Published in final edited form as:
Vaccine. 2011 October 26; 29(46): 8381–8389. doi:10.1016/j.vaccine.2011.08.058.

A naturally-derived outer-membrane vesicle vaccine protects against lethal pulmonary Burkholderia pseudomallei infection

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Abstract

B. pseudomallei, and other members of the **Burkholderia**, are among the most antibiotic-resistant bacterial species encountered in human infection. Mortality rates associated with severe **B. pseudomallei** infection approach 50% despite therapeutic treatment. A protective vaccine against **B. pseudomallei** would dramatically reduce morbidity and mortality in endemic areas and provide a safeguard for the U.S. and other countries against biological attack with this organism. In this study, we investigated the immunogenicity and protective efficacy of **B. pseudomallei**-derived outer membrane vesicles (OMVs). Vesicles are produced by Gram-negative and Gram-positive bacteria and contain many of the bacterial products recognized by the host immune system during infection. We demonstrate that subcutaneous (SC) immunization with OMVs provides significant protection against an otherwise lethal **B. pseudomallei** aerosol challenge in BALB/c mice. Mice immunized with **B. pseudomallei** OMVs displayed OMV-specific serum antibody and T-cell memory responses. Furthermore, OMV-mediated immunity appears species-specific as cross-reactive antibody and T cells were not generated in mice immunized with **E. coli**-derived OMVs. These results provide the first compelling evidence that OMVs represent a non-living vaccine formulation that is able to produce protective humoral and cellular immunity against an aerosolized intracellular bacterium. This vaccine platform constitutes a safe and inexpensive immunization strategy against **B. pseudomallei** that can be exploited for other intracellular respiratory pathogens, including other **Burkholderia** and bacteria capable of establishing persistent infection.

Keywords

aerosol; intracellular; persistence; OMV

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1. Introduction

The genus **Burkholderia** encompasses a large group of ubiquitous Gram-negative bacteria pathogenic for both plants and animals. Members of the **Burkholderia** responsible for human disease include the opportunistic **Burkholderia cepacia** complex (Bcc), including **B. cenocepacia** and **B. multivorans**, which have emerged as significant causes of fatal pulmonary infection in individuals with cystic fibrosis in the United States, Canada, and Europe [1]. **B. mallei**, the etiologic agent of glanders, is an obligate mammalian pathogen that primarily infects hoofed animals, but severe human cases have been documented [2]. Lastly, the facultative intracellular bacterium, **B. pseudomallei,** is the causative agent of melioidosis, an emerging disease responsible for significant morbidity and mortality in Southeast Asia and Northern Australia [3, 4]. While most reported cases of **B. pseudomallei** infection are restricted to these geographic regions, the organism has a much larger global distribution and human cases are likely under-reported [5]. Natural infection with the **Burkholderia** can occur through subcutaneous inoculation, ingestion, or inhalation of the bacteria. Clinical manifestations can be non-specific, widely variable, and often depend upon the route of inoculation and the immune status of the host [3]. **Burkholderia** infections are inherently difficult to treat due to their resistance to multiple antibiotics, biofilm formation, and establishment of intracellular and chronic infection in the host. Preventive measures such as active immunization could dramatically reduce the global incidence of disease; however there is currently no commercially available vaccine against any member of the **Burkholderia** [6].

In recent years, a number of vaccine strategies against **B. pseudomallei** and **B. mallei** have been explored due to the potential threat of these organisms as biological warfare agents. No ideal candidate has yet emerged from pre-clinical studies [7]. For **B. pseudomallei**, inactivated whole-cell preparations and live-attenuated strains are highly immunogenic and demonstrate partial to full protection in murine models [7–10]. However, safety concerns and contraindication for use in immunocompromised individuals limits the utility of such vaccines for human use. Safer, alternative approaches to vaccination include use of purified preparations of lipopolysaccharide (LPS), capsular polysaccharide (CPS), or protein-based subunit vaccines. Studies with **B. pseudomallei** LPS and CPS have demonstrated high degrees of antibody-mediated short-term protection with both active and passive immunization [11–14]. However, the inability of these T-cell independent antigens to confer sterilizing immunity is problematic. Polysaccharide-protein conjugate vaccines that promote T-cell-dependent immune responses may improve efficacy, but the high cost and technical expertise associated with such vaccines may explain the current absence of active immunization studies in the literature [7]. Protein subunit strategies have yielded variable degrees of protection against systemic **B. pseudomallei** infection but have proved either ineffective or have not been tested against inhalational challenge [15–18]. Pulmonary infection with **B. pseudomallei** is highly lethal in humans and animal models and has been particularly difficult to prevent by vaccination thus far [7, 19]. A successful vaccine against **B. pseudomallei**, as with other intracellular bacteria, will likely require the induction of both humoral and cellular-mediated immune (CMI) responses for complete protection and eradication of persistent bacteria [20]. Furthermore, the vaccine must be safe and efficacious against multiple routes of infection.

Here we report a promising immunization approach against **B. pseudomallei** that utilizes bacteria-derived outer membrane vesicles (OMVs). OMVs are constitutively produced by Gram-negative bacteria both **in vivo** and **in vitro** and are often enriched in virulence factors and Toll-like receptor (TLR) agonists [21–23]. Vesicle production has also been observed in fungi and Gram-positive bacteria highlighting the conservation of this process among microbes, although the mechanisms of secretion likely differ [21]. Use of membrane vesicle-

based vaccines is rapidly gaining interest, and vesicle-mediated protection against mucosal and systemic bacterial challenge has been demonstrated for **Neisseria meningitidis** [24], **Bordetella pertussis** [25], **Salmonella typhimurium** [26], **Vibrio cholerae** [27], and more recently **Bacillus anthracis** [28]. In mouse studies, efficacy of vesicle vaccines has ranged from 33% protection against **B. anthracis** [28] to nearly 100% protection against **V. cholerae** [27]. **N. meningitidis** serogroup B OMVs adsorbed to aluminum adjuvant are approved for human use and provide 80% protective efficacy against severe invasive disease [24]. In this instance, protection is mediated by serum bactericidal antibody directed against **Neisseria** surface antigens thus promoting bacterial opsonization and complement-mediated killing [29].

In this study, we demonstrate that immunization with naturally-shed **B. pseudomallei** OMVs provides significant protection against lethal aerosol challenge in a murine model of melioidosis. Membrane vesicles may represent an efficacious vaccine platform against other aerosolized intracellular pathogens, including those that establish persistent infection.

2. Materials and Methods

2.1 Bacterial strains and culture

B. pseudomallei strain 1026b was obtained from BEI Resources. **E. coli** strain M15 was obtained from Qiagen. Bacteria were cultured from glycerol stocks immediately prior to use and single colonies were selected from freshly-streaked LB agar plates. Overnight cultures were diluted 1:100 in fresh LB and incubated with shaking at 37 °C until OD_{600} reached 0.75 for challenge experiments.

2.2 Outer membrane vesicle (OMV) preparation and characterization

OMVs were purified as we previously described with minor modifications [30]. Pooled OMVs were desalted and concentrated using a 100 kDa Amicon desalting column (Millipore) following the manufacturer's protocol. OMVs were then washed and resuspended in LPS-free water. OMVs were quantified with a Bradford Protein Assay (Bio-Rad). Cryo Transmission Electron Microscopy was performed using a JEOL 2010 transmission electron microscope to visually confirm the presence and purity of OMVs.

For LC-MS analysis, 100 μg of OMVs were separated by SDS-PAGE and the gel bands were manually cut into pieces and rinsed twice with 25 mM ammonium bicarbonate in 50% acentonitrile for 20 min. Proteins were digested with trypsin (~1 μg per band) in 25 mM ammonium bicarbonate at 37 °C overnight (~16 hours). The peptides were extracted by adding 100 μl of extraction buffer (0.1% formic acid in 50% acentonitrile aqueous solution), incubating for 20 min, and collecting the supernatant. This step was repeated once, followed by incubation in 100% acetonitrile. The combined supernatants were dried down in an Eppendorf Vacufuge. Prior to LC-MS analysis, the peptides were resuspended in 10 μl of 0.1% formic acid/2% acetonitrile. All spectra were acquired on a Thermo-Fisher LTQ-XL linear ion trap mass spectrometer (Waltham, MA) coupling with an Eksigent nanoLC 2D (Dublin, CA). Peptides were loaded into a Dionex PepMap C18 trap column (300 μm id × 5 mm, 5μm particle size) and then separated by a New Objective reversed phase C18 Picofrit column/emitter (75μm id, 10 cm long, 5 μm particle size, Woburn, NJ). A gradient elution at 250 nl/min starting from 5 % to 40% buffer B in 40 min, followed by 40% to 80% buffer B in 20 min, then 80% buffer B for 10 min. Buffer A is 0.1% formic acid aqueous solution and Buffer B is 0.1% formic acid in acetonitrile. A blank run was inserted between two sample runs to reduce cross contamination. The raw data were searched against **Burkholderia pseudomallei** K96243 proteome (2009-12-06) downloaded from the **Burkholderia** Genome Database (http://www.Burkholderia.com). The search engine Bioworks 3.3.1 (Thermo-

Fisher) was used with ProteinProphet [31] /Trans Proteomic Pipeline [32]. Protein matches are reported with an error rate of 2.5% predicted by ProteinProphet as the threshold.

2.3 LPS and CPS determination

The amount of LPS in **B. pseudomallei** OMVs was determined by capture ELISA. Maxisorp immunoplates (Nunc) were coated overnight at 4 °C with 100 μl of 5 μg/ml of anti-**B. pseudomallei** LPS monoclonal antibody (Mab) (from J. Prior and S. Ngugi, Dstl, UK) in PBS. After washing with PBS/0.05% Tween 20 (PBST), plates were blocked with 3% skimmed milk in PBS. Plates were then incubated for 1 h at 25 °C with 1:2 dilutions of OMVs or purified **B. thailandensis** LPS, starting at 400 μg/ml, in 3% milk/PBS/0.05% Tween/0.8% polyvinylpyrrolidone (PVP). The anti-**B. pseudomallei** LPS Mab was biotinylated using the EZ-link micro sulfo-NHS-LC-biotinylation kit (Thermo-Pierce), following the manufacturer's recommended protocol. Biotinylated anti-**B. pseudomallei** LPS Mab in 3% milk/PBS/0.05% tween/0.8% PVP was added to plates at a concentration of 1 μg/ml and incubated for 1 h. Plates were washed in PBS/T and then incubated for 1 h at 25 °C with a streptavidin-peroxidase polymer conjugate (Sigma), diluted 1:1000 in 3% milk/PBS/0.05% tween/0.8% PVP. Plates were then washed prior to development with 1-step Ultra TMB ELISA reagent (Thermo Scientific). Plates were read at 450 nm after the addition of 2M H_2SO_4 to stop the reaction. A standard curve of A_{450} vs. LPS concentration was plotted and used to determine the LPS content of OMV samples.

The presence of CPS in OMVs was determined by Western blot using monoclonal antibody 3C5, specific for **B. pseudomallei** CPS [33]. Ten μg of OMVs, **B. pseudomallei** 1026b lysate, and **B. thailandensis** lysate were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 7.5 % polyacrylamide gel (Bio-Rad). The proteins were transferred to a nitrocellulose membrane and blocked in 1.5% BSA in TBS-T for 1 hr. The membrane was incubated with 3C5 IgG3 (1:1000 dilution) overnight at 4 °C, washed 3 times with TBS-T, and incubated with goat anti-mouse HRP-conjugated secondary antibody (Pierce, 1:1000 dilution) for 1 hr at room temperature. The membrane was washed and developed using Opti-4CN substrate (BioRad).

2.4 Animals

Female BALB/c mice 8- to 10-weeks-old were purchased from Charles River Laboratories (Wilmington, MA) and maintained 5 per cage in polystyrene microisolator units under pathogen-free conditions. Animals were fed sterile rodent chow and water **ad libitum** and allowed to acclimate 1 week prior to use. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The protocols were approved by Tulane University Health Sciences Center and Tulane National Primate Research Center Institutional Animal Care and Use Committees (protocol numbers 4048R and P0105, respectively).

2.5 Immunizations

Two independent immunization experiments were performed using separately-prepared batches of purified OMV. In the first experiment, BALB/c mice (n=10 per group) were primed subcutaneously (SC) on day 0 with 2.5 μg of **B. pseudomallei** OMVs in a final volume of 100 μl sterile saline, or intranasally (IN) with 2.5 μg **B. pseudomallei** or **E. coli** OMVs in a final volume of 7.5 μl/ nostril. Prior to IN immunization, mice were briefly anesthetized with Isoflurane (VetOne). Naïve mice did not receive any treatment. Immunized mice were boosted on days 21 and 42 with the same formulations. No adjuvant was added to the OMV preparations. One month after the last immunization, a subset of mice (n=5 per group) were utilized for measurement of antibody responses and separate groups of mice (n=5 mice group) were challenged with **B. pseudomallei** by aerosol. In the

second experiment, BALB/c mice (n=15 per group) were immunized exactly as described above. Five mice per group were utilized to determine immune correlates of protection, and ten mice per group were challenged with **B. pseudomallei** by aerosol.

2.6 Aerosol challenges

Mice were challenged with **B. pseudomallei** strain 1026b via small particle aerosol as we previously described [34]. Animal groups were randomized for experimental infection; the animal capacity for each discrete run of the inhalation system was 23; the total number of runs required was three. A dynamic nose-only inhalation exposure system (CH Technologies, Westwood, NJ) was employed for the exposures. The inhalation apparatus was housed in a Class III biological safety cabinet (GermFree Laboratories, Ormond Beach, FL) within a BSL-3 containment laboratory environment. The nose-only system was maintained at 11 lpm total flow during exposures. The aerosols were generated into the central plenum of the chamber using a three-jet collison nebulizer (BGI Inc., Waltham, MA). The experimental atmosphere was continuously sampled using an all glass impinger (AGI-4, Ace Glass, Vineland, NJ) inserted into one of the nose-only ports of the exposure plenum. The impinger contents were cultured immediately after each discrete run of the system and the bacterial colony counts were used to calculate an aerosol concentration ($\mathbf{C_a}$) of **B. pseudomallei** within the plenum of the nose-only exposure apparatus. The resultant $\mathbf{C_a}$ for each run was applied to a calculated breathing rate of the mice to attain a total respiratory volume during exposure. The resulting inhaled dose was expressed in CFU/animal. The mean inhaled dose across all experimental groups was $5.35\times10^3 \pm 3.64\times10^3$ CFU. Mice were challenged with a target dose of $5LD_{50}$ (~1000 CFU for **B. pseudomallei** 1026b as determined in pilot experiments). Two naïve mice were included in each exposure run and were euthanized immediately after challenge. Lungs were plated for determination of bacterial CFU to confirm the inoculum.

2.7 CFU recovery

Lung, spleen, and liver tissue homogenates were used to determine bacterial burden at 14 and 30 days post-infection in mice that survived aerosol challenge. Tissues were aseptically removed, weighed, and individually placed in 1 ml 0.9% NaCl and homogenized with sterile, disposable tissue grinders (Fisher Scientific). Ten-fold serial dilutions of lung homogenates were plated on **Pseudomonas** isolation agar (PIA). Colonies were counted after incubation for 3 days at 37°C and reported as CFU per organ.

2.8 Analysis of antibody response

Immunized and naïve mice were anesthetized and blood was collected by retro-orbital bleed prior to each immunization. One month after the last immunization, blood samples from immunized and naive mice were collected following euthanasia for determination of antigen-specific serum antibody concentrations. Blood was allowed to clot for 30 min at room temperature and then centrifuged at 2300 × **g**; serum was collected and stored at −80 °C until assayed. The concentrations of serum OMV-specific total IgG, IgG1, IgG2a, and IgA were analyzed by enzyme-linked immunosorbent assay (ELISA). Ninety-six-well microtiter plates were coated with 0.5 μg per well of purified **B. pseudomallei** OMVs in coating buffer (0.1 M sodium bicarbonate, 0.2 M sodium carbonate) and incubated overnight at 4°C. The plates were washed three times with PBS containing 0.05% Tween-20 (PBST). For measurement of IgA, plates were additionally blocked with 2% BSA for 1 hr followed by three washes with PBST. All plates were incubated with two-fold serial dilutions of sera samples for 2 hr at room temperature. Plates were washed three times with PBST and then incubated with either alkaline phosphatase (AP)- conjugated rat anti-mouse IgG, IgG1, IgG2a (1:300 dilution in PBST) (BD Pharmingen) or AP-conjugated goat-anti-mouse IgA (1:2000) (Invitrogen) for 1 hr at room temperature. At the end of the incubation, the plates

were washed three times with PBST and developed with SIGMA**FAST** p-Nitrophenyl phosphate tablets (Sigma, St. Louis, MO) dissolved in diethanolamine buffer (1mg/ml). After 15–30 min of incubation, reaction solutions were stopped with 2 M NaOH and read at 405 nm using a μQuant microplate reader and analyzed with Gen5 software (BioTek, Winooski, VT). The results obtained are expressed as the mean reciprocal endpoint titers for total IgG; concentrations for IgG and IgA; and ratios of IgG1 to IgG2a based upon total concentrations. Endpoint titer is defined as the greatest dilution that yielded an optical density (OD_{450}) greater than three standard deviations above the mean OD_{450} for pre-immune titers. Concentrations were determined by comparison to a standard curve as previously described [30].

2.9 Antigen restimulation assay

Restimulation assays were performed with splenocytes from immunized and naïve mice for analysis of T cell responses. Spleens were removed aseptically and single-cell splenocyte suspensions from each mouse were obtained by passing the spleens through sterile 40 μm cell strainers (Fisher Scientific). Cells were washed twice with Hank's buffered saline solution (HBSS) (ATCC). Cell pellets were resuspended in HBSS and layered onto ACK Lysing buffer (Gibco) for 4 min. Splenic mononuclear leukocyte isolation was achieved by centrifugation at 1500 × **g** for 10 min. Leukocytes were recovered at the interface, washed twice with HBSS, and resuspended in Advanced RPMI 1640 medium (ATCC) supplemented with 10% FBS (Atlanta Biologicals) and 1% antibiotic-antimycotic (Gibco). Cells were plated in a 96-well microtiter plate at 1.5×10^6 cells/well. Cell cultures were stimulated with 2 μg of **B. pseudomallei** OMVs, 1 μg ConA (Sigma), or left unstimulated as negative controls. The cultures were incubated at 37 °C in 5% CO_2, and cell culture supernatants from each treatment group were collected after 72 hr and stored at −80° until use.

2.10 Statistical analyses

All analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA). Statistical analyses were performed using a one-way or two-way ANOVA with Bonferroni's post-test. Values of $P < 0.05$ were considered statistically significant. For survival analysis, the log rank Mantel-Cox test was used.

3. Results

3.1 B. pseudomallei OMVs contain LPS, CPS, and protein antigens

We have previously shown that OMVs are abundantly shed by broth-grown **B. pseudomallei** and are easily harvested from culture supernatants using density gradient ultra-centrifugation [30]. Purified **B. pseudomallei** vesicles range in size from 50–250 nm and contain between 1 to 1.5 mg protein per liter of culture (Figure 1A). Using LC-MS analysis, we detected numerous proteins in the OMVs, including 17 putative periplasmic proteins and 12 predicted outer membrane or extracellular proteins (Table S1). Several of the proteins identified are previously characterized immunogenic proteins (Table S1; proteins highlighted in red) [30, 35]. We thus hypothesized that OMVs shed by broth-grown **B. pseudomallei** possess similar antigenic cargo as those expressed during infection **in vivo**. In order to test this hypothesis, we utilized convalescent sera from a rhesus macaque that had recovered from experimental **B. pseudomallei** aerosol infection (manuscript in preparation). As shown in Figure S1, broth-grown bacteria produce OMVs that contain numerous immunoreactive antigens expressed and recognized during **B. pseudomallei** infection in a non-human primate model of melioidosis. Due to the nature of OMV biogenesis, we also postulated that OMVs harbored LPS and CPS, both of which stimulate protective antibody responses against **B. pseudomallei** [11–13]. Limulus assay confirmed

the presence of LPS in the OMVs; OMVs contained 200 μg/ml of LPS as determined by capture ELISA. Using a monoclonal antibody directed against the CPS of **B. pseudomallei** [33], we demonstrated by Western blot that this surface antigen is also abundant in the OMVs (Figure 1B). The presence of numerous immunoreactive proteins, as well as LPS and CPS, in the **B. pseudomallei** OMVs prompted us to explore their utility as a vaccine candidate.

3.2 B. pseudomallei OMVs induce specific antibody responses without a requirement for adjuvant

OMV biogenesis generates vesicles that contain large quantities of LPS with inherent endotoxicity. Thus, vaccine preparations utilizing OMVs from Gram-negative bacteria will most often require LPS extraction or de-toxification of lipid A prior to administration [36, 37]. Furthermore, the removal of LPS from OMVs often necessitates the addition of adjuvant to restore OMV immunogenicity [37]. **B. pseudomallei** LPS is up to 1,000-fold less toxic than **Escherichia coli** LPS [38, 39] and we observed no cytotoxicity in murine macrophages co-cultured with 5 μg of **B. pseudomallei** OMVs for 72 hrs (not shown). We therefore exploited the natural adjuvanticity and low toxicity of **B. pseudomallei** LPS as a native component of the OMV preparation. Two groups of mice were immunized with 2.5 μg of **B. pseudomallei** OMVs by the intranasal (IN) or SC route and boosted on days 21 and 42. In order to examine specificity of the antibody response to the OMVs, we also purified OMVs from a non-pathogenic strain of **E. coli** as a control antigen. The **E. coli** OMVs were prepared in exactly the same manner as the **B. pseudomallei** OMVs and contained LPS. For this reason, mice were immunized with **E. coli** OMVs by the IN route only due to significant endotoxicity associated with **E. coli** LPS administered SC [40]. No additional adjuvant was added to either OMV preparation. **B. pseudomallei** OMVs administered SC or IN induced high titers of OMV-specific serum IgG after a single boost. Moreover, serum IgG titers increased approximately 1-log after a second boost and were significantly higher than pre-immune titers (Figure 2). OMV immunization generated IgG responses against multiple protein antigens in the OMV preparation (Figure S2). Furthermore, the IgG response to **B. pseudomallei** OMVs appears specific since mice immunized with **E. coli** OMVs did not generate IgG that recognized **B. pseudomallei** OMVs. This was not due to immune tolerance because **E. coli** OMV-immunized mice produced antibodies that recognized their cognate OMVs (Figure 4C,D). Naïve mice also did not possess antibody that recognized **B. pseudomallei** OMV antigens (Fig. 2 and S2).

3.3 Immunization with B. pseudomallei OMVs provides significant protection against lethal aerosol challenge

In order to determine if immunization with **B. pseudomallei** OMVs could provide protection against inhalational infection, groups of mice were immunized as above and challenged by aerosol with virulent **B. pseudomallei** strain 1026b. Two independent immunization and challenge experiments were performed with two separately prepared batches of OMV vaccine to demonstrate reproducibility. Naïve mice displayed 100% mortality by day 7 (Figure 3). In contrast, mice immunized SC with **B. pseudomallei** OMVs were significantly protected against lethal aerosol challenge ($\mathbf{P} < 0.001$). No significant protection was observed in mice immunized IN with **B. pseudomallei** OMVs or **E. coli** OMVs although a small percentage of animals survived. The composite survival data for a 2 week period is shown since no animal succumbed after day 7. In addition, a portion of surviving animals was euthanized 2 weeks post-challenge for determination of bacterial burden.

3.4 B. pseudomallei OMV immunization reduces, but does not completely eliminate, bacterial persistence

Tissues known to harbor persistent **B. pseudomallei** (lung, liver, and spleen) were harvested from survivors after 14 and 30 days of observation and plated for determination of bacterial loads. Both groups of **B. pseudomallei** OMV-immunized mice (SC and IN) demonstrated absence of bacteria in the lungs by 14 days post-aerosol challenge (Table 1). In contrast, the **E. coli** OMV-immunized mice that survived challenge contained up to 10^6 CFU in their lungs on day 14. Two out of three **B. pseudomallei** OMV SC-immunized mice showed no evidence of **B. pseudomallei** in the spleen, and very low numbers of bacteria were detected in the liver (<30 CFU). As observed in the lung, **E. coli** OMV-immunized mice had higher numbers of **B. pseudomallei** in the spleen and liver compared to **B. pseudomallei** OMV-immunized animals at 14 days post-challenge.

At 30 days post-challenge, a similar outcome was observed in that the **E. coli** OMV-immunized animal had higher CFU in all tissues compared to **B. pseudomallei** OMV immunized mice. We also noted low numbers of bacteria in the lungs of **B. pseudomallei** OMV-immunized mice that contrasts with the lack of colonization seen at 14 days in these groups. These mice were also colonized with low numbers of bacteria in the spleen and/or liver. Bacterial re-colonization of the lung from distant organs might have occurred after an extended period of infection, as **B. pseudomallei** possesses a tropism for the lung [3].

3.5 B. pseudomallei OMV immunization induces high titers of OMV-specific serum IgG and IgA

Antibody responses were measured in serum obtained from separate groups of mice one month after the last immunization in order to assess immune correlates of protection. **B. pseudomallei** OMV-specific serum IgG was significantly higher in the **B. pseudomallei** OMV SC- and IN-immunized animals than in controls (Figure 4A). The concentrations of OMV-specific IgG were not significantly different between **B. pseudomallei** OMV SC- and IN-immunized mice. Furthermore, the concentrations of IgG1 and IgG2a were not significantly different between **B. pseudomallei** SC- and IN-immunized mice (Table 2). Both **B. pseudomallei** OMV SC- and IN-immunized groups demonstrated a Type 2 immune response with IgG1:IgG2a ratios equal to 7.5 and 12.2, respectively (Table 2). **B. pseudomallei** OMV-specific serum IgA was significantly higher in **B. pseudomallei** OMV IN-immunized mice compared to control groups (Figure 4B). As noted in our initial immunogenicity studies, antibody responses to **B. pseudomallei** OMVs were specific since **E. coli** OMV-immunized mice did not produce antibodies that recognized **B. pseudomallei** OMVs, although they produced high titers of **E.coli** OMV-specific serum IgG and IgA (Figure 4 C,D). Conversely, **B. pseudomallei** OMV-immunized mice did not generate a significant antibody response to **E. coli** OMVs (Fig. 4C,D).

3.6 Immunization with B. pseudomallei OMVs induces T cell memory responses

A Th1-driven CMI response, in concert with the production of specific antibodies, is likely essential for vaccine efficacy against **B. pseudomallei** [9, 20]. To assess antigen-specific T cell responses in OMV-immunized mice, spleens were harvested one month after the last immunization and re-stimulated **ex vivo** with **B. pseudomallei** OMVs. Cell culture supernatants were assayed on day three for IFN-γ production as an indication of a Th1 memory response. Both groups of mice immunized with **B. pseudomallei** OMVs (SC and IN) produced significantly higher amounts of IFN-γ compared to control groups (Figure 5). Similar to that observed for antibody responses, T cell memory responses to **B. pseudomallei** OMV immunization appeared specific as splenocytes from **E**. **coli** OMV-immunized mice did not produce IFN-γ upon restimulation with **B. pseudomallei** OMVs.

4. Discussion

The significant morbidity and mortality associated with **Burkholderia** pulmonary infection in humans necessitates the development of a safe and efficacious vaccine against inhalational disease. Furthermore, a vaccine that provides sterile immunity would be especially useful since many members of the **Burkholderia** cause persistent infection. In this study, we present compelling evidence that a naturally-derived OMV vaccine provides inherent adjuvanticity, immunogenicity, and protective efficacy against **B. pseudomallei** pulmonary challenge. Mice immunized SC with **B. pseudomallei** OMVs demonstrated nearly 60% survival against aerosol challenge compared to 0% survival in naïve animals. To our knowledge, this is the best vaccine-mediated protection attained thus far against lethal pneumonic melioidosis in the mouse model. These results suggest that membrane vesicles may represent a promising vaccine strategy against other respiratory pathogens, including those that establish persistent pulmonary infection such as **Mycobacterium tuberculosis** or the **B. cepacia** complex. Indeed, it was recently shown that **M. tuberculosis** produces vesicles that modulate immune responses and enhance bacterial virulence via TLR2 signaling [41].

Membrane vesicle-based vaccines offer numerous advantages to traditional vaccine strategies. For example, they are easy and inexpensive to produce – particularly native vesicles that do not require chemical treatment or other artificial modes of preparation. They are non-viable yet share many of the surface antigens presented by an inactivated or live-attenuated strain without presenting the same safety concerns. Vesicles also contain numerous antigens that can influence immune responses [21, 22]. This feature could overcome limitations associated with the use of a single antigen (i.e. LPS or protein subunit) and vaccine failure due to antigenic variance among heterogenous bacterial strains [42, 43], escape mutants [44, 45], and human leukocyte haplotype (HLA) restriction [46]. Using highly sensitive LC-MS analysis, we identified numerous protein antigens in the purified vesicles (Table S1). Several proteins appear to be highly abundant and immunogenic as determined by SDS-PAGE and Western blot, respectively (Fig. S1A, S1C and S2B). Furthermore, we have purified multiple, independent batches of OMVs over a one-year period. We witness identical protein and immunogenicity profiles with each preparation which attests to the reproducibility of the product.

The safety and protective efficacy afforded by an OMV vaccine against **N**. **meningitidis** (**Nm**) serogroup B strains establishes precedence for use of such vaccines in the human population [24, 47–49]. However, unlike **B. pseudomallei** OMVs, production of **Nm**-derived OMVs requires the removal of the extremely toxic lipooligosaccharide which necessitates the addition of aluminum hydroxide adjuvant to the OMV preparation to restore immunogenicity [37]. Alum polarizes the immune response towards humoral and Th2 CMI [50], supporting the production of high titers of bactericidal antibody necessary for protection against meningococcus. Both humoral and Th1 CMI are likely essential for protection against **B. pseudomallei**. Because **B. pseudomallei** OMVs possess low toxicity yet retain adjuvanticity, we opted to utilize **B. pseudomallei** OMVs in their native form without extraction of LPS or addition of an exogenous adjuvant. We postulated that innate immune recognition of **B. pseudomallei** OMVs would mimic those to the intact organism since OMVs have been shown to contain LPS, lipoproteins, and CpG DNA [21–23] and to activate TLRs [51]. Furthermore, the particulate nature of OMVs will enable delivery of intrinsic TLR agonists and antigenic cargo to the same antigen-presenting cell, which leads to more efficient antigen presentation [52].

Our homologous prime-boost immunization studies compared the traditional parenteral route of immunization to intranasal delivery. It has been proposed that **B. pseudomallei** may

utilize the NALT as a portal of entry in murine melioidosis [53]. Initially we expected that the IN route of immunization might better prevent mucosal infections through the priming and activation of local antimicrobial immunity. The finding that significant protection was observed in mice immunized SC, but not those immunized IN, with **B. pseudomallei** OMVs was an interesting and unexpected outcome. We could not attribute differences in protection to OMV-specific serum IgG responses because the concentrations were not significantly different between the two groups. We demonstrated that purified OMVs from **B. pseudomallei** contain both LPS and CPS which may contribute to the protective efficacy of the OMV vaccine. The protective capacity of antibodies directed toward the O-antigen of LPS and CPS of **B. pseudomallei** has been demonstrated in multiple studies [11–14]. Notably, out of 47 monoclonal antibodies generated to protein, glycoprotein, and polysaccharide epitopes of **B. pseudomallei**, only those directed against LPS and CPS were strongly bactericidal and highly effective in protecting against intranasal **B. pseudomallei** infection. None of the monoclonal antibodies reacting to bacterial proteins showed prominent opsonic activity, suggesting that protein epitopes were less accessible on intact bacteria [14]. Although OMV-specific serum IgG, IgG1, and IgG2a responses were similar for IN- and SC-immunized mice, the LPS- or CPS-specific antibody induced by the OMV vaccine could vary between routes of immunization. In support of this, purified LPS from **Brucella melitensis** administered SC to mice induced higher levels of LPS-specific serum IgG and IgG3 compared to IN delivery and provided superior protection against **Brucella** infection in the lung [54]. Thus, differences in antibody concentrations or subtypes specific for the LPS and/or CPS sub-components of the OMV could account for the observed differences in vaccine efficacy.We are currently examining each of these possibilities which may help explain differences between resistant and susceptible groups of immunized mice and ultimately provide insight into mechanisms of immunity to **B. pseudomallei**.

CMI responses are also an essential component of vaccine protection against **B. pseudomallei**, particularly once the organism establishes intracellular residence [20, 55]. Histological analyses demonstrate **B. pseudomallei** within macrophages in the lung, liver, and spleen [56, 57]. Thus, vaccine-induced sterile immunity has been extremely difficult to achieve [7]. Despite the small number of animals available for tissue burden assessment, both **B. pseudomallei** OMV SC- and IN-immunized mice demonstrated a reduction in **B. pseudomallei** tissue burden compared to control **E. coli** OMV immunized mice that survived challenge. This could reflect the significant production of IFN-γ observed in restimulated splenocytes in **B. pseudomallei** OMV-immunized animals [20, 58]. Antigen-specific T cells, particularly $CD4^+$ T cells, are important sources of IFN-γ and are essential for host resistance to acute and chronic infection with **B. pseudomallei** [55]. Notably, protection could not be attributed to IFN-γ production alone since the IN group succumbed to challenge. The frequency of T cells producing multiple cytokines (IFN-γ, TNF and IL-2), rather than IFN-γ alone, has been shown to correlate with protective vaccine responses against several intracellular pathogens including **M. tuberculosis** [59], **Leishmania major** [60] and **Plasmodium falciparum** [61]. OMVs can deliver virulence factors directly into the host cytoplasm via fusion of OMVs with lipid rafts in the host plasma membrane [62]. Moreover, degradation of OMVs in lysosomal compartments has also been observed [22]. These features may facilitate antigen presentation of OMV cargo via both MHC Class I and Class II, respectively. Thus, it will be important to delineate the role of single and multi-cytokine-producing $CD8^+$ and $CD4^+$ T-cells in response to the **B. pseudomallei** OMV vaccine [63].

In conclusion, inhalation of **B. pseudomallei** is a natural route of infection, and it represents the primary route of exposure in a deliberate biological attack. A **B. pseudomallei** vaccine should therefore be efficacious against this route of infection. Immunization with OMVs provided significant protection in the BALB/c mouse model of acute pneumonic

melioidosis.We propose that naturally-derived OMVs represent a safe, inexpensive, multi-antigen vaccine strategy against **B. pseudomallei** that promotes both humoral and CMI responses. The approach utilized in this work provides a foundation to further improve the **B. pseudomallei** OMV vaccine via future optimization studies examining dose, delivery, and adjuvant formulations. Furthermore, the success achieved with non-optimized, native **B. pseudomallei** OMVs in this study highlights an opportunity to extend vesicle-based vaccines to other clinically significant intracellular pathogens that have evaded traditional vaccination efforts.

Highlights

Outer membrane vesicle (OMV) immunization protects against **B. pseudomallei**>OMVs contain protein, LPS, and CPS which may contribute to protective efficacy>OMV immunization induces antibody and cell-mediated immune responses

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Grant Number U54 AI057156 from National Institute of Allergy and Infectious Diseases (NIAID)/NIH; Louisiana Vaccine Center/South Louisiana Institute for Infectious Disease Research Cooperative Pilot Research grant to L.M.; and a Tulane Research Enhancement Grant to L.M. and C.R. D.A. is funded through a subaward from National Institute of Allergy and Infectious Diseases Grant U54AI065359. O.Q. was supported by Grant Number 1U01AI078008-3. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the RCE Programs Office, NIAID, or NIH. We would like to express sincere gratitude to Joann Prior and Sarah Ngugi from The Defence Science and Technology Laboratory (Dstl), Porton Down, UK, for the gift of the anti-**B. pseudomallei** LPS Mab. We thank Monica Embers, Betzi Norton, Hailey Petersen, Daniel Frederick, and Stephanie Killeen for technical assistance. We also thank Jibao He, supervisor of the Tulane University Coordinated Instrument Facility, for assistance with electron microscopy and Chau-Wen Chou, director of the Louisiana State University Health Sciences Center Proteomics Core, for LC-MS analysis.

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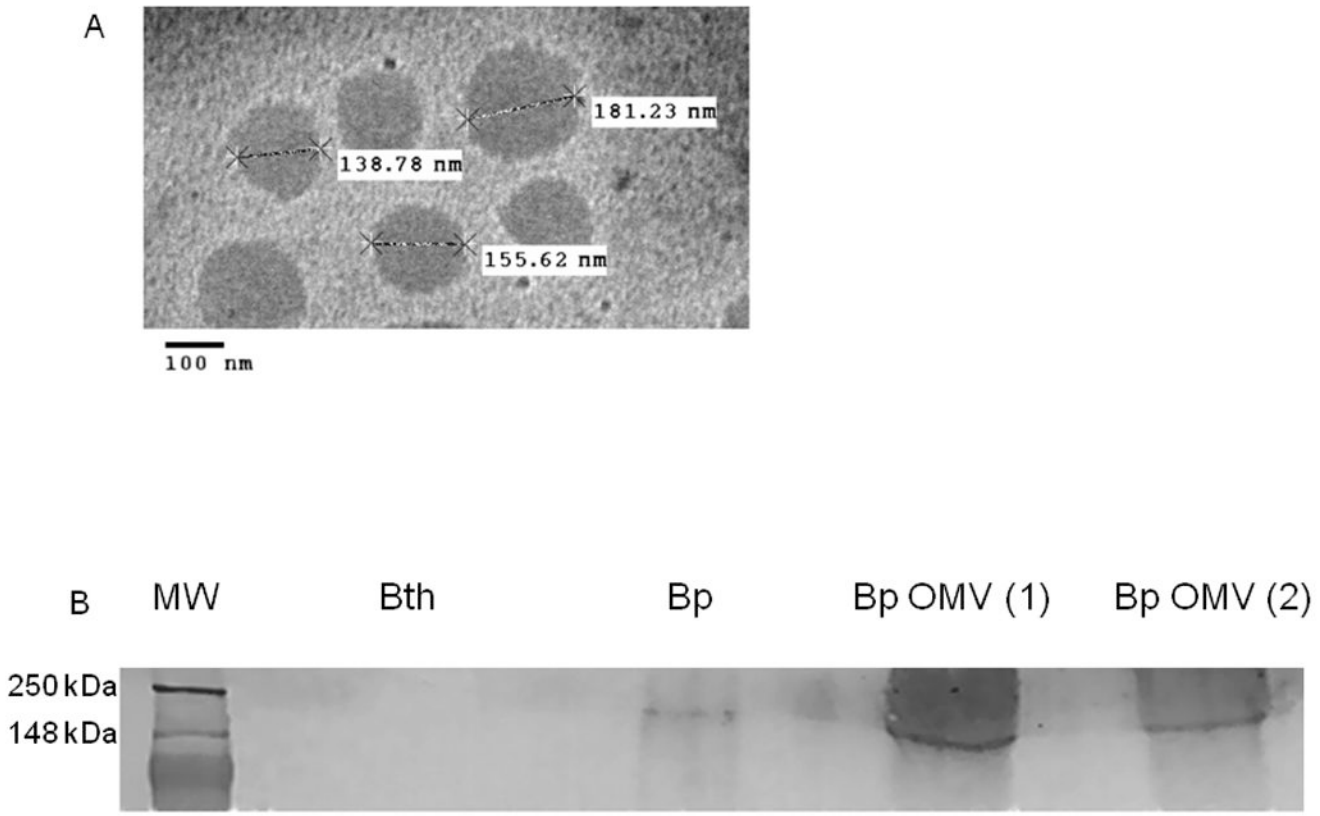

Figure 1. Characterization of B. pseudomallei OMVs

(A) Cryo-Transmission electron micrograph of **B. pseudomallei** OMVs. Purified OMVs (0.8 mg/ml) were diluted 1:10 in filtered sterile water for imaging. Image was taken using a JEOL 2010 Transmission Electron Microscope. Bar indicates 100 nm. (B) Western blot demonstrating the presence of capsular polysaccharide (CPS) in **B. pseudomallei** OMVs. Ten μg of two separate vaccine batches of Bp OMVs (1 and 2) were probed with monoclonal antibody 3C5 specific for **B. pseudomallei** CPS [33]. **B. thailandensis** (Bth), which lacks capsule, and **B. pseudomallei** 1026b (Bp) whole-cell lysates were used as negative and positive controls, respectively.

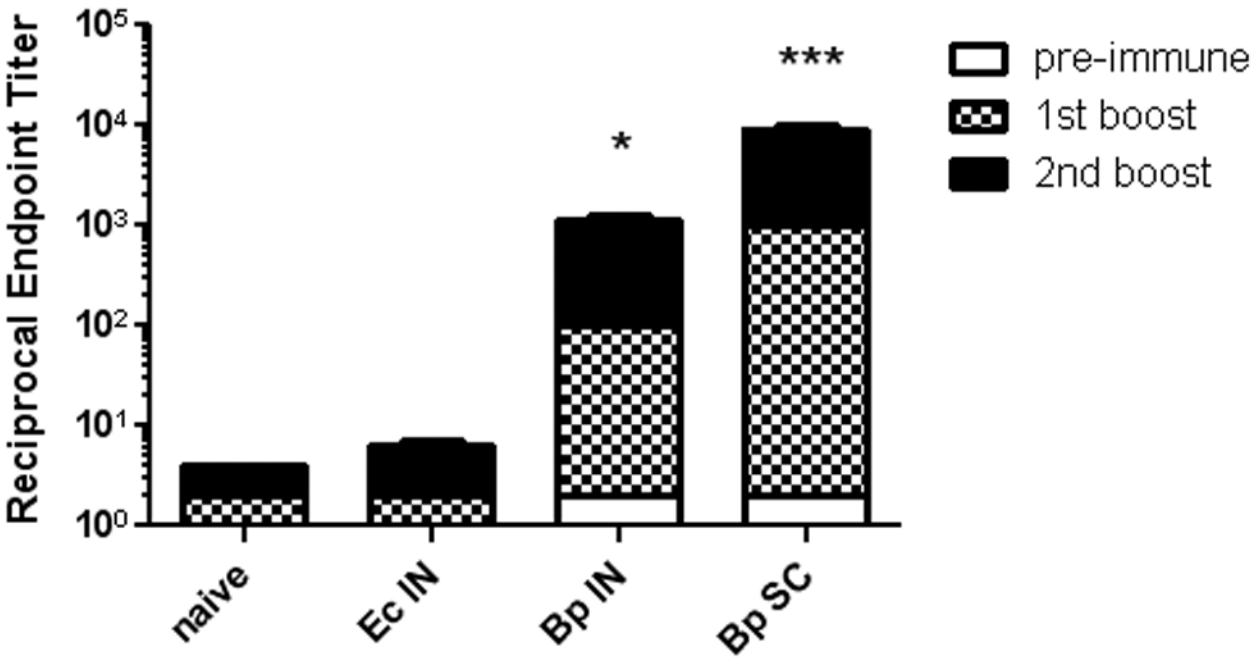

Figure 2. Serum IgG responses to B. pseudomallei OMVs are specific and do not require exogenous adjuvant

Mean reciprocal endpoint titers for **B. pseudomallei** OMV-specific serum IgG are shown for pre-immune sera, and sera obtained 3 weeks after two (1st boost) and three (2nd boost) administrations of 2.5 μg of **B. pseudomallei** or **E. coli** OMVs without exogenous adjuvant. Treatment groups (n=5 mice per group) are naïve = non-treated; Ec IN=**E. coli** OMV-immunized intranasally; **B. pseudomallei** IN=**B. pseudomallei** OMV-immunized intranasally; and **B. pseudomallei** SC=**B. pseudomallei** OMV-immunized subcutaneously. Asterisks indicate statistical difference of final endpoint titers compared to pre-immune titers within groups (*$\mathbf{P} < 0.05$, ***$\mathbf{P} < 0.001$ using a two-way ANOVA with Bonferroni's post-test).

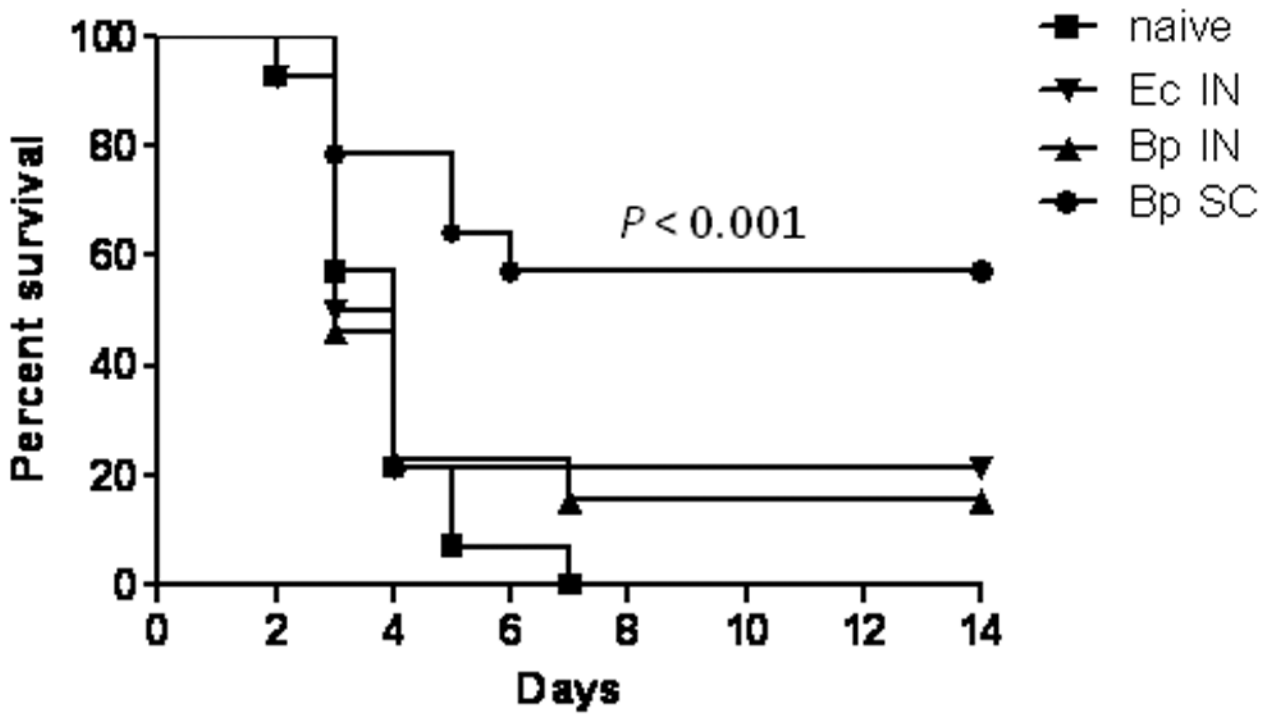

Figure 3. SC immunization with B. pseudomallei OMVs protects mice against lethal aerosol challenge

Mice (n=15 per group) were challenged with 5 LD_{50} of **B. pseudomallei** 1026b by small particle aerosol. Composite survival data from two independent experiments is shown through day 14. Mice immunized SC with **B. pseudomallei** OMVs were significantly protected (**P** < 0.001 using a log-rank Mantel-Cox survival analysis).

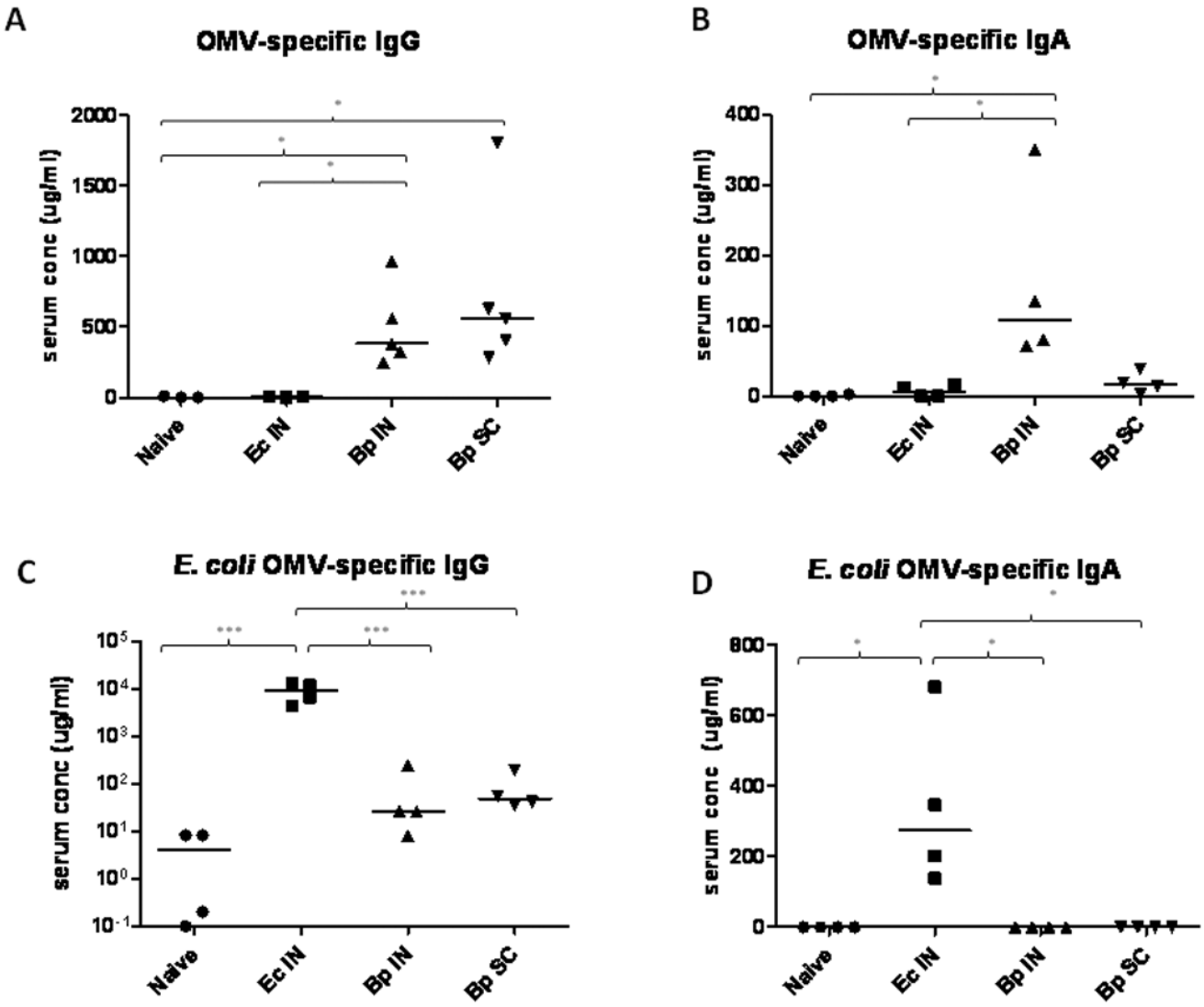

Figure 4. B. pseudomallei OMV immunization induces humoral immunity

B. pseudomallei OMV-specific serum IgG (A) and IgA (B) and **E. coli** OMV specific serum IgG (C) and IgA (D) were measured by ELISA. Microtiter plates were coated with 500 ng/well of purified **B. pseudomallei** OMVs or **E. coli** OMVs. Naïve = non-treated; Ec IN=**E. coli** OMV-immunized intranasally; Bp IN=**B. pseudomallei** OMV-immunized intranasally; and Bp SC=**B. pseudomallei** OMV-immunized subcutaneously. Horizontal line represents the median value for each group (n = 5). (***P** < 0.05, ****P** < 0.01, *****P** < 0.001 using a one-way ANOVA with Bonferroni's post-test).

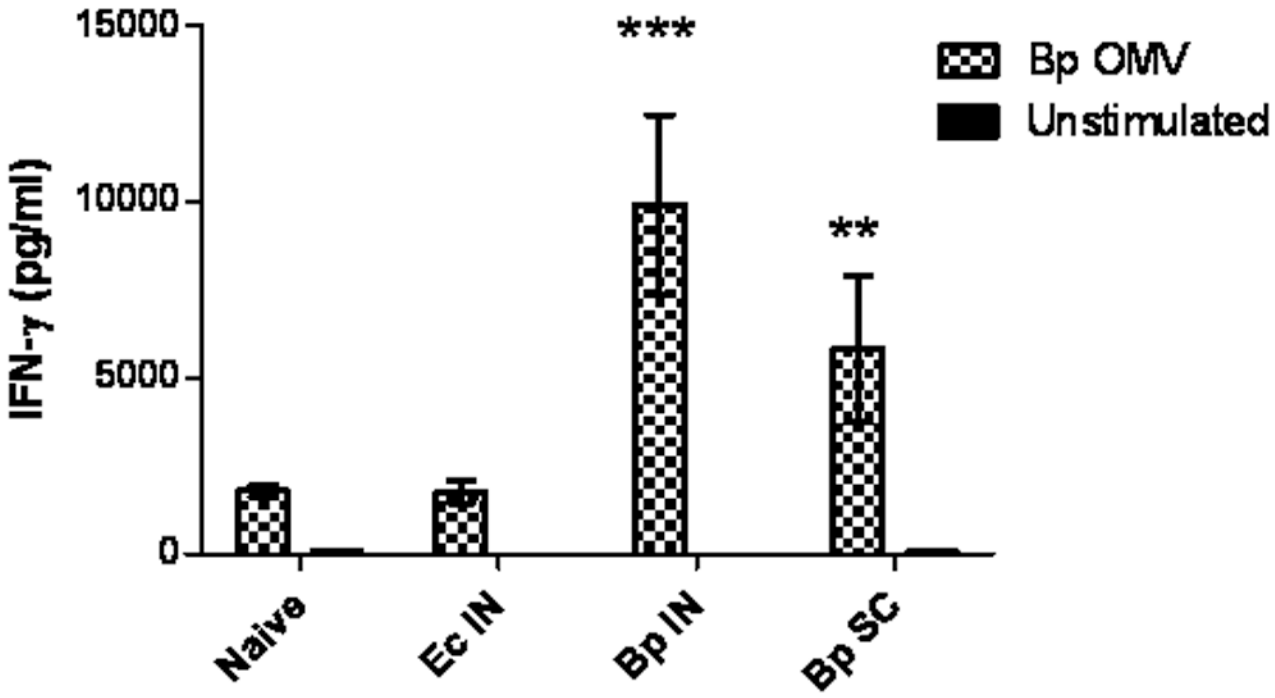

Figure 5. B. pseudomallei OMV immunization induces T cell memory responses

(A) Splenocytes from individual mice in each group (n = 3) were restimulated in triplicate with **B. pseudomallei** OMVs (2 μg) or ConA (1 μg, not shown) or left unstimulated, and cell culture supernatants were assayed in duplicate on day 3 for IFN-γ cytokine production. (****P** < 0.01, *****P** < 0.001 using a two-way ANOVA with Bonferroni's post-test).

Table 1

B. pseudomallei OMV-immunized mice demonstrate reduced bacterial burdens

Tissue bacterial burdens (CFU/organ) were determined in **E. coli** OMV- immunized (Ec IN), **B. pseudomallei** OMV IN-immunized (Bp IN), and **B. pseudomallei** OMV SC-immunized (Bp SC) mice at 14 and 30 days post-infection (p.i.). Three mice per group were utilized when possible. Number of mice (n) examined in each group is indicated in parentheses. Range in CFU recovered from replicate mice is reported.

	14 days p.i.				30 days p.i.		
Group (n)	**lung**	**liver**	**spleen**	**Group (n)**	**lung**	**liver**	**spleen**
Ec IN (2)	7×10^2 - 2×10^6	2×10^2 - 6×10^3	5×10^2 – 1×10^4	Ec IN (1)	3.5×10^2	2.6×10^3	1.2×10^2
Bp IN (1)	0	1×10^2	3×10^1	Bp IN (1)	1×10^1	3×10^1	0
Bp SC (3)[a]	0	1×10^1 - 3×10^1	3×10^3	Bp SC (3)[b]	1.3×10^2	6×10^1 – 2.9×10^2	1×10^1 – 5×10^1

[a] Only 1 mouse out of 3 was colonized in the spleen, therefore no range is provided.

[b] Only 1 mouse out of 3 was colonized in the lung, therefore no range is provided.

Table 2

Mean serum B. pseudomallei OMV-specific IgG1 and IgG2a concentrations (μg/ml) and IgG1:IgG2a ratios

Ratios > 1 indicate a type 2 humoral immune response, while ratios < 1 indicate a type 1 cellular immune response. ND = non-detectable

Group	IgG1	IgG2a	Ratio
Naïve	ND	3.7	-
Ec IN	ND	7.1	-
Bp IN	413.0	33.9	12.2
Bp SC	324.5	43.2	7.5