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## Protective response to subunit vaccination against intranasal *Burkholderia mallei* and *B. pseudomallei* challenge

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### Abstract

*Burkholderia mallei* and *B. pseudomallei* are Gram-negative pathogenic bacteria, responsible for the diseases glanders and melioidosis, respectively. Furthermore, there is currently no vaccine available against these *Burkholderia* species. In this study, we aimed to identify protective proteins against these pathogens. Immunization with recombinant *B. mallei* Hcp1 (type VI secreted/structural protein), BimA (autotransporter protein), BopA (type III secreted protein), and *B. pseudomallei* LolC (ABC transporter protein) generated significant protection against lethal inhaled *B. mallei* ATCC23344 and *B. pseudomallei* 1026b challenge. Immunization with BopA elicited the greatest protective activity, resulting in 100% and 60% survival against *B. mallei* and *B. pseudomallei* challenge, respectively. Moreover, sera from recovered mice demonstrated reactivity with the recombinant proteins. Dendritic cells stimulated with each of the different recombinant proteins showed distinct cytokine patterns. In addition, T cells from immunized mice produced IFN- $\gamma$  following *in vitro* re-stimulation. These results indicated therefore that it was possible to elicit cross-protective immunity against both *B. mallei* and *B. pseudomallei* by vaccinating animals with one or more novel recombinant proteins identified in *B. mallei*.

### Keywords

*Burkholderia*; *B. mallei*; *B. pseudomallei*; vaccine; subunit vaccination; intranasal infection

## Introduction

Vaccines are the most efficacious and cost-effective means of protecting human and animal populations from infection. At present, there are no approved vaccines available for use in protecting animals or humans against the Gram-negative bacterial pathogens *Burkholderia mallei* and *B. pseudomallei*. Therefore, we sought to develop and evaluate vaccines that might be used to generate cross-protective immunity against both pathogens.

*B. mallei* is a non-motile bacterium responsible for glanders. This disease mainly affects horses, which are considered to be the natural reservoir for infection, although mules and donkeys are also susceptible (1). Humans are accidental hosts of *B. mallei* following prolonged and close contact with infected animals. *B. mallei* infects humans by entering through open wounds and surfaces of the eyes or nose. Symptoms of glanders are dependent on the route of infection (2). *B. pseudomallei* are motile bacteria causing melioidosis (3). Melioidosis is a life-threatening disease that is mainly acquired through skin inoculation or pulmonary contamination, although other routes have been documented. This saprophyte inhabitant of soil environments is mainly encountered in Southeast Asia and Northern Australia, but is sporadically isolated in subtropical and temperate countries (4).

Both *Burkholderia* species are highly pathogenic and are classified as such in list B by the Centers for Disease Control and Prevention (5). *Burkholderia* infections are difficult to treat with antibiotics and there are several reports that indicate it is feasible to protect against melioidosis, at least in animal models of disease, with non-living vaccines (6). There has also been some progress in identifying partially protective subunits. Passively administered antisera raised against flagellin, polysaccharide, or conjugates of polysaccharide and flagellin, protect diabetic rats against challenge with *B. pseudomallei* (7–9). However, *B. mallei* are not motile and do not produce flagella. Moreover, the ability of flagellin to induce protection against an aerosol, or intranasal challenge has not been reported. Therefore, we assessed flagellin as a potential candidate for inclusion in a *Burkholderia* vaccine and found it unsuitable (our unpublished data). In contrast, all of the current evidence indicates that other surface-expressed or secreted proteins are immunogenic and structural similarity exists between the proteins in *B. pseudomallei* and *B. mallei* (10–11). In this study, we aimed to identify *Burkholderia* protective proteins that could be administered in vaccines to generate cross-protective immunity against both *B. mallei* and *B. pseudomallei*. We hypothesize that cross-protection is possible based on the similarities in antigenic composition and mechanisms of protection between these organisms, and if this is true, development of a single vaccine which stimulates T-cell and antibody responses against melioidosis and glanders-producing bacterial agents is feasible. If cross-protective immunity is observed, then it may be possible to develop a single vaccine capable of generating protection against both melioidosis and glanders.

## 2. Recombinant protein expression and purification

Bioinformatics analysis of target sequences was used to indicate the presence (or absence) of an N-terminal secretion sequence, transmembrane domains and homology to published crystal structures. The programs used were SignalP v.3.0, TMHMM v.2.0 and PHYRE v. 0.2, respectively (12–14). DNA sequences coding for *B. mallei* proteins BopA (BMA\_A1521; AA 23 – 512), BimA (BMA\_A0749; residues 19 – 265), Hcp1 (BMA\_A0742; residues 1 – 169) and the *B. pseudomallei* protein LolC (BPSL2277; residues 44 – 266) (15) were cloned into the pET28a (+) expression vector (Novagen). Primers were designed to PCR-amplify and clone the selected sequences in frame with a C-terminal 6x Histidine tag, for all four targets. Expand high fidelity DNA polymerase (Roche) was used to amplify targets from *B. mallei* ATCC 23344 or *B. pseudomallei* K96243

genomic DNA. Once ligated into pET28a (+), plasmid DNA was electroporated into *Escherichia coli* DH5 $\alpha$ . Cloned sequences were verified by DNA sequencing, using T7 promoter/terminator oligonucleotide primers.

Target protein expression in *E. coli* ( $\lambda$ DE3) Rosetta was induced by growth in Overnight Express instant TB medium (Novagen) for 18 – 20 h. Bacterial pellets were lysed using 10x CelLytic B (Sigma), and 6x His-tagged proteins were purified by Ni<sup>2+</sup> affinity chromatography. Purified proteins were dialyzed against two changes of 10 mM Hepes/150 mM NaCl, pH 7.4, aliquoted and stored at –80 °C. Protein concentrations were determined using the BCA kit (Pierce) using bovine serum albumin (BSA) as a standard, and sample purity assessed by SDS-PAGE.

### 3. Vaccination and challenge with *B. pseudomallei* or *B. mallei*

To evaluate the potential of *Burkholderia* surface expressed or secreted proteins to generate protective immunity, the purified recombinant proteins were used individually or in combination to vaccinate mice via the intranasal (i.n.) route. For immunization of mice against *B. mallei* challenge, 6–8 week old female BALB/c mice (n = 8 per group) were primed with 10  $\mu$ g of recombinant proteins mixed with adjuvant (12.5  $\mu$ g of CpG oligodeoxynucleotide (ODN) 2395 (Coley Pharmaceuticals) and mixed with 12.5  $\mu$ g immune-stimulating complex (ISCOM) AbISCO 100 (Isconova AB), followed by a 2 week boost of 5  $\mu$ g recombinant proteins with adjuvant. Four weeks post-boost, animals were infected by intranasal inoculation with 2 LD<sub>50</sub> of *B. mallei* ATCC 23344 administered i.n. to anesthetized mice. Control animals were vaccinated with non-specific protein (Bovine Serum Albumin, BSA) and adjuvant. Animals were observed closely following challenge and euthanized immediately when pre-determined endpoints were reached and these time points were used to calculate survival times. All animal studies were approved by the Institutional Animal Care and Use Committee at UTMB.

Following challenge with *B. mallei*, 12.5% of control animals survived for > 21 days (Figure 1A). In contrast, survival percentages were significantly increased to 100% up to 21 days post-infection in mice vaccinated with recombinant BimA or BopA. The surviving animals were euthanized at day 21 post-challenge, and the lungs and spleens were homogenized and bacterial counts determined. In all the surviving animals, *B. mallei* were not recovered from the lungs. However, *B. mallei* were recovered from the spleens of all surviving animals (data not shown).

To determine whether antibodies from infected mice reacted with the recombinant *Burkholderia* proteins, serum was obtained from surviving animals. These sera were then tested for recognition of the purified recombinant *Burkholderia* antigens Hcp1, BimA, BopA and LolC by Western blot. Serum from infected mice recognized each of the recombinant antigens tested except for the LolC protein (Figure 2A) and were recognized by both IgG1 and IgG2a isotypes (Figure 2B).

Experiments were also conducted to determine whether the *Burkholderia* recombinant antigens were also capable of generating protective immunity against *B. pseudomallei* challenge (Figure 1B). For these experiments BALB/c mice, (n = 5 per group) were primed by i.n. inoculation with two adjuvant systems and immunized with 2  $\mu$ g of the purified recombinant BimA, BopA, or LolC proteins given with CLDC adjuvant (cationic liposome-DNA complex), and then boosted 2 weeks later and again 2 weeks after that. The adjuvant used for these studies consisted of cationic liposome-DNA complexes, as reported previously (16) for use in non-specific immunotherapy of *B. pseudomallei*. Controls consisted of mice administered CLDC adjuvant alone, diluent only or BopA antigen alone.

Two weeks after the last immunization, mice were subjected to lethal i.n. challenge with 2 LD<sub>50</sub> of *B. pseudomallei* strain 1026b, as described previously (17). Survival times were determined as noted above, and all the *B. pseudomallei* animal challenge studies were approved by the Institutional Animal Care and Use Committee at Colorado State University.

Our data indicate that the vaccine candidates protected the animals from an initial, acute infection, but failed to confer sterilizing immunity. Our future studies are focusing on the identification of the immunogenic domains in the proteins coupled with optimization in the vaccination strategies to develop a fully protective vaccine.

We observed that immunization separately or as a combination of each of the four recombinant *Burkholderia* antigens conferred at least 75% protection against *B. mallei* infection at 21 days compared to control mice (Figures 1A and 1B). Notably, one of the vaccine antigens (BopA) also conferred significant 60% longer term (> 60 days) protection against *B. pseudomallei* challenge. Furthermore, when surviving BopA-vaccinated mice were sacrificed and lung, spleen, and liver tissues plated for detection of *B. pseudomallei*, 25% of the surviving animals were sterile, at least within the limits of detection of the organ culture (typically 50 CFU/organ).

#### 4. ELISA assay for humoral responses to vaccination

Blood was removed from the orbital veins of immunized mice 2 days post-boost. The blood was allowed to clot at room temperature prior to centrifugation at 5,000 × g. Serum was collected and stored at -20 °C. The recombinant Hcp1-, LolC-, BimA- and BopA-specific protein responses were determined by ELISA (Table 1). Briefly, microtiter plates were coated with 5 µg/ml of the appropriate recombinant protein in PBS overnight. Non-specific binding was blocked using 1% (w/v) ovalbumin in PBS (OVA-PBS) for 1 h at RT. The plates were washed three times using 0.05% (v/v) Tween 20 in PBS, and appropriate dilutions of sera in OVA-PBS were added in triplicate and incubated for 2 h at 37 °C. Following the washes, biotinylated-rat-anti-mouse IgG1 or IgG2a (BD Biosciences) diluted in OVA-PBS was added and incubated for 2 h at 37 °C. Next, HRP-conjugated Streptavidin (BD Biosciences) was added and incubated for 25 min at RT. The substrate ABTS was added and the absorbance at 405 nm was measured. Antibody concentrations (in µg/ml) were calculated from standard curves generated with IgG1 or IgG2a specific antibodies.

#### 5. Discussion

Immunization with recombinant LolC, BimA, Hcp1HCP and BopA proteins provided significant protection against *B. mallei* ATCC 23344 and *B. pseudomallei* 1026b (Figures 1A and B), in which *B. mallei* BopA gave the best results. The combination of all subunits for protection from *B. mallei* may have some utility as a combined vaccine, although not remarkably better than BopA alone. The serological results suggest that optimal level of Th1 (IgG2a) and Th2 (IgG1) responses are important for protection in *B. mallei* infection. An interesting observation is that in control group mice, which received only CpG2395 and ISCOM, we have observed over 78% survival, whereas in a previous study, mice receiving only ISCOM had ~12% survival after infection with 2 LD<sub>50</sub> of *B. mallei* ATCC 23344, indicating that CpG2395 itself also offers protection (data not shown). There are several reports showing immune-enhancing activity of CpG (18–24). Consistent with this study, all future studies will be repeated using a more prolonged time to challenge at 4 weeks to reduce background protection offered by CpG2395, and a control group without CpG treatment will be included as a control as was done in these studies.

## Acknowledgments

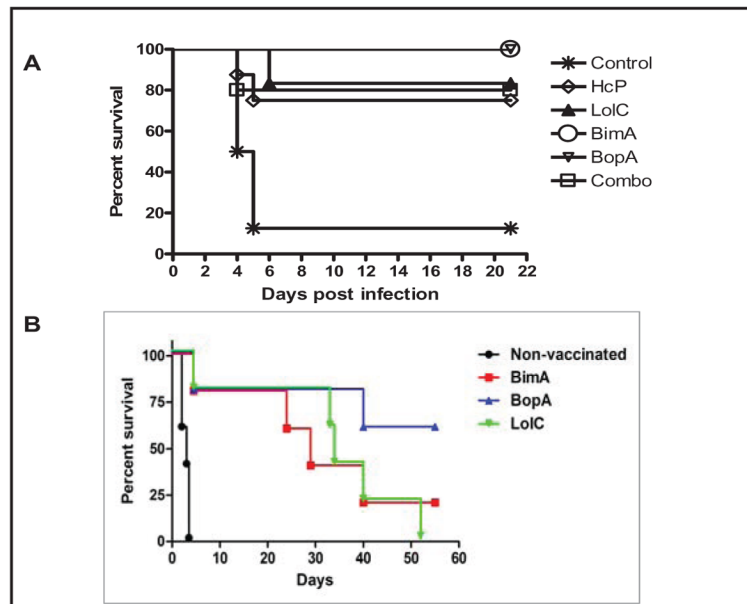
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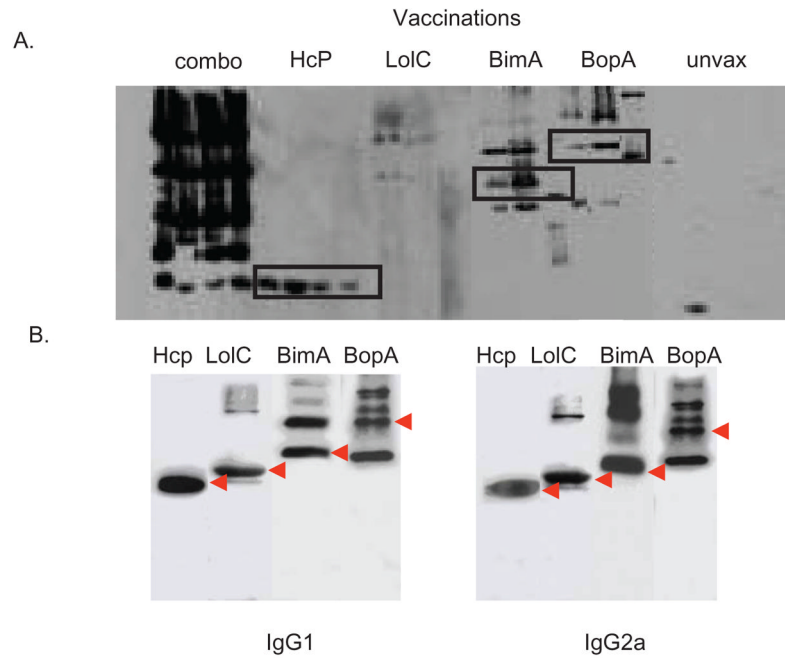




**Fig. 1.**

Survival of BALB/c mice immunized with different recombinant proteins and challenged with *B. mallei* ATCC 23344 and *B. pseudomallei* 1026b.

BALB/c mice were challenged i.n. with 2 LD<sub>50</sub> *B. mallei* 4 weeks following intranasal vaccination with BimA (n= 2), BopA (n=5), Combo (n=5), LoIC (n=6), Hcp1 (n=8) or Control (n= 8). BopA- and BimA-vaccinated animals resulted in 100% survival up to 21 days post-challenge. (B) BALB/c mice (n = 15, pooled data from 3 separate experiments) were immunized 3 times with the indicated antigens, then challenged i.n. with 2 LD<sub>50</sub> *B. pseudomallei* strain 1026b and survival times determined,.



**Fig. 2.** (A). *B. mallei* antibody response post-vaccination. Western blots were performed on sera collected 2 weeks post-boost to determine IgG reactivity. Mice vaccinated with recombinant BopA, BimA, LolC and Hcp1, individually or in combination (combo), demonstrated response to all proteins except LolC. Individually vaccinated mice produced a robust humoral response, although LolC was lacking. (B) Isotype-specific responses to the vaccine candidates (IgG1 and IgG2a) detected from vaccinated and challenged mice.



**Table 1**Immune response to Recombinant *Burkholderia* Proteins

<b>Protein</b>	<b>IgG1<sup>a</sup> (µg/ml)</b>	<b>IgG2a<sup>a</sup> (µg/ml)</b>
Hcp1	<b>95</b>	<b>40</b>
LolC	<b>0.104</b>	<b>0.902</b>
BimA	<b>2,970</b>	<b>5,700</b>
BopA	<b>2,070</b>	<b>6,080</b>

The IgG1 and IgG2a responses were analyzed by ELISA using sera removed from immunized and infected mice.