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Identification of an OmpW homologue in *Burkholderia pseudomallei*, a protective vaccine antigen against melioidosis

William T. Casey[a,1], Natasha Spink[b], Felipe Cia[b], Cassandra Collins[a], Maria Romano[c], Rita Berisio[c], Gregory J. Bancroft[b], Siobhán McClean[a,*]

[a] *Centre of Microbial Host Interactions, ITT Dublin, Tallaght, Dublin 24, Ireland*
[b] *Department of Immunology and Infection, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, United Kingdom*
[c] *Institute of Biostructures and Bioimaging, National Research Council, Via Mezzocannone 16, I-80134 Naples, Italy*

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ABSTRACT

Burkholderia pseudomallei is the causative agent of melioidosis, which is associated with a range of clinical manifestations, including sepsis and fatal pneumonia and is endemic in Southeast Asia and Northern Australia. Treatment can be challenging and control of infection involves prolonged antibiotic therapy, yet there are no approved vaccines available to prevent infection. Our aim was to develop and assess the potential of a prophylactic vaccine candidate targeted against melioidosis. The identified candidate is the 22 kDa outer membrane protein, OmpW. We previously demonstrated that this protein was immunoprotective in mouse models of *Burkholderia cepacia* complex (Bcc) infections. We cloned *Bp_ompW* in *Escherichia coli*, expressed and purified the protein. Endotoxin free protein administered with SAS adjuvant protected Balb/C mice (75% survival) relative to controls (25% survival) ($p < 0.05$). A potent serological response was observed with IgG2a to IgG1 ratio of 6.0. Furthermore C57BL/6 mice were protected for up to 80 days against a lethal dose of *B. pseudomallei* and surpassed the efficacy of the live attenuated 2D2 positive control. *BpompW* is homologous across thirteen sequenced *B. pseudomallei* strains, indicating that it should be broadly protective against *B. pseudomallei*. In conclusion, we have demonstrated that BpOmpW is able to induce protective immunity against melioidosis and is likely to be an effective vaccine antigen, possibly in combination with other subunit antigens.

1. Introduction

Burkholderia pseudomallei is a Gram negative bacterium that is classed as a category B biothreat and the cause of melioidosis, a tropical disease endemic in Southeast Asia and Northern Australia. It is reported to be responsible for 20% of all community acquired pneumonias in Northeastern Thailand [1] but is also recognised as causing fatal infections in other regions [2]. Infection can occur via inhalation, ingestion or skin abrasions. Melioidosis has a wide range of clinical presentations from acute to chronic infection. Despite antibiotic therapy, melioidosis is a life-threatening infection, with fatality rates of 43% in Thailand [3]. A fifth of infections reported in Thailand are among children under 14 years with an overall mortality of 51% [4].

In contrast, *Burkholderia cepacia* complex (Bcc), a group of 20 species [5–7], generally causes chronic life-threatening infections in CF patients and certain immunocompromised populations. Both *B. pseudomallei* and Bcc are inherently difficult to treat due to multidrug resistance, the ability to form biofilms, intracellular lifestyle and chronic infection stages in the host [8]. Both organisms have been shown to survive and replicate inside macrophages [9,10]. Prevention of infection via vaccination represents a more effective way to protect susceptible populations than antibiotics, yet there are no approved vaccines for any *Burkholderia* species to date. Pre-clinical studies indicate that protective immunity against *B. pseudomallei* may be induced by live attenuated immunogens [11], although concerns about the potential for reversion to virulence of live vaccines mean they are unlikely to be approved for use in humans [12].

Several candidates for subunit vaccines against *B. pseudomallei* have also been tested. Previous studies with highly conserved *B. pseudomallei* type III secretion system proteins BipB-N, BipC-C and BipD failed to act as protective antigens, due to the low expression of these proteins during infection [13]. Immunisation with two

* Corresponding author.
E-mail address: Siobhan.mcclean@ittdublin.ie (S. McClean).
[1] Present address: School of Biomolecular & Biomedical Sciences, University College Dublin, Belfield Dublin 4, Ireland.

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OmpA proteins showed protection (50% survival at 21 days) in mice [14] while immunisation with an OMP85 protein resulted in up to 70% protection for 15 days [15]. Several other subunit antigens have been evaluated, as recently reviewed [16], with two antigens in particular, LolC and Hcp2, independently showing efficacy, resulting in 80% survival at 42 days [8,17,18].

We have previously shown that Bcc protein, OmpW, was involved in attachment of *B. cenocepacia* and *B. multivorans* to host epithelial cells and was a protective vaccine antigen in mice challenged with either species [19]. An OmpW homologue in *B. thailandensis*, was identified as immunoreactive during immunoproteomic analysis [20]. *B. thailandensis* shares 94% identity with *B. pseudomallei* at the amino acid level and is widely used as a surrogate for *Bp* in studies [20]. *B. pseudomallei* OmpW (BpOmpW, Locus tag BPSL1552) shared 89% homology with the protective Bcc OmpW antigen [21] and is among the most abundant proteins expressed by *B. pseudomallei*, representing 5.88% of total outer membrane proteins [22]. Given the homology between these antigens and the close similarities between these organisms, we wanted to examine the efficacy of this antigen against *B. pseudomallei*.

2. Material and methods

2.1. Generation of *pRSET_*ompW *expression vector*

Genomic DNA of *B. pseudomallei* K96243 was provided by Richard Titball, University of Exeter. The *ompW* gene was amplified by PCR with the oligonucleotide primer pair: 5′ gatcggatccggcttcgcccggagagggcat and 3′ agtcaagcttctagaacttcatcccgacgc, incorporating a *Bam*HI restriction site and a *Hin*dIII site respectively. The resultant product was 0.58 kb in length and excluded the native signal peptide sequence. The purified *ompW* PCR product was ligated into the linear pGEM-T Easy vector system, according to the manufacturer's specifications (Promega, Southampton, UK). Ligation was transformed into *E. coli* TOP10 cells and screened by blue/white screening on LB plates containing (Isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.1 mM) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL, 20 μg L^{-1}). The pGEM_*ompW* plasmid was isolated and digested with *Bam*HI and *Hin*dIII, as was the expression vector pRSET_B (Invitrogen, California, USA). The digested *ompW* fragment was then ligated to the linearised pRSET vector. The resultant plasmid, pRSET_*ompW*, was transformed into *E. coli* TOP10 (Invitrogen). The construct was confirmed to be correctly orientated and in frame with the N-terminal coded His-tag, by DNA sequencing (Eurofins, Dublin, Ireland).

2.2. Expression of BpOmpW

The pRSET_B_*ompW* construct was introduced into *E. coli* BL21 STAR (Invitrogen) cells by heat shock transformation and successful transformants were selected by carbenicillin resistance (50 mg L^{-1}). *E. coli* BL21 STAR pRSET_B_*ompW* cells were grown in 1 L LB supplemented with carbenicillin (50 mg L^{-1}) at 37 °C on an orbital shaker at 200 rpm. Cells were induced with 1 mM IPTG at OD_{600} of 0.5 and incubated for a further 16–24 h.

2.3. Purification of BpOmpW by affinity chromatography

Cells were harvested by centrifugation at 3220 × *g* for 10 min. Pellets were re-suspended in 3 ml lysis buffer (50 mM potassium phosphate, pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole) and disrupted using a sonic dismembrator with four 10 s bursts. The lysates were centrifuged at 50,000 × *g*. OmpW was purified from crude lysate using HisPur Ni-NTA columns (Thermo Scientific, Loughborough, UK). Due to poor protein solubility, 8 M urea was added to all buffers to maintain BpOmpW solubility throughout. Protein purity was analysed by SDS-PAGE (12% acrylamide) [23]. Gels were stained with Coomassie Brilliant Blue overnight or were transferred to methanol pre-soaked nitrocellulose membranes by semi-dry blotting at 25 V for 50 min for western blotting. Membranes were blocked with 5% Marvel® in 0.05% PBST for 1 h at RT and washed with PBST before incubating overnight at 4 °C with HRP conjugated anti-His tag antibody in the blocking solution. Membranes were washed and incubated in luminol (Millipore) substrate for 5 min before exposing to photographic film.

2.4. Endotoxin removal from BpOmpW preparation

Endotoxin was removed using the HisPur Ni-NTA column system and an adapted washing protocol [24], involving cell lysis in the presence of 6 M guanidine-HCl, followed by washing twice with consecutive sodium phosphate/tris buffers containing 8 M urea. Organic washes containing isopropanol were applied to the column three times. The columns were washed with 1% TFA in endotoxin free water followed by elution using 250 mM imidazole, 8 M urea. Fractions were analysed by SDS-PAGE and those containing pure BpOmpW dialysed against 50 mM Tris–HCl buffer pH 7. Densitometric analysis of gels was performed using ImageJ software (http://imagej.nih.gov/ij/download.html).

2.5. Protein characterisation

Circular dichroism (CD) analysis was performed on 200 μg of purified protein at concentrations of 0.2 mg ml^{-1} in 50 mM Tris–HCl. Protein identity was confirmed with bands excised from SDS-PAGE gels and in-gel trypsin digested, analysed by MALDI-TOF Mass spectrometry (Bruker, Coventry, UK) and compared to known peptides via the MASCOT database [25]. Endotoxin levels of protein solutions were routinely assayed using the Limulus Amebocyte Lysate based endotoxin detection kit (Lonza, Basel, Switzerland) according to manufacturer's specifications.

2.6. Immunisation of mice

Female Balb/c or C57BL/6 mice (6–8 week-old; Harlan Laboratories, Bicester, Oxon, UK) were used throughout the studies. Groups of mice were given free access to food and water and subjected to a 12 h light/dark cycle. For challenge, the animals were handled under ACDP bio-safety containment level 3 conditions. All animal experiments were performed in accordance with the guidelines of the Animals (Scientific Procedures) Act of 1986 and were approved by the local ethical review committee at the London School of Hygiene and Tropical Medicine. Mice were vaccinated intraperitoneally (ip) with 50 μg of BpOmpW and either SAS (Sigma) or alum (Sigma) as adjuvant, or adjuvant alone or saline alone at week 0 with a booster of same at week 4. A group vaccinated with *B. pseudomallei ilv1* deletion mutant 2D2 (10^6 CFU) at week 4 acted as a positive control for vaccine-mediated protection. Serum samples (100 μl) were withdrawn from three mice per group for serology at week 9. The remaining mice ($n = 8$) were infected via ip injection with *B. pseudomallei* 576 (6×10^5 or 4×10^6 CFU for BALB/c and C57BL/6, respectively) at week 12 or week 9 respectively and were monitored for survival. The doses used were chosen so as to generate an acute lethal infection within 7 days in either model. For each infection, aliquots were thawed from frozen bacteria stocks and diluted in pyrogen-free saline (PFS). They were observed at least once daily and any animal reaching defined humane endpoints and deemed incapable of survival were humanely killed by cervical dislocation.

Table 1
BlastP comparison of *B. multivorans* OmpW (GI:161524680) against selected *B. pseudomallei* strains.

B. pseudomallei Strain	Score	Expect	Identities	Positives	Gaps
K96243	330 bits (845)	3e−114	161/211(76%)	181/211(85%)	0/211(0%)
668	328 bits (840)	1e−113	160/211(76%)	180/211(85%)	0/211(0%)
1710b	319 bits (818)	2e−110	154/193(80%)	174/193(90%)	0/193(0%)

2.7. Determination of antigen-specific IgG by ELISA

Microtitre plates were coated with purified endotoxin-free BpOmpW (20 ng/ml, dialysed against Tris–HCl, pH 7) in sodium bicarbonate buffer (pH 9.4) at 4 °C overnight. Coating solution was removed and plates were blocked with 10% BSA solution in PBS at RT for 1 h. Wells were washed with 0.05% Tween 20 in PBS with agitation of the plates. Serum samples were serially diluted (4-fold) in PBS containing 1% BSA and 100 μl aliquots added to wells in triplicate at RT for 2 h. Plates were washed four times as described above with PBS 0.05% Tween 20 before addition of anti-mouse IgG1, IgG2a or IgG2b HRP-conjugated antibodies (1:10,000 in 1% BSA) at RT for 2 h. After washing, TMB substrate was added and incubated in the dark until the colour developed. Reactions were stopped with 0.5 M H_2SO_4 and plates read at 450 nm. Serum antibody titres were defined as end-point titres, i.e. the reciprocal of the highest dilution of serum producing an OD above the cut-off value, where the cut-off values was determined as the OD of the corresponding dilution of control sera plus three standard deviations.

2.8. Statistical analysis

Log-Rank tests of survival data were performed using the GraphPad Prism software version 5.01 (GraphPad Software, San Diego California), $^*P < 0.05$.

3. Results

3.1. Selection of OmpW target protein

The OmpW of *Burkholderia multivorans* ATCC 17616 specifically designated as GI:161524680 (Refseq accession NC_010084.1) is a 216 amino acid protein encoded by the 651 bp gene, Bmul_1507). The protein sequence of GI:161524680 was compared to *B. pseudomallei* by BlastP to identify the most highly similar homologue among sequenced *B. pseudomallei* strains (Table 1). *B. pseudomallei* K96243 was identified as possessing an OmpW homologue (GI:52209600) with the highest similarity to the identified *B. multivorans* protein. K96243 strain is also routinely used as the standard *B. pseudomallei* and consequently, it was selected as the source strain for heterologous expression of recombinant *B. pseudomallei* OmpW (BpOmpW) in *E. coli*. In order for BpOmpW to be a subunit antigen with the potential to be broadly protective across *B. pseudomallei*, it is important that it is expressed across the many strains in the species. Homologues of OmpW were compared by BlastP comparison together with Clustal W2 alignment and Phobius analysis across the 13 sequences *B. pseudomallei* strains. The gene is located in chromosome 1 of all strains. Blast P comparisons showed a very high degree of sequence identity and conservation with ≥99 across all 13 strains (Table 2).

3.2. Recombinant BpOmpW was soluble in the presence of urea

Pellets from induced cultures of *E. coli* BL21 STAR pRSET_B_*ompW* were analysed by SDS-PAGE with the target protein identified at 24 to 25 kDa. The N-terminal tag results in a protein size of 22.5 kDa, but as has been previously shown, OmpW exhibits an upward shift during SDS-PAGE [26] and was routinely identified at 24–25 kDa (Fig. 1). In the presence of urea, a clear protein band was evident (Fig. 1A) while in the absence of urea, the protein resulted in considerably smearing on the gels (data not shown) indicating that urea is required for solubilisation of the target protein. Purified BpOmpW was positively identified by MALDI-T of MS in the sample, with high sequence coverage of 43%.

3.3. BpOmpW purification requires rigourous washing to ensure endotoxin removal

Standard affinity purification on HisPur columns of supernatants from *E. coli* BL21 pRSET_*ompW* induced cultures did not sufficiently remove endotoxin. Endotoxin removal is essential to ensure that the protein can safely be administered to mice and moreover that the responses are specific to the protein and not to LPS. To overcome endotoxin contamination, a modified rigorous washing procedure [24] was utilised during affinity purification. BpOmpW purified by this alternative method was visualised by SDS-PAGE gels following imidazole elution (Fig. 1B) and was also found to have endotoxin levels <25 EU/ml. Densitometric analysis indicated that BpOmpW had 92% purity. Full preservation of the protein structural integrity was observed after this procedure using circular dichroism (data not shown).

Table 2
Comparison of OmpW homologues among the 13 sequenced *B. pseudomallei* strains showing chromosome location, size, presence of signal peptide and BlastP result.

B. pseudomallei strain	Theoretical size (kDa)	Signal peptide	Number of homologues	Protein BLAST comparison		
				Cover (%)	Identity (%)	Positives (%)
K96243	22	Yes	4	100	100	100
1106a	22	Yes	5	100	100	100
668	22	Yes	5	100	99	99
1710b	20	No	4	90	100	100
BPC006	20	No	5	90	100	100
MSHR146	20	No	5	90	100	100
MSHR305	20	No	5	90	100	100
MSHR511	20	No	5	90	100	100
MSHR520	20	No	5	90	100	100
NAU20B-16	20	No	5	90	100	100
NCTC 13178	20	No	5	90	100	100
NCTC 13179	20	No	5	90	100	100
1026b	30	Yes	3	100	100	100

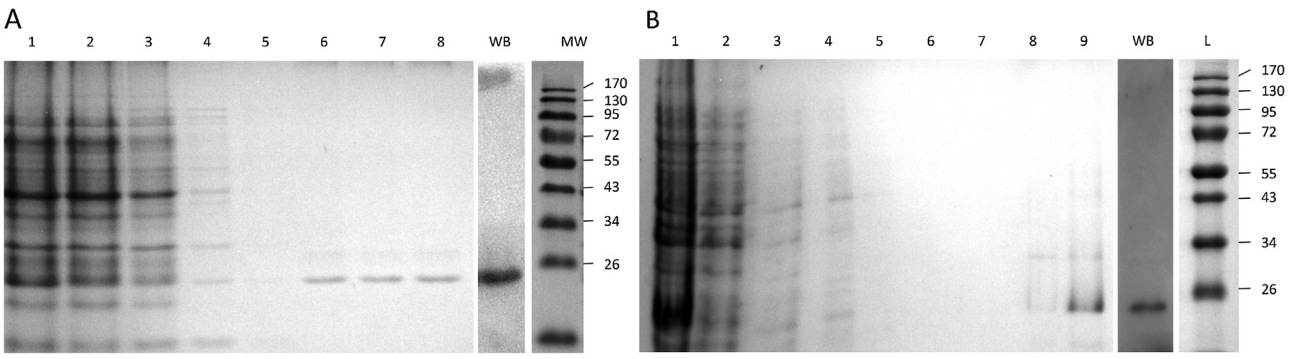

Fig. 1. Analysis of purified BpOmpW. (A) Urea based purification procedure of BpOmpW: Lane 1, cell free extract, Lane 2, HisPur column flow through, lanes 3–5, wash 1–3, Lanes 6–8, elutions 1–3; WB, western blot identification of purified BpOmpW. (B) Optimised purification for endotoxin removal: Lane 1, guanidine HCl buffer wash; lane 2–4, urea buffer washes; lanes 5–7, organic (isopropanol) washes; lane 8, 1% TFA pre-elution; lane 9, 250 mM imidazole elution showing purified protein band at 24 kDa. WB, western blot identification of purified BpOmpW. MW: molecular weight marker showing approximate protein size in kDa.

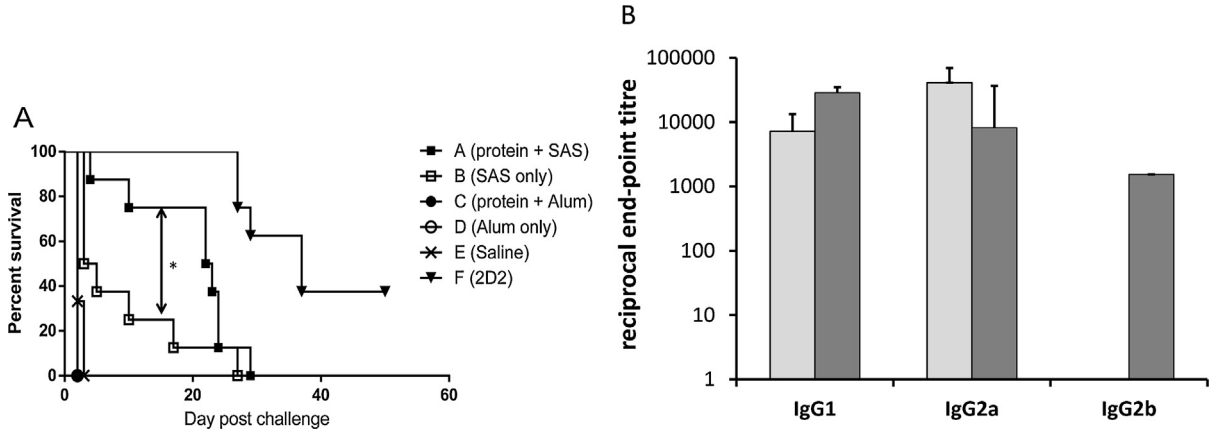

Fig. 2. Immunological responses to BpOmpW. Female BALB/c mice ($n = 11$) were i.p. vaccinated with either BpOmpW + SAS adjuvant (■), SAS only (□), BpOmpW + alum adjuvant (●), alum only (○), saline (×) or *B. pseudomallei* 2D2 mutant (▼). Mice were vaccinated twice at four week intervals followed by an eight week rest period, after which mice were challenged i.p. with 6×10^5 CFU *B. pseudomallei* 576. (A) Survival ($n = 8$); (B) Antigen-specific immunoglobulin reciprocal end-point titres as determined by ELISA from sera taken from mice ($n = 3$ per group) prior to challenge, light bars: BpOmpW with SAS; dark bars BpOmpW with alum. $^*p < 0.05$.

3.4. *BpOmpW administered with SAS as adjuvant protects against acute lethal* B. pseudomallei *infections in two mouse models*

Following a highly acute lethal challenge with *B. pseudomallei*, 75% of BALB/C mice immunised with BpOmpW with SAS as adjuvant survived up to 21 days as compared with only 12.5% control mice in the SAS treated group ($p < 0.05$), indicating that BpOmpW afforded significant protection against *B. pseudomallei* challenge (Fig. 2A). Antibody titres in immunised mice prior to challenge were determined from four independent ELISAs, relative to adjuvant controls for titre cut-off and showed strong serological responses in both BpOmpW immunised groups (Fig. 2B). The IgG2a to IgG 1 ratios in response to BpOmpW administered with SAS is indicative of a strong Th-1 response (ratio IgG2a to IgG1 = 6.0), while the serology of BpOmpW and alum group demonstrated a strong Th-2 response (IgG2a to IgG1 ratio = 0.3). No IgG2b antibody response was observed for the BpOmpW + SAS group, suggesting that IgG2a and IgG1, rather than IgG2b, contribute to vaccine-induced protection. In order to confirm that the protection by this protein is not restricted to a single MHC haplotype, we evaluated the response in the genetically resistant C57BL/6 mouse model [27]. When challenged, BpOmpW also afforded long protection over 80 days in 75% of C57BL/6 mice. This compares well with those vaccinated with the attenuated 2D2 vaccine which afforded 0% protection by day 35. Only 12.5% of the SAS only treated control mice survived to day 80 (Fig. 3).

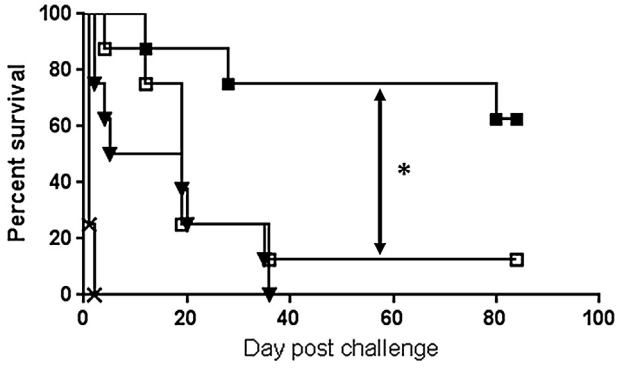

Fig. 3. Protection of C57BL/6 with BpOmpW. Survival of C57B/6 mice immunised i.p. with BpOmpW + SAS adjuvant (■), SAS only (□), saline (×) or *B. pseudomallei* 2D2 mutant (▼). Mice were vaccinated twice at four week intervals followed by a 5 week rest period before i.p. challenge with 4×10^6 CFU *B. pseudomallei* 576. $^*p < 0.05$.

4. Discussion

Subunit antigens represent a safe means of immunising against bacterial infections such as *B. pseudomallei*, however only a limited number of subunit antigens have been evaluated [8,28]. Using a proteomic approach, we previously showed that OmpW was involved in attachment of Bcc to lung epithelial cells, was immunoreactive in patients colonised with Bcc and protected mice

against Bcc challenge [19]. OmpW is also involved in colonisation and host responses to unrelated Gram negative bacteria [29]. It is involved in the attachment of *Vibrio cholerae* to mouse intestine [26] and OmpW showed considerable antigenicity in *Salmonella enterica* Enteritidis [8]. Due to the homology between BpOmpW and Bcc OmpW and the demonstrated immunoreactivity of OmpW in *B. thailandensis* infected animals [20], we wanted to examine whether BpOmpW might be protective against *B. pseudomallei*. We have successfully shown protection against an acute lethal infection of *B. pseudomallei* in both BALB/c and the more resistant C57BL/6 mice. In particular, 75% of C57BL/6 mice showed sustained protection over 80 days and compared well against the 2D2 strain, which is a stringent benchmark for a subunit antigen. Direct comparisons between preclinical studies are difficult, due to different dosing protocols. Despite this, BpOmpW showed comparable protection against acute i.p. challenge in BalbC mice than relative to other subunit antigens previously examined, for example LolC (80% survival at day 42) [17] or Omp85 (70% survival at day 15) [15] and performed better in terms of the longer time over which protection was sustained.

There is considerable similarity between experimental melioidosis and human disease. Transcriptomic analysis of murine infections and blood from infected patients demonstrated substantial commonality in the genes that are upregulated in response to infection in mice compared with human melioidosis, particularly in genes associated with interferons, neutrophils, B cells and T cells [30]. It has been well accepted that protection against *B. pseudomallei* relies on a cell-mediated immune response [8], which may explain why SAS was a much more effective adjuvant compared with alum in triggering BpOmpW induced protection in BALB/c mice. The SAS adjuvant, an MPLA/Trehalose dicorynomycolate emulsion, is a potent immunostimulatory activator which triggers a Th-1 response, triggering a potent cellular protective against *B. pseudomallei*. Although the cellular immune response was not examined in this study, the serological response (IgG2a/IgG1 = 6.0) suggested SAS stimulated a strong Th-1 response. Although SAS will need further evaluation as a vaccine adjuvant, given its amphiphilic composition, it is most likely particularly suited to membrane protein antigens.

The ability of OmpW to protect against *B. pseudomallei* and against Bcc is interesting, and the potential for cross-protection with other shared homologous proteins will need further evaluation. More “crossover” studies on both organisms are required. Importantly, BpOmpW alone protected two independent mouse models, with distinct MHC haplotypes, against melioidosis. Since combinations of several efficacious subunit antigens in a polyvalent vaccine are likely to be more effective than single subunit vaccines [28], the combination of BpOmpW with additional antigens may lead to an even more efficacious vaccine against *B. pseudomallei*.

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