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Identification of a novel virulence factor of *Burkholderia pseudomallei* and *B. mallei*, a protective vaccine antigen against melioidosis

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IMPORTANT INFORMATIVE STATEMENTS

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In conducting the research described in this report, the investigators adhered to the “Guide to the Care and Use of Experimental Animals, Vol. I, 2nd Ed.” published by the Canadian Council on Animal Care.

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Part of this work was captured in the patent: STRATILO, C.W., Jager, S.J., Recombinant Burkholderia pseudomallei adhesion protein and methods and uses thereof., U.S. Patent No. 8,420,090., 2013.

Abstract

Burkholderia pseudomallei is the causative agent of melioidosis, a severe systemic, potentially life-threatening disease with many possible manifestations in humans. This pathogen expresses a large number of virulence factors, with many found on islands of pathogenesis. Significant efforts have been invested into vaccine development; however none have offered complete protection. A unique and conserved protein of *B. pseudomallei* and *B. mallei* has been identified as the exoprotein of a two-partner secretion pathway. This exoprotein, when expressed in a recombinant form effected the formation of actin tails in macrophage cell culture, suggesting a role in intracellular spread and a possible role in virulence. A truncated recombinant form of this protein conferred protection against *B. pseudomallei* and *B. mallei* challenge in mice when used as a vaccine candidate.

Significance to defence and security

This work describes the characterization and evaluation of a vaccine candidate for *Burkholderia pseudomallei* and *Burkholderia mallei*. As no vaccine exists for these organisms, identification of potential vaccine candidates is the first step in vaccine development. This paper describes the production and testing of a recombinant vaccine against these agents.

Résumé

Burkholderia pseudomallei est l'agent étiologique de la mélioïdose, une maladie systémique grave et potentiellement mortelle qui peut se manifester de bien des façons chez l'humain. Bon nombre de facteurs de virulence entrent en jeu en présence de ce pathogène et apparaissent souvent dans des îlots de pathogénicité. Des efforts considérables ont été déployés en vue de mettre au point un vaccin; cependant, aucun n'a encore réussi à assurer une protection complète. Néanmoins, une protéine conservée unique de *B. pseudomallei* et de *B. mallei*a été identifiée comme l'exoprotéine d'une voie de sécrétion à deux partenaires. Exprimée sous forme recombinante, cette exoprotéine a exercé une influence sur la formation de queues d'actine dans des cultures cellulaires macrophages, ce qui donne à penser qu'elle pourrait jouer un rôle dans la propagation intracellulaire et possiblement dans la virulence. Dans le cadre d'un test de provocation effectué avec *B. pseudomallei* et *B. mallei*, une forme recombinante tronquée de cette protéine a été utilisée comme vaccin potentiel et a permis d'assurer une protection à des souris.

Importance pour la défense et la sécurité

Les travaux décrivent la caractérisation et l'évaluation d'un éventuel vaccin contre *Burkholderia pseudomallei* et *Burkholderia mallei*. L'identification de vaccins potentiels constitue une première étape dans la mise au point d'un vaccin contre ces pathogènes, car il n'en existe actuellement aucun. Le présent document décrit la production et la mise à l'essai d'un vaccin recombinant contre ces pathogènes.

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Acknowledgements

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1 Introduction

Burkholderia pseudomallei is a gram negative environmental saprophyte that is endemic to much of Southeast Asia and northern Australia. This bacterium causes the human disease melioidosis, which is characterized by a broad spectrum of acute and chronic symptoms including severe pulmonary disease and septicemia [1]. In northeast Thailand, melioidosis is responsible for at least 20% of all community acquired septicemias and 40% of sepsis-related mortality [2]. Although a treatment regimen including the antibiotic ceftazidime greatly reduces typically high mortality rates, multi-drug resistance continues to present a serious challenge when treating melioidosis [3, 4]. *Burkholderia mallei*, the causative agent of glanders, is an obligate mammalian pathogen that can be highly virulent in humans [5]. *B. pseudomallei* and *B. mallei* are classified as category B select agents by the US Centers for Disease Control and Prevention due to the severity of these diseases, ease of infection by inhalation and the lack of effective therapies and vaccines [6].

To date, there are no effective vaccines that protect against *B. pseudomallei* or *B. mallei* infection. As reviewed by Silva and Dow, several candidates offer significant but incomplete protection [7]. Killed whole-cell and attenuated mutants, including metabolic knockouts or those lacking various virulence factors, have also shown to be somewhat protective in both *B. mallei* and *B. pseudomallei*, [8–11]. A number of subunit vaccine candidates have been identified in *B. pseudomallei* including proteins, capsular polysaccharides, lipopolysaccharides and conjugates; none have offered complete protection [12–16].

Filamentous hemagglutinin (FHA) of *Bordetella pertussis* is the exoprotein of a two-partner secretion pathway (TPS) and is an important component of the successful acellular pertussis vaccine [17–19]. TPS systems usually consist of two proteins: a large exoprotein and a specific transporter protein involved in the exoprotein's translocation across the outer membrane of gram-negative bacteria. TPS pathway exoproteins differ in size, sequence and function. These proteins may have hemolytic/cytolytic or adhesion activity or be involved in proteolysis, systemic dissemination of bacteria, intracellular survival, biofilm formation, as well as other functions often related virulence and survival [20–25].

Based on homology to the N-terminal region of TPS exoproteins and the presence of a TPS transporter/activator in close proximity, we identified several putative TPS systems in *B. pseudomallei* that contained a FHA family N-terminal domain or HA activity domain (Pfam: PF05860). Like the first TPS system identified in *B. pseudomallei*, most genes expressing TPS proteins in *B. pseudomallei* were found on genomic islands and were not universally conserved [26, 27]. However, we identified a TPS that was consistently present in all sequenced strains of both *B. pseudomallei* and *B. mallei,* but not in other *Burkholderia* species. This paper describes the isolation, cloning and characterization of this conserved *B. mallei* and *B. pseudomallei* TPS exoprotein and its possible role in virulence and efficacy of this antigen as a vaccine.

2 Material and methods

2.1 Construction of BPSS1727 expression vectors

Genomic DNA was extracted from *B. pseudomallei* (Ashdown and K96243) using DNAeasy (Qiagen, Maryland, USA). The full length BPSS1727 gene, including native signal sequence, was PCR amplified with the primer pair BPSS1727 5′—CATATGGTCATGCAGAGGAATGAGGTC and BPSS1727 3′—CTCGAGGCGTCACTCGGATGTCCT. The resultant 2.83 kb product, full-length hemagglutinin like protein (fHLP), was gel purified and ligated into pCRT7/CT-TOPO according to the manufacturer's instructions (ThermoFisher Scientific-Invitrogen, Massachusetts, USA) generating pfHLP. The ligation was transformed into *E. coli* TOP10 cells (Invitrogen) and screened by colony PCR. A truncated BPSS1727 gene, lacking the N- and C-terminal regions, was PCR amplified with the primer pair HAD5′—AAAAAAGGTACCGGGACGGACTTGGTCAATATC and CTD3′—TTTTTTGGATCCTACTCTCGAATGGTCTGCAACTG incorporating KpnI and BamHI sites respectively. The resultant 2.28 kb product, tHLP, was gel purified, digested with KpnI/BamHI and ligated into similarly digested pCold-I (Takara Bio, California, USA) generating ptHLP. The ligation was transformed into *E. coli* NovaBlue cells (MilliporeSigma-Novagen, Massachusetts, USA) and screened by colony PCR. Sequence and orientation of pfHLP and ptHLP were verified with a Beckman Coulter CEQ-8000 Genetic Analyzer (Beckman Coulter, California, USA).

2.2 Expression of recombinant fHLP and tHLP

E. coli BL21Star(DE3) (Invitrogen) or Rossetta2(DE3) pLys (Novagen) cells harboring pfHLP or ptHLP were grown in 1L LB media supplemented with carbenicillin (50 mgL^{-1}) at 37°C with shaking at 225 rpm. fHLP cultures were induced at an OD_{600} of 0.5 with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown for an additional 3 h at 37°C. At an OD_{600} of 0.6, tHLP cultures were incubated at 15°C for 20 min tHLP cultures were then induced with 0.5 mM IPTG and incubated overnight at 15°C.

2.3 Purification of fHLP and tHLP by affinity chromatography

Cells were harvested by centrifugation at 5,000 x g for 15 min. Pellets were re-suspended in lysis buffer (5 ml BugBuster protein extraction reagent (Novagen), 10 µl Lysonase bioprocessing reagent (Novagen)/gram pellet) and incubated 20 min at room temperature (RT) with mixing. Lysates were centrifuged at 6,000 x g for 15 min. Inclusion bodies re-suspended in 10 ml wash buffer (50 mM Tri, pH8.0, 0.5 M NaCl, 1% Triton X-100) and incubated as above. Washed inclusion bodies were solubilized in 5 ml 8 M urea, 20 mM Tris, pH 10, 1 mM DTT, 1 mM n-dodecyl-β-D-maltopyranoside (DDM) and centrifuged at 50,000 x g for 30 min. tHLP was purified under denaturing conditions on Ni-NTA Superflow columns (Qiagen, Maryland, USA) according to the manufactures instructions. tHLP was refolded by rapid dilution into refolding buffer (20 mM Tris, pH 7.5, 0.5 M L-arginine, 1 mM DDM, 1 mM DTT, 1 x Halt protease inhibitor cocktail EDTA free (ThermoFisher Scientific-Pierce, Massachusetts, USA)) or matrix assisted, where the denaturing buffer was replaced over a 20 column volume gradient with 50 mM Tris pH 8, 300 mM NaCl, 50 mM urea, 0.1% n-octyl-β-D-glucopyranoside (OGP) and 10 mM imidazole. Due to the presence of an upstream stop codon, the C-terminal His tag in pfHLP is not translated. Accordingly, fHLP was refolded by rapid dilution into refolding buffer and purified under native conditions on heparin sepharose 6 Fast Flow(GE Healthcare Life Sciences, Pennsylvania, USA) columns according to manufactures instructions. Refolded and purified proteins were

concentrated and buffer exchanged into PBS, 1% DDM. Protein purity was assessed by SDS-PAGE (NuPAGE 4–12% Bis-Tris, Invitrogen) stained with SimplyBlue SafeStain (Invitrogen).

2.4 Polyclonal antisera generation

Female BALB/c mice, 5–6 weeks old, (Charles River, Massachusetts, USA) were used throughout this study. Groups of five mice were vaccinated by intraperitoneal (IP) injection (100 µl) with 20 µg tHLP mixed 1:1 with TiterMax Gold adjuvant (MilliporeSigma-Sigma) on days 0, 14 and 28. Blood was collected by tail vein on day 21. On day 42 mice were exsanguinated via cardiac puncture, and the sera were separated from red blood cells via centrifugation. Sera were used neat or polyclonal antibodies were purified using protein G columns as per manufacturer's directions (GE Healthcare Life Sciences). Sera collected from vaccinated mice were diluted 1:20000 with 1% BSA in PBS and the immunoglobulin profile was assayed with IsoStrip mouse antibody isotyping kit (SigmaMillipore-Roche Applied Science) according to the manufacturer's instructions. Densitometric analysis of the isotyping strip was performed using ImageJ software [28].

2.5 Detection of endogenous HLP antigen by ELISA and Western blot

Enzyme-linked immunosorbent assay (ELISA) was performed. microtiter plates were coated with antigen (purified tHLP, live *B. mallei* 23344 or *B. pseudomallei* (K96243 or a clinical isolate) in PBS) in sodium bicarbonate buffer (pH9.6) at 4°C overnight. Wells were washed with 0.1% Tween-20 in PBS (PBST) and blocked with 2% BSA in PBS for 2 h at RT. Sera from vaccinated mice or mice that survived challenge were used neat or diluted in blocking buffer containing 0.1% Tween-20. Microtiter plates were incubated with mouse sera for 1 h at RT followed by washing five times with PBST. Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (H+L) (Cedarlane, North Carolina, USA), was diluted as above and the microtiter plate incubated for 1 h at RT followed by washing as above. ABTS substrate was added and plates were read at 405 nm after 30 min incubation at RT. Colonies isolated from lung, liver, and spleen homogenates (from mice challenged with *B. pseudomallei* and *B. mallei*) were cultured overnight in LB media. Proteins in the culture supernatant, bacterial cell pellet or neat culture were resolved by SDS-PAGE (NuPAGE 10% Bis-Tris, Invitrogen) and transferred to nitrocellulose by semi-dry blotting at 30 V for 1 h for western blotting. Membranes were blocked with 5% skim milk, 0.1% Tween-20 in PBS for 1 h at RT before incubating overnight at 4°C with sera (from tHLP vaccinated mice) at a 1:1000 dilution in blocking buffer. Membranes were washed with PBST and incubated 1 h at RT with HRP-conjugated goat anti-mouse IgG (H+L) at 32 ng/ml in blocking buffer. Membranes were washed with PBST and incubated in Supersignal® West Pico (Pierce) before exposing to photographic film.

2.6 Creation of unmarked deletion mutants

B. pseudomallei DD2254, an *amr*RAB-*opr*A unmarked deletion mutant of 1026b, was constructed via two step overlap PCR which deleted the *amr*RAB-*opr*A while fusing the flanking regions [29]. This amplicon was cloned into a derivative of pDM4 with a mutant counter-selectable marker based on the *B. pseudomallei pheS* instead of *sacB*. The resultant plasmid was electroporated into *E. coli* S17-λpir and transferred via conjugation into *B. pseudomallei.* Transconjugants were selected on chloramphenicol-supplemented LB agar plates. Merodiploids arising from chromosomal integration of the suicide plasmid were resolved on LB media 0.1% *p*-chlorophenylalanine using the mutant counter-selectable marker based on the

B. pseudomallei pheS [30]. The *B. pseudomallei* strain isolated was susceptible to kanamycin, gentamicin, and erythromycin as described elsewhere. The zone of inhibition of the deletion mutant was 29 mm for chloramphenicol, 25 mm for kanamycin, 21 mm for gentamicin, 23 mm for erythromycin, as compared to wild type 29, 8, 7, 7 mm respectively. *Burkholderia* strains that are deletion mutants of amrRAB-*opr*A exhibit equivalence virulence WT 1026b and was confirmed in this work.

Deletion mutants of *B. mallei* 24433 and *B. pseudomallei* DD2254 were created by deleting putative fha gene BMAA1756 and BPSS1727. The deletion was constructed by double crossover allelic exchange using S17-λpir-dependent vector pDM4, which carries the negative selectable marker *sacB*. The PCR primer pairs 5′—**CTCGAG**TCGCGTTACGACAAATTCAA-3′, 5′-**CCATGGGGACGCGT**ACGACCTCATTCC TCTGCAT-3′, and 5′-**CGCGTCCCCATGG**CAGTTGCAGACCATTCGAGA-3′, 5′-**ATATG**TTTTGGTTCAGCGCACGA-3′ were used to generate a 578 and 418 base pair product, respectively. A second round of PCR was performed to fuse the two amplicons, thereby producing a 982 base pair amplicon. This PCR product was directionally cloned into pGRV2 and the resulting plasmid was designated p1756 [9]. This plasmid was mobilized into *B. mallei* via biparental mating with *E. coli* S17-1 (p1756), as previously described [31]. Pm was used to counter select *E. coli* and Gm was used to select for transconjugants containing p1756 integrated into the chromosome of *Burkholderia* via homologous recombination. Numerous Gm resistant colonies (SR1::p1756) were obtained and one was used to inoculate LBG broth without antibiotic selection. Twenty-fold dilutions of the overnight culture were spread onto LBG plates with 5% sucrose. To select for bacteria that underwent a second homologous recombination resulting in a deletion of BMAA1756, 28 sucrose resistant colonies were selected and a portion of each colony was used for whole-cell PCR with hlpflank5′ and hlpflank3′ primers to confirm the BMAA1756 deletion (ΔBMAA1756). Based on that, one of the PCR confirmed colonies was arbitrarily chosen and designated as *B. mallei* ΔBMAA1756. The same procedure was used to generate *B. pseudomallei* ΔBPSS1727.

2.7 Adherence and invasion assay

B. pseudomallei and *B. mallei* were grown in LB media and LB media supplemented with 4% glycerol respectively. RAW264.7 cells and A549 cells were grown as per ATCC procedures. RAW264.7 and A549 cells were seeded at 5 x 105 cells/well in 24-well plates and incubated overnight at 37°C with 5% CO2. The following day, bacteria and deletion mutants were diluted to an OD600 of 0.1 in fresh tissue culture medium, and the diluted cultures were added to each well (MOI of 100). Plates were incubated for 2 h, and each well was washed thoroughly with fresh culture medium. When performing the adherence assay, mammalian cells were immediately lysed using 1% Triton X-100. For the invasion assay, cells were incubated an additional 1.5 h in fresh culture medium supplemented with gentamicin (90 mgL-1) before being washed thoroughly with fresh culture medium and lysed with 1% Triton X-100. Lysates for the assays were diluted and plated on LB agar to determine the total colony forming units (CFU) in each well.

2.8 Characterization of fHLP induced cellular phenotype

Confluent monolayers of RAW264.7 macrophages on glass coverslips were treated with purified fHLA of *B. pseudomallei* and co-incubated for 24 h. Coverslips were then washed in sterile PBS before fixing with 4% paraformaldehyde (w/v) in PBS for 15 min. Coverslips were then washed in PBS and immersed in a fresh solution of 13.3 mg/ml ammonium chloride in PBS for 15 minutes at RT, followed by a final wash in PBS. Cells were permeabilised with 0.2% Triton X-100 in PBS for 15 minutes. Staining of the

filamentous actin cytoskeleton was carried out with Alexa Fluor 488 phalloidin (Molecular Probes, Invitrogen). After actin staining, the cover slips were washed 3x5 minutes in PBS, with the first wash containing DAPI (Sigma) to stain the nuclei. Coverslips were briefly immersed 2X in distilled water, mounted onto slides using ProLong Gold Antifade (Molecular Probes, Invitrogen), and visualized on a confocal microscope.

2.9 Mouse immunization and challenge

Purified tHLP protein was used with or without adjuvant as a vaccine against *B. pseudomallei* K96243 (data not shown), *B. pseudomallei* (Ashdown) and *B. mallei* 23344. Groups of five mice (20g) were vaccinated by IP injection (100 µl) with 20µg tHLP alone or with adjuvant (TiterMax Gold) or PBS alone on day 0 with a booster at day 21. Twenty-one days post boost, the animals were challenged via the intranasal route with between 3 x 10^3 and 5 x 10^3 CFU of *B. pseudomallei* K96243 or 1 x 10^5 CFU of *B. mallei* 23344 and monitored daily over 25–31 days for signs of morbidity and mortality. Immunization and bacterial challenge were performed over three independent experiments for *B. pseudomallei* K96243 and two experiments for *B. mallei*. Challenge controls and survivors were euthanized, and the lung, liver, and spleen aseptically removed and individually homogenized in 10ml of PBS for the enumeration of viable bacteria on LB agar plates counted as CFU/organ.

3 Results

3.1 Identification and purification of hemagglutination activity domain proteins in *B. pseudomallei* and *B. mallei*

The genome sequences of ten *B. pseudomallei* strains were searched via BLAST for genes with HA-like domains [32]. BPSS1727 of *B. pseudomallei* K96243 strain and BMAA1727 of *B. mallei* 23344 strain encodes for the HA-like protein (HLP) YP_111733, which shares 98–100% homology with a specific HA-like protein found in all *B. pseudomallei* and *B. mallei* strains sequenced (Figure 1), but shows little to no conservation with HA-like proteins found in other *Burkholderia* species including *B. thailandensis*. The characterization of this protein was initially reported in a patent by Stratilo and Jager (U.S. Patent No. 8,420,090, 2013). The carboxyl terminal end of this protein has homology to other inositol polyphosphate kinase (IPK). The unique structure and lack of homologous proteins in other species suggests that BPSS1727 and BMAA1756 may be functionally unique to *B. pseudomallei* and *B. mallei*.

Analysis of the amino acid sequence revealed a putative signal sequence at the amino terminal end and a hydrophobic region at the carboxy terminal end of HLP. A full-length protein with the natural signal sequence (fHLP) was generated (Figure 1A) as was a truncated version of HLP omitting the signal sequence and the hydrophobic region of the full-length protein (tHLP). These proteins were expressed in *E. coli* and were purified to ~95% homogeneity (Figure 1B and C). It is noted that fHLP run larger than expected 94.3kDA.

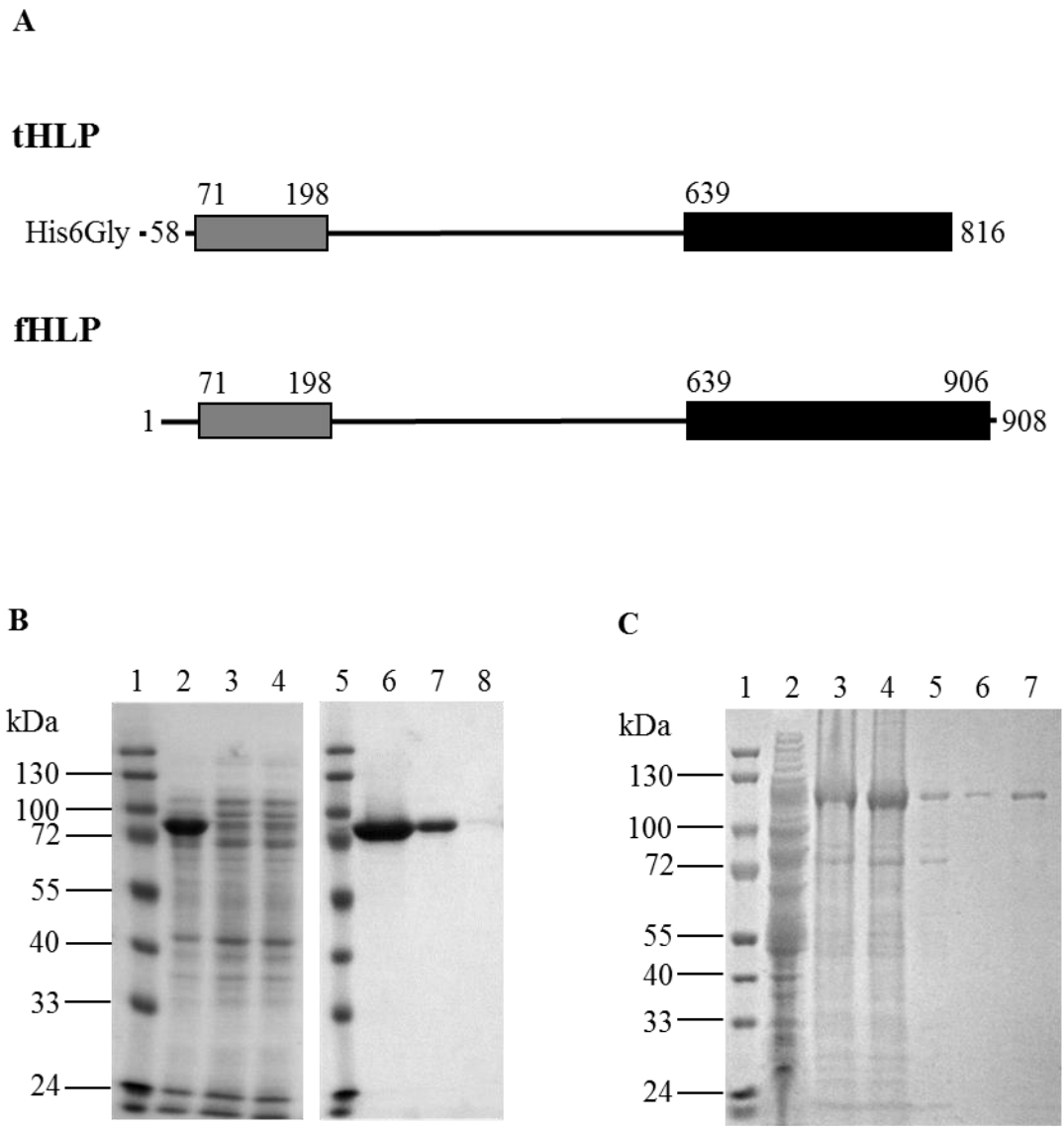

Figure 1: *Depiction of Recombinant proteins tHLP and fHLP and purification of these proteins. (A) Depiction of the tHLP (truncated) and fHLP (full length) recombinant proteins showing the putative hemagglutinin-like domain (shaded grey) and inositol polyphosphate kinase domain (shaded black). (B) Purification of an 81kDa protein corresponding to tHLP was purified to >95% homogeneity using a Nickle NTA column under denaturing conditions (lanes 1&5 = MW standard; lane 2 = start lysate; lanes 3 = pass through: lane 4 wash; lanes 6–8 = eluted tHLP). (C) Purification of an 94.3kDa protein corresponding to fHLP was purified to >90% homogeneity using a heparin column (lane 1 = MW standard; lane 2 = starting lysate; lane 3 = Insoluble pass fraction; lane 4 pass through; lane 5–7 eluted fHLP.*

3.2 HLP-induced actin tails in macrophages

Dowling et al. (2010) identified a gene cluster (BPSS1721-BPSS1735) encoding anti-macrophage virulence factors that produce several cellular phenotypes following infection of macrophages, including the formation of actin tails [33]. To determine whether HLP had any effect on macrophage cellular phenotype, we treated the macrophage cell line RAW264.7 with purified fHLP and then imaged actin and nuclear stained cells on a confocal microscope (Figure 2). Treatment of macrophages with the fHLP alone is sufficient to stimulate actin rearrangement and the formation of actin tails; sham treated cells did not demonstrate these cellular phenotypes. This would identify BPSS1727 as a putative anti-macrophage virulence factor.

Due to homology with other adhesions HLP was investigated for involvement in adhesion and invasion [14, 29–31]. To do so, we created a *B. mallei* mutant lacking BMAA1756 and *B. pseudomallei* lacking BPSS1727. These mutants did not demonstrate significant reduced efficacy in adhesion or invasion assays as compared to wild-type *B. mallei* (data not shown). Pathogenesis comparison between these mutants and wildtype *B. mallei* and *B. pseudomallei* demonstrated a non-significant difference in mean time to death. Deletion mutants had a delay in mean time to death of approximately 3–6 days compared to 3–4 days (data not shown) for control animals. Together, these data suggest that the homologous protein encoded by BMAA1756 and BPSS1727 play a role in actin rearrangement, and the formation of actin tails, but does not play an essential role in adhesion and invasion of host cells by *B. pseudomallei* from the extracellular milieu nor is essential in the pathogenesis of these organisms in a mouse model.

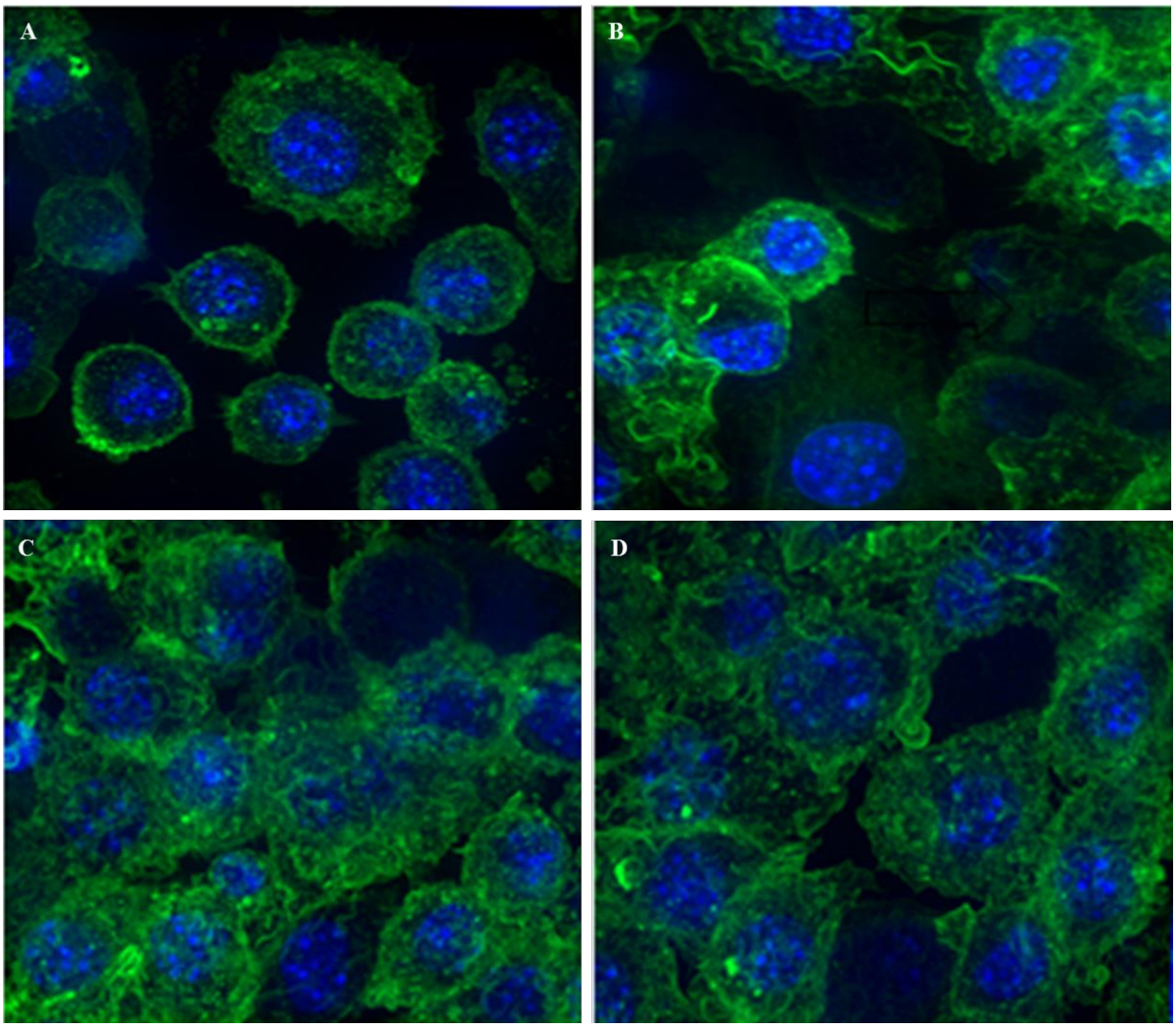

Figure 2: *Cultured macrophages treated with fHLP stimulates the formation of actin tails and actin rearrangement. Confocal microscope images of RAW264.7 cells obtained after a 24 hour incubation with 75ng/ml of fHLP showing the overlay of the actin stain (green) and the DAPI nuclear stain (blue) in (A) sham treated cells and (B–D) fHLP treated cells. Representative images from three experiments.*

3.3 Western blot analysis shows that polyclonal sera against tHLP binds to endogenous HLP *B. pseudomallei* and *B. mallei* protein

Antibodies were generated against recombinant tHLP by injecting the purified soluble protein into mice and collecting sera. To demonstrate that this serum binds to corresponding endogenous antigens, we subjected *B. pseudomallei* and *B. mallei* lysates from infected and uninfected mouse tissues to Western blot analysis (Figure 3). Bands corresponding to the full-length endogenous protein were detected in the lysates and are comparable to the purified fHLP (lane 10) demonstrating that the recombinant protein elicited an immune response specific to the endogenous *B. pseudomallei* and *B. mallei* hemagglutinin-like proteins. It is noted that the native protein does run larger than the predicted 94kDa.

Polyclonal serum was reactive against endogenous HLP produced by *B. pseudomallei*. Polyclonal sera from tHLP-immunized mice bound to tHLP (Figure 4A) and both *B. pseudomallei* and *B. mallei* in an indirect ELISA. (Figure 4B). These data show that mice immunized with tHLP generated polyclonal antibodies that are reactive and specific towards recombinant HLP, and endogenous HLP expressed in cultured bacteria and infected tissues.

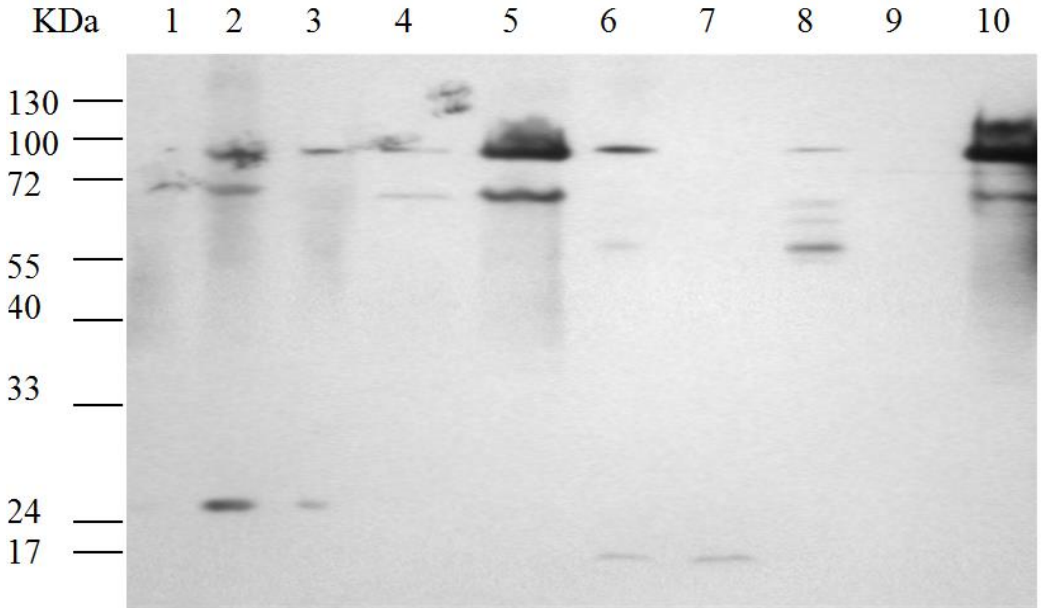

Figure 3*: Western blot analysis of polyclonal antibodies produced against tHLP. Polyclonal antibodies raised against tHLP specifically bind to the corresponding native B. pseudomallei and B. mallei proteins as detected by Western blot analysis. Lysates prepared from infected and uninfected mouse tissues, and cultures of B. pseudomallei and mallei were separated on a 10% NuPage gel and transferred to a nitrocellulose membrane. The membrane was incubated with 1:1000 dilution of protein G purified sera collected from mice immunized with tHLP. Reactive proteins were detected with 1:30000 dilution of goat anti-mouse HRP conjugated antibody and Supersignal West Pico chemiluminescent substrate. Samples were loaded as follows: lane 1 = spleen; lane 2 = liver; lane 3= lungs; lane 4 = brain; lane 5 = B. pseudomallei colony; lane 6 = B. mallei colony; lane 7 = spleen; lane 8 = lungs; lane 9 = brain; lane 10 = purified fHLP. Lanes 1–4 were from infected mice and lanes 7–9 were from uninfected mice. The predicted size of endogenous HLP is ~94 kDa.*

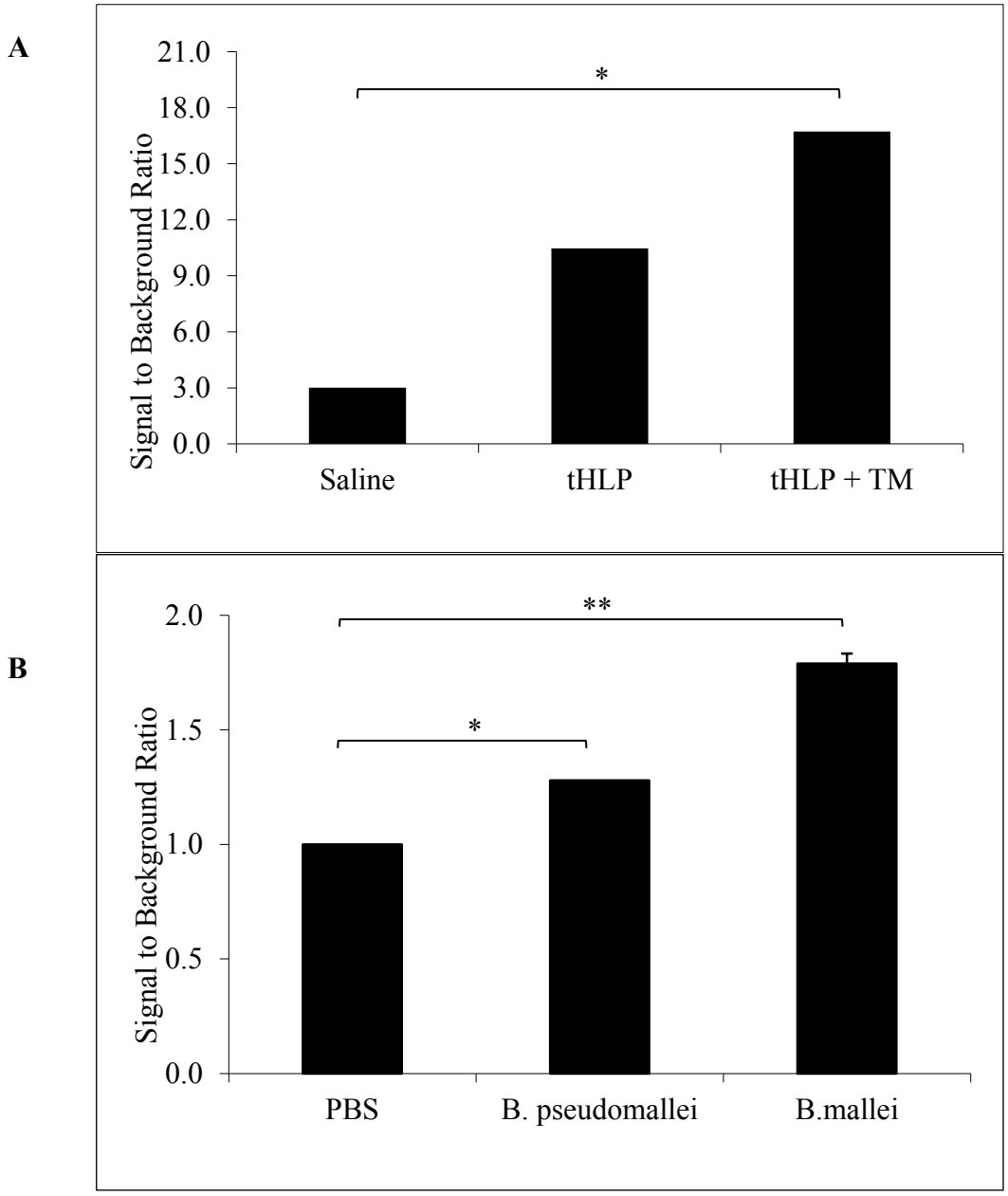

Figure 4: *Polyclonal sera from mice vaccinated with tHLP specifically binds to recombinant and endogenously expressed HLP. ELISA data showing that (A) polyclonal sera from mice vaccinated with tHLPand tHLP with adjuvant Titre Max Gold (TM) identifies recombinant tHLP (* p=0.04), and (B) polyclonal sera from mice vaccinated with tHLP identifies to endogenous HLP expressed by live B. pseudomallei and B. mallei cells (* p=0.007, ** p=0.002). Data expressed as mean plus or minus SEM and analyzed with a Student's t-test.*

3.4 Recombinant HLP provides protection from lethal challenge with *B. pseudomallei*

The recombinant tHLP was used as a component in a vaccine along with adjuvants and administered IP to several groups of mice, followed by a boost at 21 days. Twenty-one days post-boost, the mice were challenged with between 3 x 10^3 and 5 x 10^3 CFUs of *B. pseudomallei* (depending on replicate of experiment) of K96243 or Ashdown strain. When the subunit vaccine was administered with adjuvants, > 80% of the mice survived the lethal challenge, whereas control mice succumbed in ~3 days (Figure 5). The same survival was noted when challenged with K96243 strain of *B. pseudomallei*. Bacterial organ loads at the end of experiment demonstrated that bacteria were still present in the spleens of approximately half of the vaccinated animals (data not shown). The control animals that succumb to the disease had bacteria isolated in their liver, lungs and spleens. When vaccinated mice were challenged with *B. mallei* strain 23344 (1 x 10^5 CFUs), 40 percent of the mice survived asymptomatically until the end of the experiment (data not shown).

These results demonstrate that the protein encoded by BPSS1727 can be formulated into an effective subunit vaccine that is protective against a lethal challenge of *B. pseudomallei*.

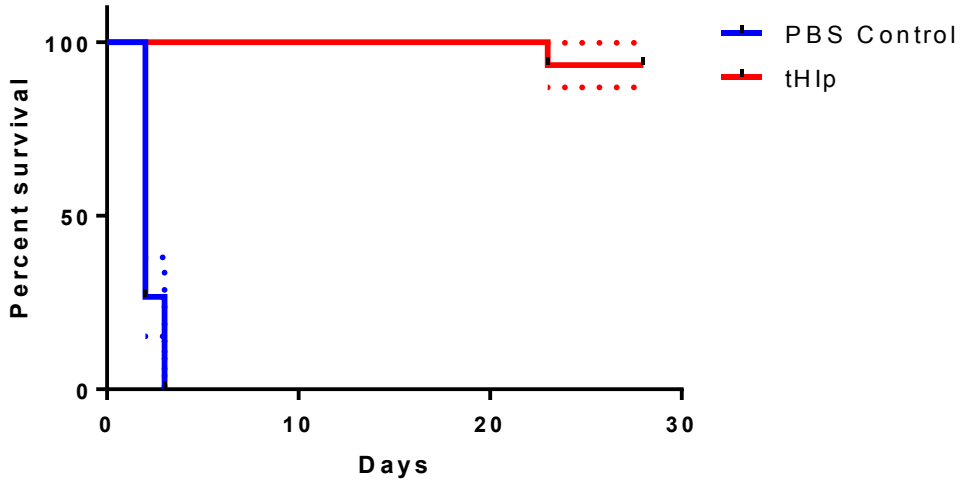

Figure 5: *Survival of BALB/c mice challenged with Burkholderia pseudomallei (3–5 x 10^3 CFUs B. pseudomallei Ashdown). Mice were vaccinated, 21 days later boosted, 21 days after boost they were challenged and monitored for 28 days. Mean of three replicate experiments with STEM shown (dots are standard error). Groups of 5 mice were vaccinated intraperitoneal (IP) and challenged (IN). Group 1 = Mice vaccinated and boosted with phosphate saline (Control); Group 2 = mice vaccinated and boosted with tHLP.*

4 Discussion

In this study, we examined a novel HLP in *Burkholderia* as a potential vaccine. BPSS1727 and its adjacent partner, BPSS1728 share similarities with other TPS systems leading us to hypothesize that the BPSS1727/1728 pair form a novel TPS system. Analysis of the BPSS1728 protein sequence suggests this protein belongs to a family of TpsB transporter proteins that contain a polypeptide-transport-associated (POTRA) domain and an FhaC domain that activates and secretes its cognate partner [34]. This protein shares a high level of homology with hemolysin activator protein precursor HpmB of *Proteus mirabilis* and to hemolysin activator-like protein FhaC of *Bordetella pertussis* precursor, both of which are part of TSP systems. The genome of *B. pseudomallei* has a number of proteins with hemagglutinin-like domains. BPSS2053 was the only *fhaB* gene that has been previously examined and shown to be involved in adhesion and invasion [35].

Several prior studies have briefly looked at BPSS1727 and HLP function, however this study is the first to characterize HLP as a vaccine candidate against *B. pseudomallei/mallei* [33, 36–38]. BPSS1727 was one of 49 antigens identified that were sera diagnostic for melioidosis infection, from screening 747 individual human sera [39]. Polyclonal antibodies produced from tHLP bind to endogenous HLP of *B. pseudomalle*i and *B. mallei* cells as an antigen in an indirect ELISA demonstrating that HLP is present on the surface of live bacterial cells (Figure 4B). The antibody detects the presence of *B. pseudomallei* tissue of infected animals and may be a useful diagnostic reagent, since diagnosis remains to be one of the greatest problems with *B. pseudomallei*/*mallei* infection (Figure 3).

After identifying the highly conserved hemagglutinin-like homologues BPSS1727 (tHLP) from *B. pseudomallei* and BMAA1756 (fHLP) from *B. mallei*, we cloned and expressed these genes in *E. coli* allowing for large-scale isolation of high quality protein (Figure 1). Mice vaccinated with a subunit vaccine containing purified tHLP plus adjuvants were protected (>80%) from a lethal challenge of wild-type *B. pseudomallei*, while control mice succumbed to infection within 3 days (Figure 5). These data demonstrate that tHLP plus adjuvant offered significant protection against a lethal challenge of *B. pseudomallei*.

The function of this protein has not been fully characterized but it is hypothesized that the hemagglutination activity domain of the protein may bind to host cell structure via its heparin binding site [34, 40–43]. Notably, we verified heparin binding of fHLP by purifying the protein on a heparin-sepharose column (data not shown). The IPK domain of HLP may play a role in actin rearrangement and the formation of actin tails. IP3KA activity in human neurons stimulates the formation of branched actin structures and linear actin bundles [44]. We have observed in our studies an increased branched actin structure formation in RAW264.7 macrophages following incubation with purified fHLP (Figure 2). Dowling et al. observed this phenotype when they exposed J774-2 macrophages to crude *E. coli* lysate overexpressing the region of BPSS1720-BPSS1728 [33]. Manipulation of host cell inositol phosphates are utilized by several intracellular bacteria for actin cytoskeletal rearrangement [45–47]. A number of *Burkholderia* proteins have been implicated in actin rearrangement and intercellular spread including BimA, which mediates actin-based mobility by mimicking host ENA/VASP to initiate and elongate actin filaments [48–50]. Expression of BPSS1727/1728 and BimA has been shown to increase inside macrophage cells [36]. The expression of BPSS1727/1728 has similar expression profiles as type six effector proteins (T6SS-5), Bpss1728 has a >20 fold increase expression during growth inside macrophages [36]. T6SS-5 has been shown to be crucial for intercellular spread and the VgrG-5 protein is essential for

membrane fusion and multinucleated giant cell formation [51]. Actin manipulation by *B. pseudomallei* appears to be an essential part of invasion, intracellular and intercellular spread of *B. pseudomallei,* the present results would indicate a possible role for BPSS1727 in actin manipulation [52].

This novel protein, HLP, is found only in *B. pseudomallei* and *B. mallei* as an additional virulence factor that in its truncated forms is an effective vaccine candidate against *B. pseudomallei*. HLP used as part of a polyvalent vaccine with other effective subunit vaccines may offer even greater efficacy against *B. pseudomallei*.

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Burkholderia pseudomallei is the causative agent of melioidosis, a severe systemic, potentially life-threatening disease with many possible manifestations in humans. This pathogen expresses a large number of virulence factors, with many found on islands of pathogenesis. Significant efforts have been invested into vaccine development; however none have offered complete protection. A unique and conserved protein of *B. pseudomallei* and *B. mallei* has been identified as the exoprotein of a two-partner secretion pathway. This exoprotein, when expressed in a recombinant form effected the formation of actin tails in macrophage cell culture, suggesting a role in intracellular spread and a possible role in virulence. A truncated recombinant form of this protein conferred protection against *B. pseudomallei* and *B. mallei* challenge in mice when used as a vaccine candidate.

Burkholderia pseudomallei est l'agent étiologique de la mélioïdose, une maladie systémique grave et potentiellement mortelle qui peut se manifester de bien des façons chez l'humain. Bon nombre de facteurs de virulence entrent en jeu en présence de ce pathogène et apparaissent souvent dans des îlots de pathogénicité. Des efforts considérables ont été déployés en vue de mettre au point un vaccin; cependant, aucun n'a encore réussi à assurer une protection complète. Néanmoins, une protéine conservée unique de *B. pseudomallei* et de *B. mallei*a été identifiée comme l'exoprotéine d'une voie de sécrétion à deux partenaires. Exprimée sous forme recombinante, cette exoprotéine a exercé une influence sur la formation de queues d'actine dans des cultures cellulaires macrophages, ce qui donne à penser qu'elle pourrait jouer un rôle dans la propagation intracellulaire et possiblement dans la virulence. Dans le cadre d'un test de provocation effectué avec *B. pseudomallei* et *B. mallei*, une forme recombinante tronquée de cette protéine a été utilisée comme vaccin potentiel et a permis d'assurer une protection à des souris.