDI-TOF) mass spectrometry (UTMB Mass Spectrometry Core) to
confirm the protein identity.

Recombinant protein purification and refolding. To induce the expression of protein inclusion bodies,
overnight cultures were diluted 1:100 in 500 ml LB broth, grown to an OD$_{600}$ of ~0.5 and induced with
1 mM IPTG. Cultures were grown for 3 h at 37°C with agitation and pelleted (6000 × g for 15 min).
Pellets were frozen at −20°C prior to protein purification. To purify inclusion bodies, 1 g of the cell pellets
was resuspended in 10 ml 1× Celllytic B (Sigma) in phosphate-buffered saline (PBS) together with 0.2
mg/ml lysozyme and 1 ml EDTA-free protease inhibitor cocktail (Roche Diagnostics). This solution was
incubated with shaking for 15 min and centrifuged (16,000 × g for 15 min) to pellet insoluble material.
The insoluble pellets were resuspended in 10 ml 1× Celllytic B (Sigma) and vortexed for 2 min prior to the
addition of 0.2 mg/ml lysozyme (Sigma). This solution was incubated for 10 min at room temperature
and centrifuged (16,000 × g for 5 min). To fully remove all soluble material from the inclusion bodies,
the insoluble pellets were washed 10 times with Celllytic B (Sigma) diluted 1:100 in PBS, with centrifuging
(16,000 × g for 5 min) between washes. To solubilize inclusion bodies, 1 g of insoluble material was
resuspended in 8 ml of Celllytic B (Sigma) and incubated with shaking for 30 min at room temperature.
After incubation, the solutions were centrifuged (16,000 × g for 15 min) to pellet cell debris. Superna-
tants containing solubilized inclusion bodies were assayed via bicinchoninic acid (BCA) (Pierce) to
determine protein concentrations and stored at −20°C until use. Solubilized inclusion bodies were
adjusted to 1 mg/ml in buffer containing 8 M urea, 100 mM NaH$_2$PO$_4$, and 10 mM Tris-HCl (pH 7.3).
Adjusted proteins were added dropwise to a 50× volume of 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 M dithiothreitol (DTT; Athena ES). These solutions were dialyzed against 4 to 6 liters of 2
PBS performed at 8-h intervals. All proteins were dialyzed against PBS, except for Hcp1 (BMAA0742 or
BPSS1498), which was dialyzed against decreasing concentrations of Tris-HCl from 50 mM to 20 mM,
with exchanges performed as described above. The resulting protein solutions were concentrated via filter centrifugation (EMD Millipore Amicon Ultra-15, 10-kDa molecular mass cutoff), assayed via BCA to determine the protein
concentrations, and stored at −80°C until use.

Validation of immunogenicity predictions. To validate our in silico predictions, we confirmed
seroreactivities with both anti-B. pseudomallei murine sera and convalescent human melioidosis sera.
Convalescent-phase sera were obtained from seropositive volunteers (Northeastern Thailand) with
informed written consent according to the Khon Kaen University ethics committee for human research.
Deidentified human sera were received, and studies at UTMB were excluded from IRB review. Anti-B.
pseudomallei sera were obtained from C57BL/6 mice 35 days postinfection with a sublethal dose of B.
pseudomallei K96243, and sera from nonimmunized control mice were used as a control. To determine
IgG antibody titers and evaluate seroreactivity,Coming high-binding polystyrene plates (Fisher) were
coated overnight with 10 μg/ml recombinant protein in PBS. After coating, plates were washed twice
with wash buffer (PBS containing 0.05% Tween 20) and blocked in blocking solution (0.1% Tween 20 and
2% bovine serum albumin) for 2 h. After blocking, plates were washed twice prior to the addition of
serum samples. Sera were diluted 1:25 in sample diluent (PBS solution containing 0.01% Tween 20 and
1% BSA), added to the plate, and serially diluted. After the addition of sera, goat anti-mouse IgG
polyclonal antibody (1:500; Abcam) was added to the plate and incubated for 3 h at room temperature
with shaking. After incubating, the plate was washed four times with wash solution prior to the addition
of a tetramethylbenzidine (TMB; eBioscience) substrate for 15 min at room temperature. The reactions
were stopped with 2 N H$_2$SO$_4$ and the plates were read with an Epoch nanospectrophotometer at 450
to 570 nm. Endpoint titers were determined to be the OD$_{450-570}$ value equivalent to twice the standard
deviation from those of naïve sera.
To evaluate seroreactivity with convalescent human sera, 1 to 2 μg of recombinant proteins were diluted in Laemmli buffer (Bio-Rad) and heated at 95°C for 5 min. Samples were run by SDS-PAGE (10% or 4 to 20% gels; Bio-Rad) at 100 V for 1.5 h. Proteins were transferred to Immobilon PVDF membranes (EMD Millipore) using a Trans-Blot SD semi-dry transfer cell (Bio-Rad) at 15 V for 30 min. The membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.001% Tween 20 (TBST) solution containing 5% powdered milk and 1% BSA. After blocking, the membranes were probed with human sera (diluted 1:500 to 1:1000) or goat anti-His antibody (diluted 1:10,000) and incubated overnight at 4°C with rocking. After incubating, the membranes were washed three times with TBST (10 min each) prior to the addition of goat anti-mouse IgG diluted 1:5,000 to 1:10,000 for 1 h at room temperature. The membranes were washed three times with TBST prior to the addition of ECL 2 chemiluminescent substrate (Pierce) and were imaged with an ImageQuant LAS 4000 system.

**Lipopoly saccharide purification.** Lipopolysaccharide was purified from *Burkholderia thailandensis* as previously described (20). Briefly, *B. thailandensis* E264 was inoculated into 2.5 liters of LB broth and allowed to grow for ~20 h at 37°C with agitation (200 rpm). Then, the bacterial pellet was obtained via centrifugation (6,000 × g for 15 min) and resuspended in a 1:1 mixture of phenol and water. After heating to 80°C, the solution was cooled and dialyzed against 4 to 5 exchanges of water. This solution was clarified via centrifugation (6,000 × g for 15 min) and lyophilized. The lyophilized substance was resuspended in an aqueous solution containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM CaCl₂ and digested with RNase, DNase I, and proteinase K (50 μg/ml each). A gel-like pellet of LPS was obtained via ultracentrifugation (100,000 × g for 3 h) and lyophilized. For additional purification, the sample was washed 4 to 5 times with 90% ethanol and lyophilized. The final pellet was weighed, resuspended in PBS or water to 1 mg/ml, and stored at −80°C until use. LPS purity was assessed via SDS-PAGE and Western blotting (not shown).

**Construction of AuNP glycoconjugates.** Gold nanoparticle (AuNP) glycoconjugates were synthesized as previously described (17), with modifications. First, 15-nm spherical AuNPs were synthesized via the reduction of 1 mM gold(III) chloride trihydrate with 90 mM sodium citrate dihydrate, according to the method described by Turkevich et al. (49). Particle sizes and shapes were confirmed via transmission electron microscopy (TEM). 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 2 h of incubation, this solution was filter centrifuged (EMD Millipore Amicon Ultra-15, 3-kDa molecular mass cutoff) and the process repeated to ensure complete coverage. The attachment of 16-MHDA was confirmed by measuring plasmon resonance via UV-Vis spectroscopy (data not shown). To facilitate conjugation, 0.1 mM 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM; Sigma), 0.1% Tween 20, and 20 mM hydroxylysine ester (SATA; Sigma) was added to protein-conjugated AuNPs and allowed to incubate overnight at room temperature with agitation. To prevent the aggregation of AuNPs, conjugation reactions were performed in either 1× PBS (pH 7.5) or 0.1 M borate (pH 8.6) buffer. LPS was conjugated to AuNPs using a modified thio-maleimide 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 and allowed to incubate for 15 min. Next, 10.9 μl of 800 μM 6-maleimidocapric acid hydrazide, trifluoroacetic acid salt (EMCH) was added, and the mixture was incubated an additional 15 min. Then, the pH of the LPS solution was adjusted to 7.0 with weak NaOH and incubated for 1 h at room temperature with rocking, followed by filter centrifugation and desalting into 5 mM EDTA. In the meantime, 25.4 μl of 250 μM 5-acetyltetraylglucyclic acid N-hydroxysuccinimide ester (SATA; Sigma) was added to protein-conjugated AuNPs and incubated for 1 h at room temperature. The reaction was quenched with 50 μl 50% (vol/vol) hydroxylamine and 50 μl of 5 mM EDTA. Then, the protein-conjugated AuNPs were filter centrifuged and resuspended in an LPS-EDTA solution for 4 h, 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 (with filter centrifuging between washes) and resuspended to a final desired volume. Conjugation was confirmed by SDS-PAGE (not shown).

**Mouse immunization studies.** Six- to eight-week-old female C57BL/6 mice (Charles River) were housed in microisolator cages under pathogen-free conditions, were provided with rodent feed and water ad libitum, and were maintained on a 12-h light cycle. 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 animal care and use committee of the University of Texas Medical Branch (protocol number 0503014B). To evaluate immunogenicity in an animal model, mice were immunized subcutaneously (s.c.) at 2-week intervals with a prime and 2 boosts of AuNP glycoconjugate vaccine formulation containing 10 μg of protein (Bsa, hemagglutinin, Hcp1, or FlgI) and 10 μg LPS in PBS, together with 500 μg Alhydrogel and 30 μg poly(I·C) adjuvants (InvivoGen). For the combination group (AuNP-combo-LPS), mice received equal parts AuNP-hemagglutinin-LPS, AuNP-FlgI-LPS, and AuNP-Hcp1-LPS (3.33 μg each for 10 μg total protein concentration). Control groups received adjuvant alone or in combination with 10 μg LPS. To evaluate antibody titers, blood was drawn retro-orbitally 2 weeks following the last boost. To isolate sera, blood was incubated for 30 min at room temperature to allow for clotting and was centrifuged (10,000 × g for 10 min). Sera were removed and stored at −20°C until use. For ELISAs and serum assays, individual sera from each immunization group were pooled (n = 10).

**Bacterial growth conditions.** All bacterial infections were performed with *B. pseudomallei* K96243 or *B. mallei* ATCC 23344 in CDC-approved and registered biosafety level 3 (BSL3) and animal biosafety level 3 (ABS3L) facilities at the University of Texas Medical Branch. All experiments were performed in...
accordance with select agent standard operating practices. Prior to the animal challenge, B. pseudomallei K96243 freezer stocks were streaked onto an LB agar plate and incubated for 36 h at 37°C. For liquid cultures, 3 to 5 colonies were selected and inoculated in 20 ml LB broth and grown for 12 h at 37°C with agitation. Challenge doses were prepared from overnight cultures diluted in PBS.

**LD₅₀ determination.** Six- to eight-week-old female C57BL/6 mice (Charles River) were acclimated for 1 week prior to the bacterial challenge. The 50% lethal dose (LD₅₀) for B. pseudomallei K96243 was determined by administering i.n. with doses of 3 × 10⁵, 1.5 × 10⁵, and 3 × 10⁴ CFU/ml to groups of 10 mice. Mice were monitored for survival and the LD₅₀ was calculated as previously described (50).

**Survival study.** Three weeks after final immunization, mice (n = 10 per vaccination group) were anesthetized and challenged i.n. with 3.4 LD₅₀ (1.06 × 10⁶ CFU/ml) of B. pseudomallei K96243 in a total volume of 50 μl PBS. Animals were monitored daily for 35 days postinfection; then, surviving animals were euthanized and their lungs, livers, and spleens were harvested for CFU enumeration. The survival curve was generated using the Kaplan-Meier method.

**CFU enumeration.** The lungs, livers, and spleens of surviving animals were weighed and homogenized in 1 ml PBS using a tissue grinder (Covidien, Mansfield, MA). Homogenate samples were serially diluted in PBS, plated on LB agar plates, and incubated for 48 h at 37°C. Bacterial colonies were counted and reported as CFU per gram of tissue. The limit of detection for bacterial detection was approximately 10 CFU per organ.

**Statistical analyses.** Survival and organ colonization graphs were generated using GraphPad Prism V7.0. Significant differences in survival data were analyzed using a log-rank test (Mantel-Cox). Bacterial colonization data and LPS-specific IgG titers were analyzed via one-way analyses of variance (ANOVARs) followed by Tukey’s post hoc tests.

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