



# Functional Activities of O-Polysaccharide and Hemolysin Coregulated Protein 1 Specific Antibodies Isolated from Melioidosis Patients

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**ABSTRACT** Melioidosis is a fatal tropical disease caused by the environmental Gram-negative bacterium, *Burkholderia pseudomallei*. This bacterium is intrinsically resistant to several antibiotics and treatment of melioidosis requires prolonged antibiotic administration. To date, there are no vaccines available for melioidosis. Previous studies have shown that humoral immunity is critical for surviving melioidosis and that O-polysaccharide (OPS) and hemolysin coregulated protein 1 (Hcp1) are important protective antigens in animal models of melioidosis. Our previous studies revealed that melioidosis patients had high levels of OPS- and Hcp1-specific antibodies and that IgG against OPS (IgG-OPS) and Hcp1 (IgG-Hcp1) were associated with patient survival. In this study, we characterized the potential function(s) of IgG-OPS and IgG-Hcp1 from melioidosis patients. IgG-OPS and IgG-Hcp1 were purified from pooled serum obtained from melioidosis patients using immuno-affinity chromatography. Antibody-dependent cellular phagocytosis assays were performed with pooled serum from melioidosis patients and compared with serum obtained from healthy controls. Serum from melioidosis patients significantly enhanced *B. pseudomallei* uptake into the human monocytic cell line THP-1 compared with pooled serum from healthy donors. Enhanced opsonization was observed with IgG-OPS and IgG-Hcp1 in a dose-dependent manner. Antibody-dependent complement deposition assays were performed with IgG-OPS and IgG-Hcp1 using flow cytometry and showed that there was enhanced C3b deposition on the surface of *B. pseudomallei* treated with IgG-OPS but to a lesser degree with IgG-Hcp1. This study provides insight into the function of IgG-OPS and IgG-Hcp1 in human melioidosis and supports that OPS and Hcp1 are potential vaccine antigens for immunization against melioidosis.

**KEYWORDS** *Burkholderia pseudomallei*, O-polysaccharide, Hcp1, antibody function, ADCP, ADCD, vaccine, melioidosis, antigen, opsonization

Melioidosis, caused by the Gram-negative bacterium *Burkholderia pseudomallei*, is commonly associated with community acquired septicemia in regions of endemicity. The disease is typically found in tropical countries and is especially prevalent in northeast Thailand and northern Australia (1, 2). Worldwide, there is estimated to be 165,000 cases of melioidosis per year and 89,000 deaths (3). Clinical manifestations of melioidosis range from mild infections to severe sepsis. The majority of melioidosis patients present with bacteremia and pneumonia. In northeast Thailand and Southeast Asia, mortality rates can be as high as 40 to 50% (4, 5).

*B. pseudomallei* is an encapsulated bacterium and is intrinsically resistant to several antibiotics. Prolonged regimens that include intravenous antibiotics such as ceftazidime or carbapenems followed by oral trimethoprim-co-trimoxazole are required for

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the treatment of melioidosis. Although, vaccines that stimulate capsule-specific antibody responses are effective for other encapsulated bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae* type b, and *Neisseria meningitidis* (6–8), there are currently no vaccines available for protection against melioidosis. Therefore, there is a need to identify potential antigens for vaccine development.

O-polysaccharide (OPS) and hemolysin coregulated protein 1 (Hcp1) are important virulence factors expressed by *B. pseudomallei* and are considered to be promising vaccine candidates (9–11). OPS is a component of lipopolysaccharide (LPS) located on outer membrane of bacteria. Hcp1 is a protein component of the cluster 1 type VI secretion system (T6SS) that plays a role in the intracellular lifestyle of *B. pseudomallei* (9, 12–14). Both OPS and Hcp1 are recognized by the immune systems of melioidosis patients (15). Our previous studies demonstrated by ELISAs that melioidosis patients produced high levels of IgG against OPS and Hcp1 antigens (16, 17). OPS induced high levels of IgG1 and IgG2 subclasses while Hcp1 predominantly induced high levels of IgG1 (18).

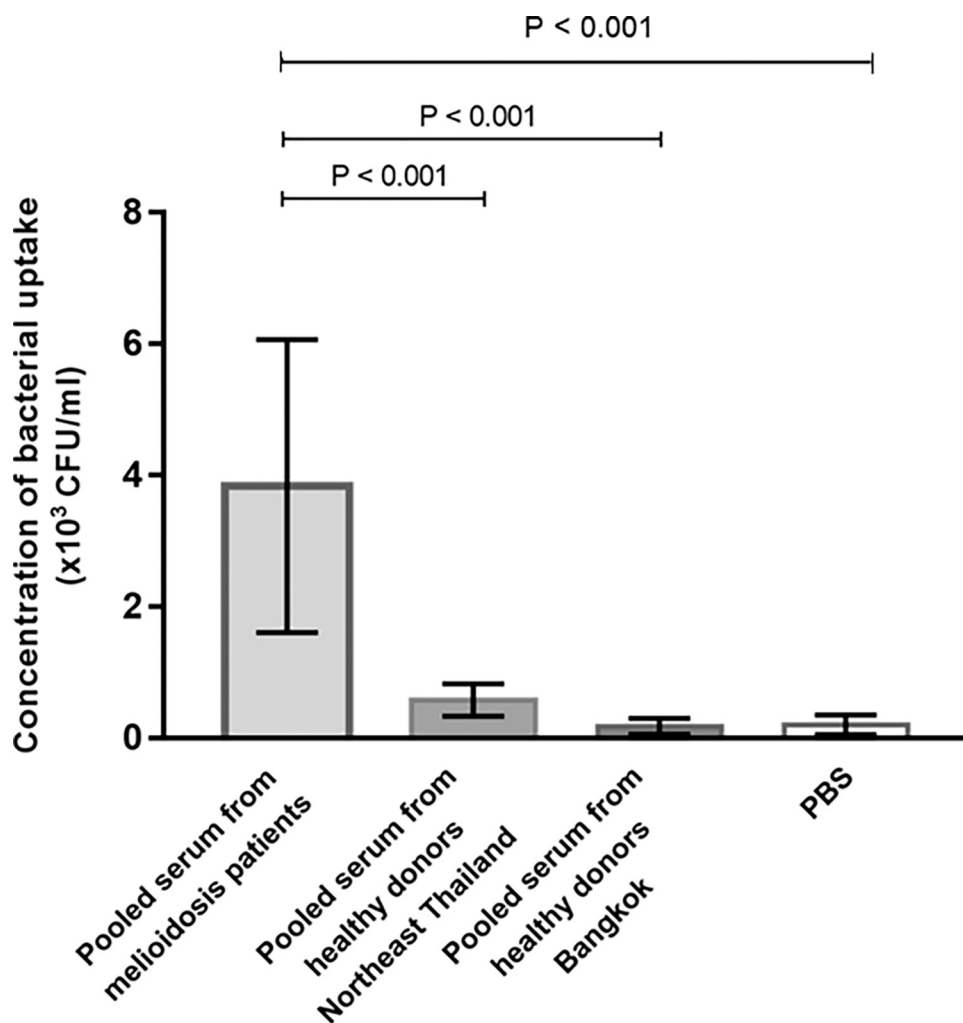
Many studies in animal models have demonstrated the association between antibody levels and protection from melioidosis but the mechanisms of protection have not been well investigated (10, 19–22). A study in human melioidosis showed a lower mortality rate was associated with seropositivity against crude *B. pseudomallei* antigen preparations (23). We previously reported an association of survival with high levels of IgG against OPS and Hcp1 in melioidosis patients (18, 24) suggesting a potential functional role for these antibodies in protection against disease (25). In addition, Chaichana et al. demonstrated that serum from survivors of melioidosis enhanced bacterial uptake compared to serum from nonsurvivors (25). The same study demonstrated that purified IgG against whole-cell antigen promotes antibody-dependent cellular phagocytosis (ADCP). However, this study did not characterize what antigen-specific antibodies in the patient serum samples were associated with the ADCP (26).

We hypothesized that specific IgG antibodies against OPS (IgG-OPS) and Hcp1 (IgG-Hcp1) in human melioidosis cases could contribute to enhanced phagocytosis and complement deposition on *B. pseudomallei*. The aim of this study was to evaluate the roles of IgG-OPS and IgG-Hcp1 in ADCP and antibody-dependent complement deposition (ADCD) assays. We purified IgG antibodies from pooled serum obtained from melioidosis patients and healthy donors using immuno-affinity chromatography. We evaluated the ADCP activities of IgG-OPS and IgG-Hcp1 for their ability to promote bacterial uptake and survival in THP-1 cells. ADCD associated with IgG-OPS and IgG-Hcp1 was also determined by assessing C3b deposition on *B. pseudomallei* K96243 via flow cytometry. Results of these studies indicated that in both instances higher activity was associated with IgG-OPS compared to IgG-Hcp1.

## RESULTS

**Serum from human melioidosis patients enhances bacterial uptake into THP-1 cells.** The opsonophagocytic activity of pooled serum from melioidosis patients and healthy controls was determined using THP-1 cells. We observed that pooled melioidosis serum at dilution of 1:10 significantly enhanced bacterial uptake into THP-1 cells compared with pooled serum from healthy donors from endemic and non-areas of endemicity. Results showed that mean concentrations  $\pm$  standard deviation (SD) of bacterial uptake for pooled melioidosis serum was  $3.83 \pm 2.30 \times 10^3$  CFU/mL, pooled healthy donor serum from areas of endemicity was  $0.58 \pm 0.25 \times 10^3$  CFU/mL and pooled healthy donor serum from non-areas of endemicity was  $0.18 \pm 0.12 \times 10^3$  CFU/mL (Fig. 1). We examined live/dead THP1 cells using an inverted light microscope, but we did not observe the difference in live/dead cells among cells incubated with different serum groups and PBS control.

**Purification and analysis of IgG-OPS and IgG-Hcp1 antibodies.** IgG-OPS and IgG-Hcp1 samples were obtained from pooled melioidosis patient serum using a two-step approach. The total amount of IgG-OPS and IgG-Hcp1 isolated from 400 mL of the pooled patient serum (290 mL for IgG-OPS and 110 mL for IgG-Hcp1) was 3.92 mg and 6.25 mg, respectively. As expected, SDS-PAGE analysis of the pooled and depleted patient serum samples demonstrated a complex mixture of proteins. In contrast, the purified total Ig and

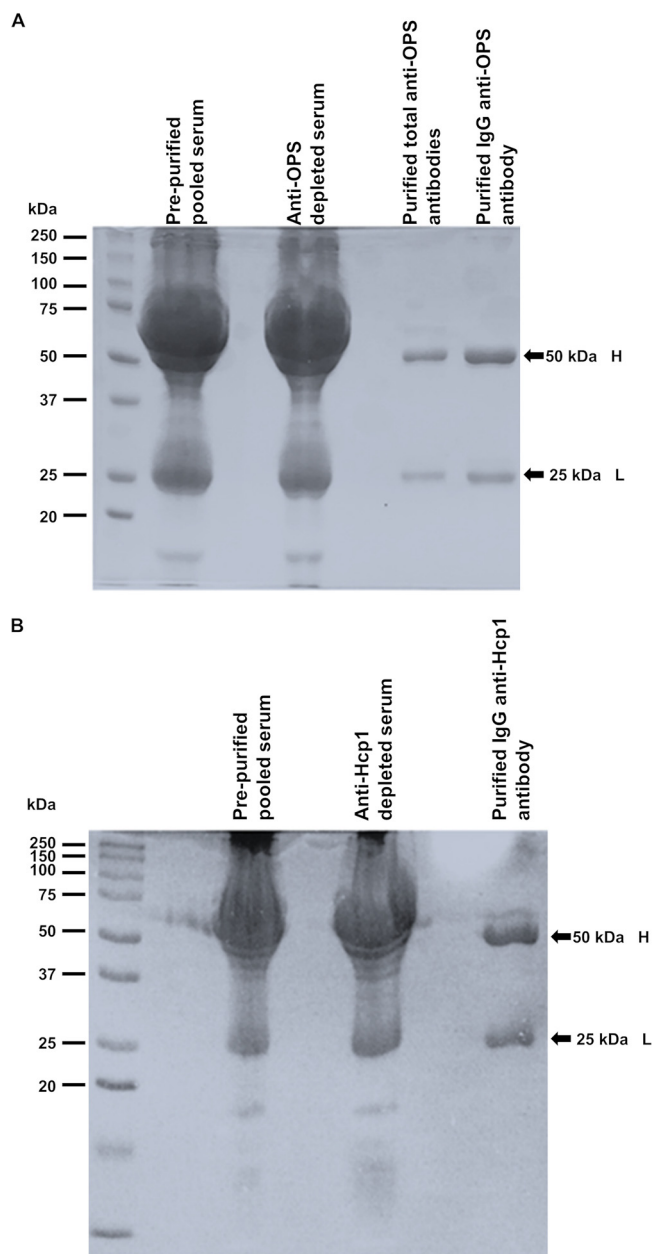


**FIG 1** Antibody-dependent cellular phagocytosis (ADCP) activity from pooled serum from melioidosis patients compared to healthy donors from Northeast Thailand, healthy donors from Bangkok and PBS control in THP-1 cells. The ADCP activity was determined by colony count method to enumerate the live bacteria in THP-1 cells. Three-independent experiments were performed.

IgG fractions revealed the presence of two major bands of ~50 kDa and ~25 kDa (Fig. 2). These observations are consistent with the molecular weights associated with IgG heavy and light chains in their reduced forms, respectively.

We next evaluated the reactivity of the various pooled serum samples and purified IgG fractions by ELISA. To facilitate these studies, all samples were adjusted to 1  $\mu$ g/mL of protein. As expected, analysis of the prepurified serum samples demonstrated that they had strong reactivity with OPS or Hcp1 while noticeable decreases in reactivity were observed for their paired depleted samples (Fig. 3). Interestingly, depletion of total Ig-OPS from its paired prepurified sample appeared to be more efficient than depletion of total Ig-Hcp1 from its paired prepurified sample with the OPS depleted sample reaching reactivity levels similar to the negative control. Importantly, however, these analyses indicated that we were successful in isolating highly purified OPS- and Hcp1-reactive IgG for use functional assays.

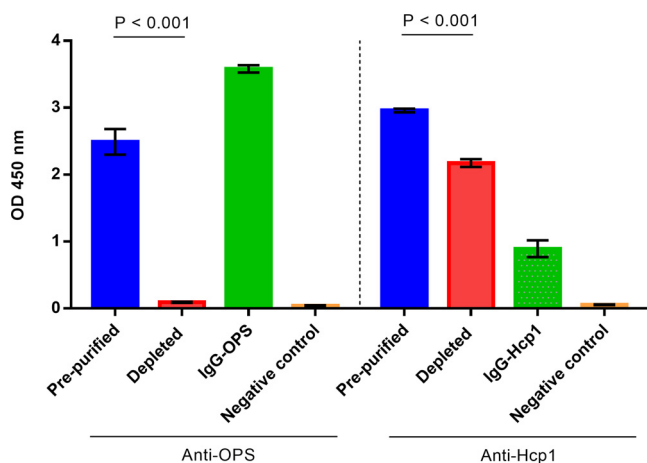
**IgG-OPS and IgG-Hcp1 antibodies enhance ADCP activities.** To assess the functional activity of IgG-OPS and IgG-Hcp1, we used human leukemic monocyte cell line THP-1 to conduct ADCP assays. Results of these studies demonstrated significantly higher opsonizing activity associated with IgG-OPS compared to PBS and the IgG control at all concentrations tested except for 0.008 mg/mL (Fig. 4). ADCP testing of IgG-Hcp1 also



**FIG 2** SDS-PAGE and Coomassie blue staining of prepurified pooled serum, depleted serum, purified anti-OPS antibodies, purified IgG anti-OPS and purified IgG anti-Hcp1. SDS-PAGE was performed on 14% gel. The separated proteins were stained with Coomassie blue. Size of proteins were compared to protein markers (kDa). Numbers on the left represent molecular weight markers. (A) Prepurified pooled serum, anti-OPS depleted serum, purified anti-OPS antibodies, and purified IgG anti-OPS. (B) Prepurified pooled serum, anti-Hcp1 depleted serum, and purified IgG anti-Hcp1. H, heavy chain; L, light chain.

showed significantly higher activity than PBS and the IgG control but lower activity than IgG-OPS (Fig. 4) for IgG-Hcp1 concentrations of 0.157 and 0.078 mg/mL. IgG-OPS antibodies at concentrations of 0.157 and 0.078 mg/mL enhanced bacterial uptake by approximately 3 times compared to the IgG-Hcp1 antibody at the same concentrations ( $P = 0.026$  and  $P = 0.002$ , respectively). We did not observe the difference in live/dead cells among THP1 cells incubated with various IgG concentrations and PBS control. These results suggest IgG-OPS and IgG-Hcp1 enhance ADCP activities in human monocytic cells.

**IgG-OPS enhances C3b deposition on *B. pseudomallei* K96243.** To study antibody function in enhancing complement deposition on the bacterial surface, C3b deposition



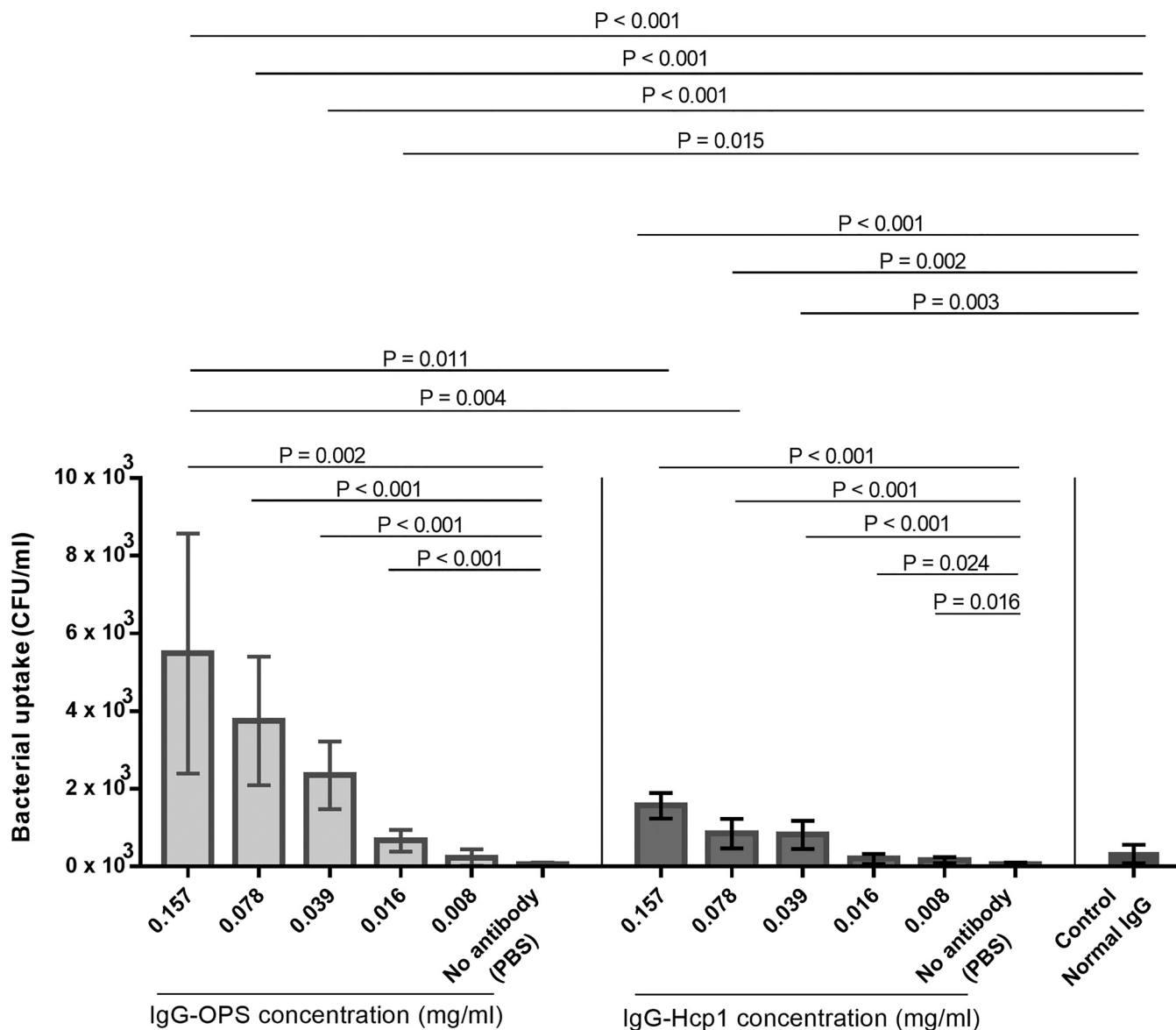
**FIG 3** Antibody activity of pre-purified pooled serum, depleted serum, purified IgG anti-OPS (IgG-OPS) and purified IgG anti-Hcp1 (IgG-Hcp1) against OPS or Hcp1 antigen. The antibody activity was determined by ELISA using serum concentration at 1  $\mu\text{g}/\text{mL}$  and antigen concentration at 2.5  $\mu\text{g}/\text{mL}$  for Hcp1 and 1.0  $\mu\text{g}/\text{mL}$  for OPS. Negative control was pooled serum samples from healthy donors in non-area of endemicity. Two independent experiments were performed.

was assessed by flow cytometry and is represented as mean fluorescent index (MFI) (Fig. 5). MFI of *B. pseudomallei* and IgG-OPS or IgG-Hcp1 incubated with fresh healthy donor serum (fresh serum) as a source of complement was compared with bacteria incubated with heat-inactivated serum (HI) or PBS as controls. Results demonstrated significantly higher C3b deposition on *B. pseudomallei* K96243 incubated with IgG-OPS and fresh serum (MFI, 7806), compared with the incubation of bacteria with HI (MFI, 3768) or PBS (MFI, 6.45) (fresh serum versus HI,  $P < 0.001$ ) (Fig. 5A and C). A similar pattern, but considerably lower MFI, was observed with *B. pseudomallei* treated with IgG-Hcp1 and fresh serum. The MFI of K96243 and IgG-Hcp1 incubated with fresh serum, HI, and the PBS control were 1483, 1167 and 6.45, respectively (fresh serum versus HI,  $P = 0.02$ ) (Fig. 5B and C). Collectively, these results demonstrate that IgG-OPS is superior to IgG-Hcp1 in promoting the deposition of C3b on the surface of *B. pseudomallei*.

## DISCUSSION

Currently, there is no vaccine for melioidosis. Understanding humoral immune responses against different *B. pseudomallei* antigens is important for vaccine development. In this study, we purified and characterized the functional activities of specific antibodies against two vaccine candidate antigens, OPS and Hcp1, using pooled serum samples from acute melioidosis patients and healthy donors living in Northeast Thailand (area of endemicity) and in Bangkok (non-area of endemicity). We observed that the ADCP activity of pooled serum from acute melioidosis patients for *B. pseudomallei* were significantly higher than the activity of serum obtained from healthy donors. We successfully purified specific IgG antibodies against OPS and Hcp1 antigens from acute melioidosis patient serum and healthy donors. In human melioidosis, we observed that ADCP activity of both antigen-specific IgGs were correlated with concentrations in dose-dependent manner. We also showed that the functional activities of purified IgG-OPS from melioidosis patient serum was significantly greater than the activity of IgG-Hcp1.

Our ADCP results in this study confirmed findings from a previous report by Chaichana et al. that serum from melioidosis patients can enhance opsonization of *B. pseudomallei* in a THP-1 cell derived macrophage model (25). Although a follow-up study by Chaichana et al. demonstrated that high levels of total IgG2 and the expression of Fc $\gamma$ R11a-H/R131, an intermediate-affinity IgG2 receptor, were associated with protection against death in acute melioidosis (26), they did not identify the specific target antigen for IgG2 as they used whole cells of *B. pseudomallei* in their assay. Herein, we purified IgG against OPS and Hcp1 antigens and demonstrated that these specific antibodies promoted phagocytosis and

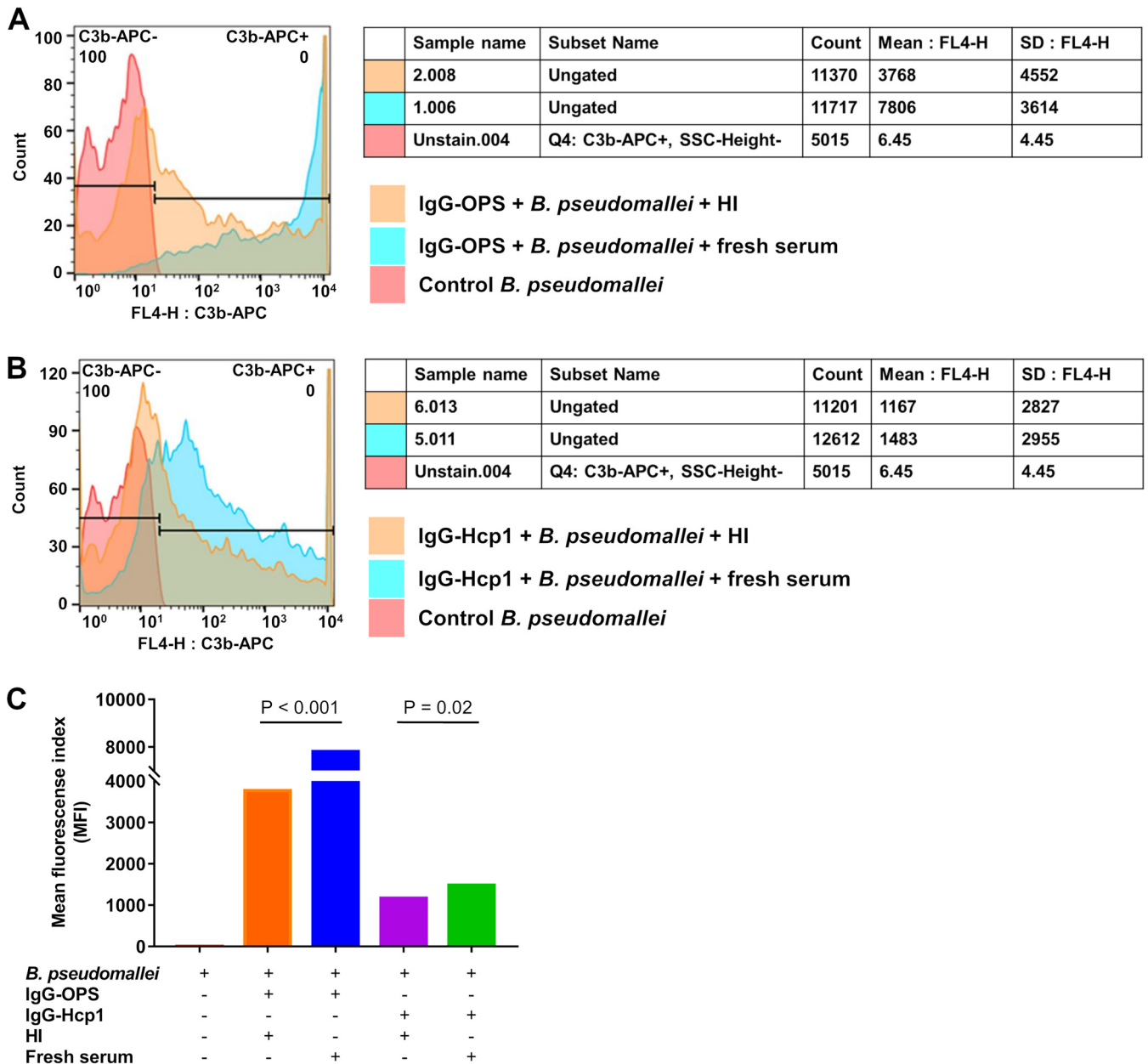


**FIG 4** Antibody-dependent cellular phagocytosis (ADCP) activity of purified IgG anti-OPS (IgG-OPS) and IgG anti-Hcp1 (IgG-Hcp1) at different concentrations. PBS and IgG from healthy donors (normal IgG) were controls. The ADCP activity was determined by colony count to enumerate live bacteria in THP-1 cells. Three-independent experiments were performed.

complement fixation. The results of this study suggest that IgG-OPS enhanced phagocytosis of and C3b deposition on *B. pseudomallei* K96243 compared to IgG-Hcp1. This might be explained by the nature of polysaccharide (OPS) versus protein antigen (Hcp1) that induced different subclasses of IgG antibodies. Indeed, our previous study showed that IgG1 and IgG2 were major subclasses of IgG antibody against OPS whereas IgG1 was a major IgG subclass for Hcp1 in serum samples from acute melioidosis patients (17). It is possible that these antibodies enhance bacterial internalization and cells-to-cells spreading but the bacteria were not killed by the host cells due to the inability of *B. pseudomallei*-infected macrophages to produce IFN- $\gamma$  and IFN- $\beta$ . However, a study has demonstrated that stimulation of infected macrophages with either of these interferons can result in increased iNOS expression and reduced intracellular survival of *B. pseudomallei* (27).

ADCD can be associated with both classical and alternative complement pathways. It has been previously reported that the capsule of *B. pseudomallei* can prevent C3b deposition on the surface of bacteria (28). However, our study indicates an important role for IgG-OPS in C3b deposition on *B. pseudomallei* and suggests that high levels of antibodies may





**FIG 5** Antibody-dependent complement deposition (ADCD) of *B. pseudomallei* K96243 surface opsonized with IgG anti-OPS (IgG-OPS) and IgG anti-Hcp1 (IgG-Hcp1). ADCD was assessed by the measurement of complement component C3b on the surface of target cells. A fresh healthy donor serum sample was used as a complement source. Negative controls were heat-inactivated serum sample (HI) (no complement) and unopsonized *B. pseudomallei* K96243. C3b deposition on bacterial cells was examined by probing with allophycocyanin (APC) conjugated anti-human C3b. Complement deposition was detected using flow cytometry and data are shown as histograms and mean fluorescence index (MFI). (A) Deposition of C3b complement component on *B. pseudomallei* K96243 opsonized with IgG-OPS. (B) Deposition of C3b complement component on *B. pseudomallei* K96243 opsonized with IgG-Hcp1. (C) Data are presented as mean fluorescence index (MFI). Three-independent experiments were performed.

play a role in controlling *B. pseudomallei* infections. The role of specific antibodies for complement activation against other bacterial and viral infections have been demonstrated previously, for example, in *Neisseria meningitidis* serogroup B (29), *Escherichia coli* O111B4 (30), *Salmonella* Typhi (31), immunodeficiency virus (HIV) antibodies (32). These studies show human monoclonal antibodies to specific antigens are most efficient in cell lysis and C3 deposition on infected cells (32). However, we did not measure the role of antibodies beyond the C3b deposition, and this requires further study.

Several studies have demonstrated the protective capacity of OPS-specific monoclonal and polyclonal antibodies in animal models of melioidosis (20, 33, 34). Studies have also

shown that antibodies against *B. pseudomallei* LPS II (also known as type A LPS) are significantly higher in patients who survived than in those who died as well as in patients with nonsepticemic versus septicemic melioidosis (24). Furthermore, studies have described correlations between OPS- and Hcp1-specific IgG subclasses and improved outcomes for melioidosis patients (11, 18). Our current study represents the first of its kind to demonstrate specific functional activities associated with affinity-purified human OPS- and Hcp1-specific antibodies which helps to better establish correlates of antigen-induced immunity against *B. pseudomallei*. Thus, this study provides valuable insights toward the development of OPS- and Hcp1-based vaccines to combat melioidosis.

There were some limitations to this study. We did not use fresh serum samples from melioidosis patients therefore antibody functions may be partly degraded during long-term storage. It is known that a number of healthy people who live in areas of endemicity have high antibody levels against *B. pseudomallei* culture filtrate antigen (CFA) (35, 36). This study did not test the differences in functional activity of specific antibodies due to the limited amounts of serum samples. Further studies are required to investigate the potential role(s) of antibodies against OPS and Hcp1 in healthy individuals.

In conclusion, the results of this study confirm that melioidosis patients have high levels of antibodies to OPS and Hcp1 and suggest the functional roles of purified human IgG specific for these antigens enhances bacterial uptake into human monocyte cells and C3b deposition on the *B. pseudomallei* K96243 cell surface. These results provide insights into the functional activities of antibodies against these two potential vaccine candidates and supports using OPS and Hcp1 antigens as targets for vaccine development.

## MATERIALS AND METHODS

**Serum preparation.** Left-over acute and convalescent-phase sera from 100 melioidosis patients who were culture positive for *B. pseudomallei* were pooled and used for purification of OPS- or Hcp1-specific antibodies. A total of 400 mL of pooled serum was obtained from melioidosis patients. A total of 3.5 mL of pooled serum from 7 healthy donors in Udon Thani in northeast Thailand (an area of endemicity of melioidosis) and 2.5 mL of pooled serum from 5 healthy donors in Bangkok (non-area of endemicity of melioidosis) were used as controls. The serum samples were passed through gauze and then filtered through 0.20  $\mu$ m filters (Acrodisc, PR, USA).

**Coupling of *B. pseudomallei* OPS to UltraLink Hydrazide resin.** To facilitate purification of specific antibodies against *B. pseudomallei* OPS, type A LPS was extracted from the select agent excluded strain *B. pseudomallei* RR2808 using a modified hot phenol method (37, 38). Purified OPS antigens were then obtained by acid hydrolysis and size exclusion chromatography essentially as previously described (37, 39). The OPS was then oxidized with sodium metaperiodate as previously described (37) and conjugated to UltraLink Hydrazide resin (Thermo Scientific, USA) via reductive amination following the manufacturer's instructions. The resulting OPS-coupled resin was stored at 4°C until use.

**Coupling of Hcp1 to UltraLink Biosupport resin.** We have previously reported that *B. pseudomallei* Hcp1 and *B. mallei* Hcp1 are 99.4% identical at the amino acid level (e.g., they differ by only one amino acid; [17]). Furthermore, studies in our lab have demonstrated that *B. pseudomallei* Hcp1 and *B. mallei* Hcp1 can be used interchangeably as ELISA coating antigens and that human immune serum samples react similarly with both proteins. To facilitate the purification of Hcp1-specific antibodies, recombinant *B. mallei* Hcp1 was purified from *E. coli* as previously described (17). The purified Hcp1 was then coupled to UltraLink Biosupport resin (Thermo Scientific, USA) following the manufacturer's instructions. The resulting Hcp1-coupled resin was stored at 4°C until use.

**Purification of OPS- and Hcp1-specific antibodies from pooled human melioidosis serum.** To purify OPS- or Hcp1-specific antibodies from human immune serum, the pooled serum was diluted 1:1 with binding buffer, 0.5  $\times$  BupH PBS pH 7.2 (Thermo Scientific, USA) for anti-OPS and 0.6 M sodium citrate and 0.1 M MOPH (pH 7.5) for anti-Hcp1. The buffered serum was then passed over 1 mL of the OPS or Hcp1 coated beads which were preequilibrated with binding buffer, washed with 15 resin volumes of binding buffer following which the antibodies were stripped from the resin with elution buffer, 100 mM glycine-HCl (pH 2.5) for anti-OPS and 100 mM glycine, 2% acetic acid (pH 2.2) for anti-Hcp1. The eluate was collected into a 1/10 volume of 1 M Tris pH 9.0. The sample was then dialyzed against multiple changes of 0.5  $\times$  BupH<sup>+</sup> PBS for anti-OPS and 20 mM Sodium phosphate, pH 7.0 for anti-Hcp1. Precipitates were removed by centrifugation and the filter sterilized material was passed over Protein G Hi-Trap columns (GE Healthcare Bio-Sciences, Sweden) to isolate purified IgG.

**Purification of IgG-OPS and IgG-Hcp1.** Protein G columns (GE Healthcare Bio-Sciences, Sweden) were used to purify IgG-OPS or IgG-Hcp1 from total anti-OPS or total anti-Hcp1 antibodies. Collection tubes were prepared by adding 60  $\mu$ L of 1 M Tris-HCl, pH 9.0 per mL for each fraction to be collected. The columns were washed with 10 column volumes of binding buffer, 20 mM Sodium phosphate (pH 7.0) at 1 mL/min. A total of 5 mL sample was applied to the columns followed by washing with 10 mL of binding buffer. The flow through



from the samples (anti-OPS IgG or anti-Hcp1 IgG depleted samples) was then collected. IgG-OPS or IgG-Hcp1 were eluted with 10 column volumes of elution buffer, 0.1 M Glycine-HCl (pH 2.7) and collected as 10 individual fractions. The purified fractions were buffered exchanged by dialysis against PBS. Normal human serum IgG used as IgG controls were prepared from 3.5 mL pooled serum samples from 7 healthy donors from Northeast Thailand and 5 healthy donors from Bangkok Thailand. The protein concentrations were determined using a BCA protein assay kit (Pierce, Thermo Fisher Scientific, USA). The yield of IgG was 7 mL with concentration of 3.947 mg/mL. The protein purity was determined by SDS-PAGE and Coomassie blue staining as previously described by Laemmli et al. (40). SDS-PAGE was performed with 14% gel.

**Determination of antibody activity.** The reactivity of serum and IgG against OPS and Hcp1 antigens was determined by Enzyme-linked Immunosorbent assay (ELISA) as previously described (16, 17). Samples were diluted in assay diluent and assays performed in duplicate. Briefly, 50  $\mu$ L of 2.5  $\mu$ g/mL of Hcp1 or 1.0  $\mu$ g/mL of OPS was coated on 96 microwell plate (Nunc, Sigma-Aldrich, Germany) at 4°C overnight. The plate was washed with PBS containing 0.05% Tween, then blocked with skim milk at 37°C for 2 h. 50  $\mu$ L of the concentration of 1  $\mu$ g/mL of prepurified serum, IgG-OPS, IgG-Hcp1, depleted serum, and negative control (pooled serum from healthy donors) were added and the plate was incubated at RT for 30 min. After washing with PBS, 50  $\mu$ L of 1:2000 dilution of HRP-conjugated rabbit anti-human IgG (Dako) was added and incubated at RT for 30 min. The plate was washed and the color was developed using 50  $\mu$ L of 3,3',5,5' tetramethylbenzidine (TMB) with peroxidase (Novex, Life Technologies, MD, USA). The reaction was quenched using 1N HCl. The absorbance was measured at an optical density (OD) of 450 nm using microplate reader (Sunrise, Tecan, Switzerland).

**Preparation of human monocytic cell line THP-1.** The opsonizing activities of purified IgG-OPS and IgG-Hcp1 antibodies from melioidosis patients were determined using the human monocytic cell line THP-1 (ATCC TIB 202). The THP-1 cells were cultivated in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco, MS, SA) with 10% fetal bovine serum (10% FBS) and incubated at 37°C with 5% CO<sub>2</sub> for 2 days prior to use. Cells were harvested, washed twice with RPMI by centrifugation at 200  $\times$  g for 5 min, and counted. The cells were diluted in RPMI to obtain a concentration of 1  $\times$  10<sup>6</sup> cells/mL and incubated at 37°C with 5% CO<sub>2</sub> before use in ADCP assays.

**Bacterial culture and opsonization.** To prepare bacterial inoculums, *B. pseudomallei* strain K96243 from a frozen stock was cultured on LB agar and incubated at 37°C overnight. Isolated colonies were picked, inoculated in 20 mL of LB broth and incubated with shaking at 37°C, 200 rpm overnight. The bacteria were diluted with LB broth and adjusted to OD<sub>600</sub> nm = 0.18 to obtain a concentration of 5  $\times$  10<sup>6</sup> CFU/mL. All experiments involving *B. pseudomallei* were performed in a biosafety level 3 laboratory.

Purified antibodies (IgG-OPS and IgG-Hcp1) were diluted with sterile PBS to concentrations of 0.157, 0.078, 0.039, 0.016, and 0.008 mg/mL. Purified IgG controls from healthy donors were diluted in PBS to 0.157 mg/mL. Pooled serum samples were diluted with RPMI at 1:5 before use.

**Antibody-dependent cellular phagocytosis (ADCP) assay.** ADCP assays were performed using THP-1 cells in suspension and centrifuged during the washing steps at multiplicity of infection (MOI) of 5 bacteria per cell (CFU/cell). A total of 200  $\mu$ L of *B. pseudomallei* K96243 at 1  $\times$  10<sup>6</sup> CFU/mL were incubated with 20  $\mu$ L of diluted antibody in PBS or diluted pooled serum in RPMI (5  $\mu$ L serum in 45  $\mu$ L RPMI) in a 1.5-mL microtube (Axygen, Corning, USA) at 37°C for 1 h. The opsonized bacteria were then washed with RPMI twice by centrifugation at 4,000  $\times$  g for 15 min. The pellet was resuspended with 220  $\mu$ L of RPMI, then transferred into 96-well U bottom plate with THP-1 cells and incubated at 37°C with 5% CO<sub>2</sub> for 2 h. Each sample was performed in triplicate (25, 26).

Opsonized *B. pseudomallei* K96243 was prepared as described above and incubated with THP-1 cells for 2 h. Extracellular bacteria were removed by washing with RPMI 3 times. THP-1 cells were treated with 500  $\mu$ g/mL of kanamycin for 2 h. Live and dead THP1 cells were examined using an inverted light microscope. The cells were washed 3 times with PBS and 1 mL of 0.1% Triton X-100 (Thermo Fisher Scientific, USA) in PBS was added and incubated at RT for 10 min. Cell lysates were diluted in PBS and 10  $\mu$ L was dropped on LB agar. The plates were incubated at 37°C for 24–48 h to determine colony counts (41).

**Antibody-dependent complement deposition (ADCD) assay.** ADCD was assessed by measuring deposition of complement component C3b on the surface of target cells as previously described (42). *B. pseudomallei* K96243 was incubated with 0.784  $\mu$ g/mL of IgG-OPS or IgG-Hcp1 antibodies at 37°C for 30 min. The opsonized bacteria were washed with PBS. A fresh healthy donor serum sample diluted in veronal buffer (1:10 dilution) was added as a complement source and incubated at 37°C for 30 min, then washed with 15 mM EDTA in PBS. Heat-inactivated serum (56°C for 30 min) was used as a negative control. C3b deposition on bacterial cells was probed with allophycocyanin (APC) conjugated anti-human C3b, clone: 1H8 (Mouse IgG2a) (Cedarlane, CA) at RT for 15 min in the dark and fixed with 4% paraformaldehyde. Complement deposition was detected using flow cytometry (FACSCalibur flow cytometer, Becton, Dickinson, CA). ADCD assays were performed in triplicate. Data were analyzed and shown as histograms and mean fluorescence index (MFI).

**Statistical analysis.** The data were analyzed using GraphPad Prism software version 7.0 (GraphPad Software Inc, La Jolla, CA). The data are presented as bar graphs with mean  $\pm$  standard deviation (SD). Student's *t* test was used to compare the quantitative data between two groups. One-way ANOVA were used to compare the quantitative data of three or more groups. Two or three independent experiments were performed. A statistically significant difference was *P* value < 0.05.

**Ethics statement.** This study was approved by the ethics committee of Faculty of Tropical Medicine, Mahidol University (MUTM 2019-035-03) and the biosafety committee of the Faculty of Tropical Medicine, Mahidol University (MU 2021-010). The study was conducted under the principles of Declaration of Helsinki (2008) and Good Clinical Practice (GCP) guidelines. Written informed consent was obtained from all participants or their relatives.

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A.P. and S.P. performed the experiments. A.P. and N.C. analyzed and interpreted data and wrote the manuscript. N.C. and P.J.B. designed experiments and were responsible for funding acquisition, project administration, and supervision. M.N.B., P.J.B., and N.C. reviewed and edited manuscript.

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## REFERENCES

1. Chaowagul W, White NJ, Dance DA, Wattanagoon Y, Naigowit P, Davis TM, Looareesuwan S, Pitakwathana N. 1989. Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand. *J Infect Dis* 159: 890–899. <https://doi.org/10.1093/infdis/159.5.890>.
2. Currie BJ, Fisher DA, Anstey NM, Jacups SP. 2000. Melioidosis: acute and chronic disease, relapse and re-activation. *Trans R Soc Trop Med Hyg* 94: 301–304. [https://doi.org/10.1016/S0035-9203\(00\)90333-x](https://doi.org/10.1016/S0035-9203(00)90333-x).
3. Limmathurotsakul D, Golding N, Dance DA, Messina JP, Pigott DM, Moyes CL, Rolim DB, Bertherat E, Day NP, Peacock SJ, Hay SI. 2016. Predicted global distribution of *Burkholderia pseudomallei* and burden of melioidosis. *Nat Microbiol* 1:15008. <https://doi.org/10.1038/nmicrobiol.2015.8>.
4. Limmathurotsakul D, Wongratanaheewin S, Teerawattanasook N, Wongsuvan G, Chaisuksant S, Chetchotisakd P, Chaowagul W, Day NPJ, Peacock SJ. 2010. Increasing incidence of human melioidosis in Northeast Thailand. *Am J Trop Med Hyg* 82:1113–1117. <https://doi.org/10.4269/ajtmh.2010.10-0038>.
5. Currie BJ. 2015. Melioidosis: evolving concepts in epidemiology, pathogenesis, and treatment. *Semin Respir Crit Care Med* 36:111–125. <https://doi.org/10.1055/s-0034-1398389>.
6. Snape MD, Kelly DF, Lewis S, Banner C, Kibwana L, Moore CE, Diggle L, John T, Yu LM, Borrow R, Borkowski A, Nau C, Pollard AJ. 2008. Seroprotection against serogroup C meningococcal disease in adolescents in the United Kingdom: observational study. *Brmj* 336:1487–1491. <https://doi.org/10.1136/bmj.39563.545255.AE>.
7. Pollard AJ, Perrett KP, Beverley PC. 2009. Maintaining protection against invasive bacteria with protein-polysaccharide conjugate vaccines. *Nat Rev Immunol* 9:213–220. <https://doi.org/10.1038/nri2494>.
8. Wahid R, Zafar SJ, McArthur MA, Pasetti MF, Levine MM, Sztein MB. 2014. Live oral *Salmonella enterica* serovar Typhi vaccines Ty21a and CVD 909 induce opsonophagocytic functional antibodies in humans that cross-react with *S. Paratyphi A* and *S. Paratyphi B*. *Clin Vaccine Immunol* 21: 427–434. <https://doi.org/10.1128/CI.00786-13>.
9. Burtneck MN, Brett PJ, Harding SV, Ngugi SA, Ribot WJ, Chantratita N, Scorpio A, Milne TS, Dean RE, Fritz DL, Peacock SJ, Prior JL, Atkins TP, Deshazer D. 2011. The cluster 1 type VI secretion system is a major virulence determinant in *Burkholderia pseudomallei*. *Infect Immun* 79:1512–1525. <https://doi.org/10.1128/IAI.01218-10>.
10. Burtneck MN, Shaffer TL, Ross BN, Muruato LA, Sbrana E, DeShazer D, Torres AG, Brett PJ. 2018. Development of subunit vaccines that provide high-level protection and sterilizing immunity against acute inhalational melioidosis. *Infect Immun* 86:e00724-17. <https://doi.org/10.1128/IAI.00724-17>.
11. Ho M, Schollaardt T, Smith MD, Perry MB, Brett PJ, Chaowagul W, Bryan LE. 1997. Specificity and functional activity of anti-*Burkholderia pseudomallei* polysaccharide antibodies. *Infect Immun* 65:3648–3653. <https://doi.org/10.1128/iai.65.9.3648-3653.1997>.
12. Burtneck MN, DeShazer D, Nair V, Gherardini FC, Brett PJ. 2010. *Burkholderia mallei* cluster 1 type VI secretion mutants exhibit growth and actin polymerization defects in RAW 264.7 murine macrophages. *Infect Immun* 78:88–99. <https://doi.org/10.1128/IAI.00985-09>.
13. Lim YT, Jobichen C, Wong J, Limmathurotsakul D, Li S, Chen Y, Raida M, Srinivasan N, MacAry PA, Sivaraman J, Gan YH. 2015. Extended loop region of Hcp1 is critical for the assembly and function of type VI secretion system in *Burkholderia pseudomallei*. *Sci Rep* 5:8235. <https://doi.org/10.1038/srep08235>.
14. Chieng S, Mohamed R, Nathan S. 2015. Transcriptome analysis of *Burkholderia pseudomallei* T65S identifies Hcp1 as a potential serodiagnostic marker. *Microb Pathog* 79:47–56. <https://doi.org/10.1016/j.micpath.2015.01.006>.
15. Sengyee S, Yarasai A, Janon R, Morakot C, Ottiwet O, Schmidt LK, West TE, Burtneck MN, Chantratita N, Brett PJ. 2021. Melioidosis patient survival correlates with strong IFN- $\gamma$  secreting T cell responses against Hcp1 and TssM. *Front Immunol* 12:698303. <https://doi.org/10.3389/fimmu.2021.698303>.
16. Suttisunhakul V, Wuthiekanun V, Brett PJ, Khusmith S, Day NP, Burtneck MN, Limmathurotsakul D, Chantratita N. 2016. Development of rapid enzyme-linked immunosorbent assays for detection of antibodies to *Burkholderia pseudomallei*. *J Clin Microbiol* 54:1259–1268. <https://doi.org/10.1128/JCM.02856-15>.
17. Pumpuang A, Dunachie SJ, Phokrai P, Jenjaroen K, Sintiprungrat K, Boonsilp S, Brett PJ, Burtneck MN, Chantratita N. 2017. Comparison of O-polysaccharide and hemolysin co-regulated protein as target antigens for serodiagnosis of melioidosis. *PLoS Negl Trop Dis* 11:e0005499. <https://doi.org/10.1371/journal.pntd.0005499>.
18. Pumpuang A, Phunpang R, Ekcharyawat P, Dulsuk A, Loupha S, Kwawong K, Charoensawat Y, Thiansukhon E, Day NPJ, Burtneck MN, Brett PJ, West TE, Chantratita N. 2019. Distinct classes and subclasses of antibodies to hemolysin co-regulated protein 1 and O-polysaccharide and correlation with clinical characteristics of melioidosis patients. *Sci Rep* 9:13972. <https://doi.org/10.1038/s41598-019-48828-4>.
19. Scott AE, Laws TR, D'Elia RV, Stokes MG, Nandi T, Williamson ED, Tan P, Prior JL, Atkins TP. 2013. Protection against experimental melioidosis following immunization with live *Burkholderia thailandensis* expressing a manno-heptose capsule. *Clin Vaccine Immunol* 20:1041–1047. <https://doi.org/10.1128/CI.00113-13>.
20. Scott AE, Burtneck MN, Stokes MG, Whelan AO, Williamson ED, Atkins TP, Prior JL, Brett PJ. 2014. *Burkholderia pseudomallei* capsular polysaccharide conjugates provide protection against acute melioidosis. *Infect Immun* 82:3206–3213. <https://doi.org/10.1128/IAI.01847-14>.
21. Silva EB, Goodyear A, Sutherland MD, Podnecky NL, Gonzalez-Juarrero M, Schweizer HP, Dow SW. 2013. Correlates of immune protection following cutaneous immunization with an attenuated *Burkholderia pseudomallei* vaccine. *Infect Immun* 81:4626–4634. <https://doi.org/10.1128/IAI.00915-13>.
22. Khakhum N, Bharaj P, Myers JN, Tapia D, Kilgore PB, Ross BN, Walker DH, Endsley JJ, Torres AG. 2019. *Burkholderia pseudomallei*  $\Delta$ tonB  $\Delta$ hcp1 live attenuated vaccine strain elicits full protective immunity against aerosolized melioidosis infection. *mSphere* 4. <https://doi.org/10.1128/mSphere.00570-18>.
23. Chaichana P, Jenjaroen K, Amornchai P, Chumseng S, Langla S, Rongkard P, Sumonwiriya M, Jeeyapant A, Chantratita N, Teparrukkul P, Limmathurotsakul D, Day NPJ, Wuthiekanun V, Dunachie SJ. 2018. Antibodies in melioidosis: the role of the indirect hemagglutination assay in evaluating patients and exposed populations. *Am J Trop Med Hyg* 99:1378–1385. <https://doi.org/10.4269/ajtmh.17-0998>.

24. Charuchaimontri C, Suputtamongkol Y, Nilakul C, Chaowagul W, Chetchotisakd P, Lertpatanasuwun N, Intaranongpai S, Brett PJ, Woods DE. 1999. Antilipopolsaccharide II: an antibody protective against fatal melioidosis. *Clin Infect Dis* 29: 813–818. <https://doi.org/10.1086/520441>.
25. Chaichana P, Kronsteiner B, Rongkard P, Teparrukkul P, Limmathurotsakul D, Chantratita N, Day NPJ, Fletcher HA, Dunachie SJ. 2020. Serum from melioidosis survivors diminished intracellular *Burkholderia pseudomallei* growth in macrophages: a Brief Research Report. *Front Cell Infect Microbiol* 10:442. <https://doi.org/10.3389/fcimb.2020.00442>.
26. Chaichana P, Jenjaroen K, Chumseng S, Sumonwiriya M, Rongkard P, Kronsteiner B, Teparrukkul P, Limmathurotsakul D, Day NPJ, Chantratita N, Dunachie SJ. 2021. Role of *Burkholderia pseudomallei*-specific IgG2 in adults with acute melioidosis, Thailand. *Emerg Infect Dis* 27:463–470. <https://doi.org/10.3201/eid2702.200213>.
27. Utaisincharoen P, Anuntagool N, Arjcharoen S, Limposuwan K, Chaisuriya P, Sirisinha S. 2004. Induction of iNOS expression and antimicrobial activity by interferon (IFN)- $\beta$  is distinct from IFN- $\gamma$  in *Burkholderia pseudomallei*-infected mouse macrophages. *Clin Exp Immunol* 136:277–283. <https://doi.org/10.1111/j.1365-2249.2004.02445.x>.
28. DeShazer D, Brett PJ, Woods DE. 1998. The type II O-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. *Mol Microbiol* 30:1081–1100. <https://doi.org/10.1046/j.1365-2958.1998.01139.x>.
29. Drogari-Apiranthitou M, Kuijper EJ, Dekker N, Dankert J. 2002. Complement activation and formation of the membrane attack complex on serogroup B *Neisseria meningitidis* in the presence or absence of serum bactericidal activity. *Infect Immun* 70:3752–3758. <https://doi.org/10.1128/IAI.70.7.3752-3758.2002>.
30. Joiner KA, Goldman RC, Hammer CH, Leive L, Frank MM. 1983. Studies of the mechanism of bacterial resistance to complement-mediated killing. V. IgG and F(ab) $_2$  mediate killing of *E. coli* 0111B4 by the alternative complement pathway without increasing C5b-9 deposition. *J Immunol* 131: 2563–2569.
31. Dahora LC, Verheul MK, Williams KL, Jin C, Stockdale L, Cavet G, Giladi E, Hill J, Kim D, Leung Y, Bobay BG, Spicer LD, Sawant S, Rijpkema S, Dennison SM, Alam SM, Pollard AJ, Tomaras GD. 2021. *Salmonella* Typhi Vi capsule prime-boost vaccination induces convergent and functional antibody responses. *Sci Immunol* 6:eabj1181. <https://doi.org/10.1126/sciimmunol.abj1181>.
32. Spear GT, Takefman DM, Sullivan BL, Landay AL, Zolla-Pazner S. 1993. Complement activation by human monoclonal antibodies to human immunodeficiency virus. *J Virol* 67:53–59. <https://doi.org/10.1128/JVI.67.1.53-59.1993>.
33. Bryan LE, Wong S, Woods DE, Dance DA, Chaowagul W. 1994. Passive protection of diabetic rats with antisera specific for the polysaccharide portion of the lipopolysaccharide isolated from *Pseudomonas pseudomallei*. *Can J Infect Dis* 5:170–178. <https://doi.org/10.1155/1994/856850>.
34. AuCoin DP, Reed DE, Marlenee NL, Bowen RA, Thorkildson P, Judy BM, Torres AG, Kozel TR. 2012. Polysaccharide specific monoclonal antibodies provide passive protection against intranasal challenge with *Burkholderia pseudomallei*. *PLoS One* 7:e35386. <https://doi.org/10.1371/journal.pone.0035386>.
35. Rolim DB, Vilar DC, de Goes Cavalcanti LP, Freitas LB, Inglis TJ, Nobre Rodrigues JL, Nagao-Dias AT. 2011. *Burkholderia pseudomallei* antibodies in individuals living in endemic regions in Northeastern Brazil. *Am J Trop Med Hyg* 84:302–305. <https://doi.org/10.4269/ajtmh.2011.10-0220>.
36. Chenthamarakshan V, Vadivelu J, Puthuchery SD. 2001. Detection of immunoglobulins M and G using culture filtrate antigen of *Burkholderia pseudomallei*. *Diagn Microbiol Infect Dis* 39:1–7. [https://doi.org/10.1016/S0732-8893\(00\)00212-1](https://doi.org/10.1016/S0732-8893(00)00212-1).
37. Heiss C, Burtnick MN, Roberts RA, Black I, Azadi P, Brett PJ. 2013. Revised structures for the predominant O-polysaccharides expressed by *Burkholderia pseudomallei* and *Burkholderia mallei*. *Carbohydr Res* 381:6–11. <https://doi.org/10.1016/j.carres.2013.08.013>.
38. Perry MB, MacLean LL, Schollaardt T, Bryan LE, Ho M. 1995. Structural characterization of the lipopolysaccharide O antigens of *Burkholderia pseudomallei*. *Infect Immun* 63:3348–3352. <https://doi.org/10.1128/iai.63.9.3348-3352.1995>.
39. Burtnick MN, Heiss C, Roberts RA, Schweizer HP, Azadi P, Brett PJ. 2012. Development of capsular polysaccharide-based glycoconjugates for immunization against melioidosis and glanders. *Front Cell Infect Microbiol* 2:108. <https://doi.org/10.3389/fcimb.2012.00108>.
40. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685. <https://doi.org/10.1038/227680a0>.
41. Sangsri T, Saiprom N, Tubsuwan A, Monk P, Partridge LJ, Chantratita N. 2020. Tetraspanins are involved in *Burkholderia pseudomallei*-induced cell-to-cell fusion of phagocytic and non-phagocytic cells. *Sci Rep* 10:17972. <https://doi.org/10.1038/s41598-020-74737-y>.
42. Woodman ME, Worth RG, Wooten RM. 2012. Capsule influences the deposition of critical complement C3 levels required for the killing of *Burkholderia pseudomallei* via NADPH-oxidase induction by human neutrophils. *PLoS One* 7:e52276. <https://doi.org/10.1371/journal.pone.0052276>.