**Evaluation of a biodegradable microparticulate polymer as a carrier for
Burkholderia psuedomallei subunit vaccines in a mouse model of melioidosis.**

Schully K.L.¹, Bell M.G.¹, Prouty A.M.¹, Gallovic M.D.², Gautam S.³, Peine K.J.⁴, Sharma S.³, Bachelder E.M.⁴, Pesce J.T.¹, Elberson M.A.¹, Ainslie K.M.^{4*}, Keane-Myers A.¹

¹Vaccines and Medical Countermeasures, Biological Defense Research Directorate,
Naval Medical Research Center-Frederick, Ft Detrick, Maryland 21702, USA.

²William G. Lowrie Department of Chemical and Biomolecular Engineering, College of
Engineering, The Ohio State University, Columbus, Ohio, 43210, United States

³Department of Pharmaceutical Sciences, College of Pharmacy, The Ohio State
University, Columbus, Ohio, 43210, United States

⁴Division of Molecular Pharmaceutics, Eshelman School of Pharmacy, University of
North Carolina, Chapel Hill, North Carolina 27599, United States

*Corresponding Author Information:

Kristy M. Ainslie, PhD

Associate Professor

UNC Eshelman School of Pharmacy

Division Molecular Pharmaceutics

4211 Marsico Hall

125 Mason Farm Rd

Chapel Hill, NC 27599

Ph: 919-962-4556

Fax: 919-962-9922

Email: ainsliek@email.unc.edu

Keywords: subunit vaccine, biodegradable polymer, acid-labile, bacterial vaccine,
bioterrorism agent

ABSTRACT (250 words)

Melioidosis, a potentially lethal disease of humans and animals, is caused by the soil-dwelling bacterium *Burkholderia pseudomallei*. Due to *B. pseudomallei*'s classification as a Tier 1 Select Agent, there is substantial interest in the development of an effective vaccine. Yet, despite decades of research, no effective target, adjuvant or delivery vehicle has been identified capable of inducing protective immunity against *B. pseudomallei* infection. We propose a microparticulate delivery vehicle comprised of the novel polymer acetalated dextran (Ac-DEX). Ac-DEX is an acid-sensitive biodegradable carrier that can be fabricated into microparticles (MPs) that are relatively stable at pH 7.4, but rapidly degrade after phagocytosis by antigen presenting cells where the pH can drop to 5.0. As compared to other biomaterials, this acid sensitivity has been shown to enhance cross presentation of subunit antigens. To evaluate this platform as a delivery system for a melioidosis vaccine, BALB/c mice were vaccinated with Ac-DEX MPs encapsulating *B. pseudomallei* whole cell lysate and the toll-like receptor (TLR) 7/8 agonist resiquimod. This vaccine elicited a robust antibody response that included both Th1 and Th2 immunity. Following lethal intraperitoneal challenge with *B. pseudomallei* 1026b, vaccinated mice demonstrated a significant delay to time of death compared to untreated mice. The formulation demonstrated incomplete protection however, indicating that lysate protein offers limited value as an antigen. Nevertheless, our Ac-DEX MPs may offer an effective delivery vehicle for a subunit *B. pseudomallei* vaccine.

51 INTRODUCTION

52 Melioidosis (reviewed in (Dance; Dance; Vietri and DeShazer)) is caused by
53 infection with the bacterium *Burkholderia pseudomallei*. This bacterium is ubiquitous in
54 the soil in much of Southeast Asia and Northern Australia, and exposure is thought to be
55 through inhalation, ingestion, or direct inoculation through the skin. Infection from *B.*
56 *pseudomallei* is the third highest cause of death from infectious diseases in northeast
57 Thailand (after HIV/AIDS and tuberculosis) and is the most common cause of
58 community-acquired pneumonia in parts of Northern Australia. Additionally, *B.*
59 *pseudomallei* is considered a biothreat agent and has been designated a Tier 1 Select
60 Agent by the Centers for Disease Control and Prevention (CDC) . Clinical manifestations
61 can be acute or chronic and can express across a wide spectrum including lethal sepsis,
62 localized abscess formation or latent infection. Melioidosis typically presents with non-
63 specific, flu-like symptoms, and even in endemic areas it is frequently misdiagnosed.
64 The acute disease can have a rapid onset and progression, but gradually developing
65 chronic cases are also common. Relapse and reactivation from latent foci can also occur
66 many decades after initial infection, despite prolonged antibiotic treatment .

67 To date, a vaccine capable of conferring a protective and sterilizing immunity
68 against *B. pseudomallei* has yet to be developed. Most vaccine strategies that have
69 proven effective against other pathogens have resulted in little to no success as a
70 melioidosis vaccine. For example, live attenuated vaccines, which have proven effective
71 for a variety of human pathogens (Liljeqvist and Stahl; Pirofski and Casadevall; Titball),
72 have been attempted for immunization against *B. pseudomallei* over the past 60 years
73 (Atkins et al.; Breitbach et al.; Dannenberg and Scott, b; Druar et al.; Haque et al.; Pilatz
74 et al.; Stevens et al.). Invariably, protection is incomplete and survivors often remain
75 colonized with *B. pseudomallei*. The same is true for killed whole cell vaccines, and

subunit vaccines as well (Patel et al.). Novel approaches such as immunization with outer membrane vesicles (OMV) derived from *B. pseudomallei* have proven somewhat effective (60% protection) in murine models (Nieves et al., 2014) and immunogenic and safe in non-human primate models (Petersen et al.). With this in mind, microencapsulation of antigens and adjuvants may prove to be a promising strategy towards the development of a melioidosis vaccine.

Nano/microparticles (NP/MPs) derived from biomaterials such as polyesters (e.g. poly(lactic-co-glycolic acid) (PLGA), polylactic acid (PLA), poly- ϵ -caprolactone (PCL) (reviewed in (Mahapatro and Singh)), liposomes (Peine et al.) , lipid–polymer hybrid nanoparticles (Hadinoto et al.) and acetalated dextran (Ac-DEX) (Bachelder et al.; Bachelder et al.) are currently being explored as carriers for vaccines against a variety of diseases. Encapsulation in these vehicles has demonstrated enhanced antigen presentation (Audran et al.; Hamdy et al.; Shen et al.; Waeckerle-Men et al.) and may provide effective vehicles for vaccines (Joshi et al.). PLGA is the most widely used polymeric carrier because it is FDA-approved for subcutaneous long-acting delivery, however, issues with the development of a low microenvironmental pH resulting from its acidic degradation byproducts (Ding and Schwendeman, 2008; Li and Schwendeman, 2005) can be damaging to antigen payloads, requiring the addition of basic neutralizing agents (Jiang and Schwendeman, 2008). Whereas polyester-based particles have concerns with acidic hydrolytic byproducts, lipid-based particles have limited long-term stability and cannot be stored outside the cold chain (Andresen et al., 2005). In contrast to these drawbacks, the newly developed Ac-DEX has tunable degradation with pH-neutral hydrolytic byproducts (Bachelder et al.; Broaders et al.), and can stabilize proteins during storage outside the cold chain (Kanthamneni et al., 2012). We have also recently described the use of Ac-DEX MPs encapsulating an anthrax vaccine to rapidly

protect mice against a lethal inhalational challenge with *Bacillus anthracis* (Schully et al.).

In addition to the aforementioned advantages of Ac-DEX, we previously demonstrated that Ac-DEX MPs are capable of activating a significantly larger population of CD8⁺ T cells specific for encapsulated antigens than other biomaterials such as non-pH-sensitive PLGA (Broaders et al.). This feature is advantageous for a melioidosis vaccine because there is a strong correlation between the resolution of clinical melioidosis and T cell responses (Barnes et al.; Healey et al.; Ketheesan et al.; Ramsay et al.; Tipayawat et al., 2011), and it is highly likely that cytotoxic T lymphocytes (CTLs) will play an important role in generating a protective immune response (Bondi and Goldberg, 2008; Haque et al., 2006b). CD8⁺ T cells and natural killer (NK) cells control various pathogens by secreting effector cytokines and cytolytic granules containing perforin and various granzymes that together disrupt cell membranes and incite apoptotic death (Lauw et al., 2000; Orange and Ballas, 2006; Smith et al., 2009). Their ability to produce INF- γ , form antigen-specific memory cells, and destroy infected cells makes CTLs a prime candidate for a vaccine to elicit. The ability of Ac-DEX MPs to elicit larger CTL populations would suggest Ac-DEX MPs the ideal delivery vehicle for eliciting a strong CTL response.

Here, we describe the first *B. pseudomallei* vaccine preparation encapsulated in a biodegradable polymer and evaluate the murine immune response to encapsulated antigens. In this study we utilized Ac-DEX MPs encapsulating both resiquimod and a whole cell lysate of *B. pseudomallei* for immunization of BALB/c mice. We characterized the immune response by analyzing systemic antibody levels and *ex vivo* cytokine production following antigen restimulation of splenocytes. Cellular responses were also evaluated through flow cytometry. Finally, we evaluated the ability of the vaccine formulations to protect mice from lethal challenge with *B. pseudomallei*. Overall, we

report a new microparticulate formulation for a *B. pseudomallei* vaccine delivery platform.

MATERIALS AND METHODS

All materials were purchased from Sigma (St. Louis, MO) and used as purchased, unless otherwise indicated.

Bacterial Strains

Burkholderia pseudomallei strain 1026b was obtained through the Critical Reagents Program, JPM-MCS, JPEO, DoD (cat # BURK003).

***B. pseudomallei* Whole Cell Lysate Preparation**

To prepare the whole cell *B. pseudomallei* lysate, strain 1026b was inoculated in 25 mL of Luria Bertani Broth (LB) – Lennox formulation, and incubated at 37°C while shaking for 24 hours. One mL of the resulting culture was used to inoculate four 2 L flasks containing 500 mL of LB which were incubated for an additional 24 hours. The resulting bacteria were then harvested by centrifugation, frozen at -80°C and exposed to 2.5×10^6 rad of ionizing radiation in a ^{60}Co irradiator for sterilization. Sterility was confirmed through bacterial culture (negative for growth) and frozen before processing into a whole cell lysate.

The sterilized pellet was resuspended in approximately 45 mL of phosphate buffered saline. The resulting slurry was passed through an M110P-20 Microfluidizer (Microfluidics, Westwood, MA) twice to lyse the bacteria at 20,000 psi, 7°C. During microfluidization, the slurry was diluted to 220 mL with 50 mM HEPES, 15 mM EDTA, and 100 mM NaCl, pH 7.4. The lysate was depth (0.5/1.0 micron) filtered to remove

particulates. Three Triton X-114 phase extractions were performed to remove endotoxin which was later determined to be 6.8×10^3 EU/mg by LAL assay (Lonza).

Ac-DEX Synthesis

Dextran (molecular weight = 10 or 71 kDa) was used to synthesize Ac-DEX (Bachelder et al., 2010a; Bachelder et al., 2010b; Kauffman et al., 2012; Meenach et al., 2012). Briefly, dextran was dissolved in anhydrous dimethyl sulfoxide (DMSO) under nitrogen with the acid catalyst pyridinium p-toluenesulfonate, reacted with 2-methoxypropene for a predetermined time (four hours or five minutes for 10 or 71 kDa dextran, respectively) at room temperature, and quenched with triethylamine (TEA). The product was precipitated drop-wise in water made basic (basic water; 0.02% v/v TEA in water; pH 9.0), filtered under vacuum, and freeze-dried. To purify the product, it was then dissolved in ethanol, any undissolved solids were removed using an Allegra 25R Centrifuge (10 min, 10,000 RPM, Beckman Coulter, Los Angeles, CA, USA), the supernatant was re-precipitated in basic water, and the product was again filtered under vacuum and lyophilized to yield purified Ac-DEX.

Ac-DEX NMR Analysis

The NMR spectrum of Ac-DEX was recorded with an AV300 ^1H -NMR (Bruker Biosciences Corporation, Billerica, MA) after suspending ~10 mg of the product in 700 μL of deuterium oxide and rapidly dissolving it with 30 μL deuterium chloride. The cyclic:acyclic acetal ratio was determined from the ratio of the acetone to ethanol peaks found on the NMR spectra (Broaders et al., 2009). The Ac-DEX used for encapsulation of *B. pseudomallei* lysate was prepared from 10 kDa dextran reacted for four hours (relative cyclic:acyclic acetal coverage [CAC] of 56.7%) and for encapsulation of resiquimod, 71 kDa dextran reacted for five minutes was used (relative CAC of 38.6%).

These different dextran molecules were used to maximize the encapsulation efficiency of the respective compound.

Resiquimod Particle Fabrication

Resiquimod was purchased from Alexis Biochemicals, Enzo Life Sciences (Farmingdale, NY). 3% w/w of resiquimod in Ac-DEX was co-dissolved in dichloromethane (DCM), combined with 3% polyvinyl alcohol (PVA) in phosphate buffer solution (PBS), vortexed for 2 minutes to create an initial macroscopic emulsion, and then ultrasonicated for 30 seconds using a Misonix Ultrasonic Liquid Processor (Qsonica, LLC, Newtown, CT) at 20 W and a 50% duty cycle. The emulsion was then added to 0.3% PVA in PBS and spun on a stir plate for three hours. PVA and unencapsulated resiquimod were then washed away by tangential flow filtration. This was done by continuously flowing the particle suspension through a cycle consisting of hollow fiber filter modules (Spectrum Laboratories, Inc., Rancho Dominguez, CA) until the particles were concentrated and most of the water removed. This cycle was performed five times with the addition of a fresh 50 mL of basic water after each concentrating step. The modules possess a molecular weight cut off greater than the MW of both PVA and resiquimod, allowing these components to permeate through the pores of the filter and be washed away in the aqueous phase. The size of the particles allowed them to be held in the retentate and recovered at the end of the procedure with another aliquot of 50 mL basic water. The particles were frozen on dry ice and then lyophilized without the addition of any additional cryoprotectants. Blank MPs were made following the same procedure but without adding resiquimod.

Lysate Protein Content Determination

Initially, the protein content of the lysate was characterized using a bicinchoninic acid assay (BCA assay; Thermo Fisher Scientific, Rockford, IL) with bovine serum

albumin as a standard. Since the working reagent in the assay cross-reacts with dextran, protein concentration was confirmed with a fluorescamine (4-phenyl-spiro [furan-2(3H), 1'-phthalan] -3,3' -dione) assay (Udenfriend et al., 1972), as it would be later used to calculate the lysate encapsulation in Ac-DEX MPs.

Lysate Particle Fabrication

Ac-DEX was first dissolved in DCM (5% w/v). Lysate was dissolved in PBS and 100 μ L was added to the DCM solution such that the protein was added at 5.0% w/w. The initial water in oil (W/O) emulsion was homogenized for 30 seconds at 20,000 RPM using a T25 Digital Ultra-Turrax (IKA Works, Wilmington, NC), 3% PVA solution was then added to the primary emulsion, and homogenized again for 30 seconds to create a water in oil in water (W/O/W) emulsion. This was immediately added to a 0.3% PVA in PBS solution and stirred for three hours. The suspension was then centrifuged (10 min, 10,000 rpm, 4°C) and the supernatant discarded. Particles were washed three times with basic water to remove PVA and unencapsulated lysate, and then suspended in basic water and freeze-dried. Blank MPs were made following the same procedure but without adding lysate.

Encapsulation Efficiency

The encapsulation efficiency (EE) of resiquimod was determined by dissolving loaded particles at 1 mg/mL in DMSO and measuring resiquimod's innate fluorescence (Ex: 260nm / Em: 360nm) on a Spectra Max Gemini XS microplate reader (Molecular Devices, Sunnyvale, CA). Fluorescence values were compared to a standard curve of resiquimod. Blank particles were treated the same way and used for background subtraction.

Lysate EE was determined using a fluorescamine assay (Udenfriend et al.,

1972). The loaded particles were prepared at a concentration of 1 mg/mL. This was done by first suspending the loaded particles in 99% v/v PBS and then adding a 0.5% v/v solution of 50% v/v formic acid (in water) to create an acidic solution (~pH 3.0). The particles were allowed to degrade overnight at 37°C. The following day, the pH of the solution was adjusted to ~7.4 with a 0.5% v/v solution of 13.25 M NaOH to create the total 1 mg/mL solution. 150 µL of the sample solutions were reacted with 50 µL of 3 mg/mL fluorescamine in acetone and the resulting fluorescence (Ex: 390nm / Em: 460nm) was read on a microplate reader. Fluorescence values were compared to a standard curve of lysate. Blank particles were treated the same way and acted as a background subtraction.

Scanning Electron Microscopy

Electron micrographs using scanning electron microscopy (SEM) were obtained using a Nova NanoSEM 400 (Field Emission, Inc., Hillsboro, OR). To prepare the samples, a 10 mg/mL suspension of particles in basic water was pipetted onto an aluminum pin stub specimen mount (Ted Pella; Redding, CA) and the samples were left to air dry at room temperature. After sputter coating with a layer of gold-palladium alloy for 120 seconds using a Sputter Coater 108 (Cressington Scientific Instruments, Watford, England), the samples were analyzed with SEM.

Dynamic Light Scattering

Resiquimod and lysate particles were prepared in basic water (pH 9.0) and diluted to a concentration such that the particle suspension displayed a reading between 50 and 200 kilocounts per second (kcps) when read on a BI-200SM Dynamic Light Scattering (DLS) system (Brookhaven Instruments, Holtsville, NY). The detector, set at a 90° angle of the laser source, was used with a wavelength of 633 nm and pinhole setting

of 200. The size of the particles (number weighted) was obtained and the average of five readings (2 minute collection time per reading).

Vaccine Preparations

B. pseudomallei lysate protein was prepared as described above and where appropriate 10 µg was administered as either free protein or protein encapsulated in Ac-DEX MPs (Lysate/MP). Protein was absorbed onto alum by mixing with 10 µg of lysate protein with Imject (Thermo Scientific) at a 1:1 (vol:vol) ratio and vigorously mixing on a vortex for 30 minutes (Lysate + Alum). Resiquimod encapsulated in Ac-DEX MPs (Resiq/MPs) was administered at 8 µg per mouse.

***In vivo* studies**

The experiments reported herein were approved by our Institutional Animal Care and Use Committees and conducted in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facilities where this research was conducted are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adhere to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011. Female BALB/c mice were 6-8 weeks old at the time of arrival and were allowed one week for acclimation. They were maintained on a 12-hour light cycle and had access to rodent feed and water ad libitum.

Immunization schedules and animal numbers are described in the text. Two immunizations were prepared as described above and administered subcutaneously in the combinations outlined in Table 1. 200 µL of each vaccine preparation was administered under the scruff of the neck with a 25G 5/8" needle.

271 BALB/c mice were challenged by an intraperitoneal (ip) injection of *B.*
272 *psuedomallei* 1026b. When necessary, moribund mice were euthanized under deep
273 ketamine/xylazine anesthesia. Those mice which survived to the study endpoint were
274 euthanized by administration of deep ketamine/xylazine anesthesia followed by
275 exsanguination via cardiac puncture. The lungs, liver and spleen were harvested and
276 homogenized in 1 mL of PBS and the resulting suspensions were serially diluted and
277 plated on LB agar (Lennox) supplemented with kanamycin (5 µg/mL). Prior to clotting,
278 blood was also serially diluted and plated.

279 **Antibody and Subclass Determination**

280 Prior to each immunization and challenge, blood was collected from the lateral
281 tail vein. Whole blood was allowed to clot at room temperature before centrifugation at
282 10,000 rpm for 10 minutes. Sera was removed to a fresh tube and stored at -80°C until
283 use. Total IgG and subclass (IgG1 and IgG2a) concentrations were determined by
284 Enzyme Linked Immunosorbant Assays (ELISA) as described previously (Schully et al.)
285 except that the capture antigen was the *B. psuedomallei* lysate protein utilized during
286 vaccination. Briefly, Immulon 4HBX 96 well microtiter plates were coated with lysate
287 protein (10 µg/mL) in 0.2 M carbonate coating buffer (pH 7.4) overnight. Plates were
288 blocked with 5% skim milk in phosphate buffered saline plus 0.1% Tween-20 (PBST) for
289 one hour, washed three times with PBST and two-fold serial dilutions of serum were
290 added to the coated plates followed by incubation for one hour. The plates were washed
291 three times with PBST and the horse radish peroxidase-conjugated secondary antibody
292 was added. Goat-anti-mouse IgG (KPL, Gaithersburg, MD), goat-anti-mouse IgG1 (Life
293 Technologies, Grand Island, NY) or goat-anti-mouse IgG2a (Life Technologies) were
294 diluted 1:12,800 and incubated for 1 hour at 37° C, washed three times with PBST and
295 again with PBS. Plates were developed by the addition of ABTS one-component

substrate (KPL, Gaithersburg, MD) and incubated for 30 minutes. The reaction was stopped with the addition of 1% SDS and the absorbance at 405 nm (A405) was recorded by a standard plate reader. Antibody titers are expressed as the reciprocal endpoint titers of serum dilutions that exhibited an A405 greater than the cutoff value of three standard deviations above the mean for control wells.

Antigen Recall and Cytokine Analysis

Antigen specific recall of vaccinated splenocytes was performed as described previously (Schully et al.) except that 5 µg/mL of the lysate protein prepared above was used to stimulate the splenocytes. Briefly, 28 days post vaccination, mice were euthanized by the administration of deep ketamine/xylazine anesthesia followed by exsanguination. Spleens were removed aseptically and disrupted by pushing them through a 100 µm nylon cell strainer (Fisher). The cells were then resuspended in 5 mL of complete RPMI medium (RPMI 1640+GlutaMax with penicillin-streptomycin (5 mL/500 mL), L-glutamine (5 mL/500 mL), 10% Hyclone FBS heat inactivated (56° for 30+ min, 50 mL/500 mL), and 0.1% 2-mercaptoethanol (0.5 mL/500 mL)). Cells were centrifuged for 10 min at 1500 RPM, 4°C. Red blood cells were lysed with the addition of two milliliters of ice cold Ammonium-Chloride-Potassium (ACK) lysis buffer and incubated on ice for two minutes. The cells were then washed by resuspending in 10 mL of complete RPMI, and centrifuged for 10 min at 1500 rpm at 4°C. The supernatant was removed and the cells were resuspended in 5 mL of complete RPMI. Finally, 1×10^6 viable splenocytes (as determined by trypan blue exclusion) from each spleen were added to a 96-well tissue culture plate. The splenocytes were then left unstimulated or stimulated in triplicate with either concanavalin A (5 µg/mL) or *B. pseudomallei* lysate protein (5 µg/mL) in RPMI for 72 h at 37°C in 5% CO₂. Following incubation, the concentration of cytokines secreted into culture supernatants were quantified using Bio-Plex Pro (Bio-

Rad) multiplex cytokine assay kits customized for quantifying IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, TNF- α , GM-CSF, IL-17a and IFN- γ and measured on a Bio-Plex200 (Bio-Rad) per the manufacturer's instructions.

Flow Cytometry and De-Granulation Assay

Post-vaccination antigen specific CTL activity of CD8⁺ cells in splenocytes was measured using CD107a (LAMP-1) as a marker of de-granulation. Splenocytes were harvested and cultured in the presence of 5 μ g/mL lysate protein for 4 hours. PBS was used as the negative control. Splenocytes were stained for CD107a, CD8, CD3 and CD4 cell surface markers (Fischer Scientific) and flow cytometric acquisition and analysis was done using a BD FACS Caliber instrument (BD Biosciences, San Jose, CA). The de-granulation protocol was followed as described elsewhere (Gautam et al.; Smith et al., 2009).

Statistics

All data was entered into Graphpad Prism V5 and analyzed using the appropriate statistical package. Survival curves were analyzed using the Gehan-Breslow-Wilcoxon test weighted for the rapid death of control animals and length of the study (post challenge). Antibody titers were analyzed using Wilcoxon matched-pairs signed rank test. Cytokine analysis was performed using a one-way ANOVA followed by Tukey's post-hoc multiple comparison test to determine statistical significance.

RESULTS and DISCUSSION

Particle Preparation and Characterization

Because a specific vaccine antigen(s) has yet to be identified for *Burkholderia* pathogens (Peacock et al., 2012), we chose to encapsulate a whole-cell lysate of *B.*

psuedomallei 1026b in Ac-DEX MPs. The TLR 7/8 agonist resiquimod (R-848) was used as the adjuvant. Resiquimod, a member of the imidazoquinoline family of compounds, has previously been shown to be a potent activator of antigen presenting cells (Wu et al., 2004) and functions well as a vaccine adjuvant when encapsulated in Ac-DEX (Schully et al.). Ac-DEX encapsulated vaccine preparations have previously been shown to provide rapid protection against multiple *Bacillus anthracis* challenges (Schully et al.). For the current study, we encapsulated *B. psuedomallei* lysate and resiquimod into separate Ac-DEX MPs (Figure 1). Optimization of the encapsulation efficiency of resiquimod requires a high energy ultrasonication method. In contrast, to maintain the integrity of the lysate proteins, lower energy homogenization was used to encapsulate the lysate (Kanthamneni et al., 2012). These different fabrication methods resulted in differently sized particles (Table 2). The encapsulation efficiency of the components (Table 2) was well within the range we have observed with other protein encapsulates (Bachelder et al., 2008; Broaders et al., 2009; Schully et al., 2013) and resiquimod (Duong et al., 2013; Schully et al., 2013). The final loading of the *B. psuedomallei* lysate components was 13.4 and 4.2 µg /mg Ac-DEX for lysate protein and resiquimod, respectively (Table 2). This led to a total particle mass for injection of 0.8 mg for the Lysate/MP group and 2.6 mg for the co-delivered group (Lysate/MP + Resiq/MP) (Table 1).

Ac-DEX encapsulated *B. psuedomallei* antigens elicit rapid partial protection and extends time to death following lethal challenge.

Ac-DEX encapsulated vaccine preparations have previously been shown to provide rapid protection against vigorous Anthrax challenge (Schully et al.). In an effort to determine if Ac-DEX encapsulated *B. psuedomallei* antigens and resiquimod could protect against a lethal challenge with *B. psuedomallei*, vaccine preparations described in Table 2 were screened using BALB/c mice. In this trial, immunization schedules and

animal numbers (n=25) were established by our funding agency – The Defense Advanced Research Projects Agency (DARPA). As depicted in Figure 2, two vaccinations were administered seven days apart followed by intraperitoneal challenge with 1.4×10^6 cfu per mouse seven days after the second vaccination. Challenged mice were observed for 26 days post challenge. At the study endpoint, surviving mice were euthanized as described and their blood, lungs, livers, and spleens were harvested for *B. pseudomallei* colonization assessment.

As shown in Figure 3, 96% of control mice (i.e., those vaccinated with PBS or Empty MP) succumbed to infection by day two, yet all other groups persisted. Mice vaccinated with free lysate adjuvanted with encapsulated resiquimod (Lysate + Resiq/MP) showed 100% survival for 10 days which diminished to 88% by day 14. The 14 day survival for mice vaccinated with encapsulated protein and encapsulated resiquimod (Lysate/MP + Resiq/MP) was 72%. Mice were monitored for an additional 12 days during which time progressive death was observed in all groups. Ultimately, 16% of mice vaccinated with Lysate + Resiq/MP, 12% of mice vaccinated with Lysate/MP + Resiq/MP, and 8% of mice vaccinated with lysate protein adsorbed on alum with encapsulated resiquimod (Resiq/MP + Lysate + Alum) survived to the study endpoint of 26 days whereas no negative control mouse (i.e., PBS or Empty MP) survived beyond 20 days ($p < 0.0001$). Survival rates in mice immunized with Ac-DEX vaccine preparations significantly differed ($p < 0.0001$ for Lysate + Resiq/MP and Lysate/MP + Resiq/MP; $p = 0.0033$ for Resiq/MP + Lysate + Alum) from adjuvant only controls (Resiq/MP). Finally, the survival rates in mice immunized with Ac-DEX vaccine preparations performed significantly better ($p < 0.001$ for Lysate + Resiq/MP and Lysate/MP + Resiq/MP; $p = 0.0202$ for Resiq/MP + Lysate + Alum) when compared to a more traditional vaccine preparation such as protein lysate adsorbed on alum (Lysate + Alum). Upon necropsy of

surviving mice, lungs, livers, spleens, and blood were harvested and analyzed for the presence of *B. pseudomallei* using standard bacteriological methods. No *B. pseudomallei* was detectable in the tissues of some survivors (Figure 4) suggesting sterilizing immunity was achieved in a fraction of all the mice immunized with encapsulated resiquimod: Resiq/MP (1 of 2 survivors), Lysate + Resiq/MP (2 of 4 survivors), Lysate/MP + Resiq/MP (2 of 3 survivors), and Resiq/MP + Lysate + Alum (2 of 2 survivors)

Incomplete protection and host-organ colonization are major obstacles of *B. pseudomallei* vaccine research. Short-term survival followed by progressive death is a common occurrence and has been observed by numerous investigators (Sarkar-Tyson and Titball). While some studies have observed low levels of sterilizing immunity (Whitlock et al.), even in the presence of significant protection, organ burdens are often reduced rather than cleared (Nieves et al.; Scott et al.). Significantly increased times to death coupled to the observation of sterile animals suggest our Ac-DEX encapsulation approach holds promise for future *B. pseudomallei* vaccines.

In the present study, we attempted to remove the endotoxin to prevent its presence from confounding the conclusions about our choice of adjuvant (resiquimod). We were able to reduce endotoxin levels significantly to 6.8×10^3 EU/mg of lysate, which equates to 68 EUs of endotoxin containing approximately 6.8 ng of LPS per vaccination. Considering the rapid deterioration of groups vaccinated with either lysate protein alone (Figure 3, Lysate) or Ac-DEX encapsulated lysate (Figure 3, Lysate MP), it is doubtful that this level of endotoxin contamination had any antigenic or adjuvanting effects or contributed to survival.

Resiquimod is a toll-like receptor (TLR) 7/8 agonist that activates NF- κ B and other signaling via a MyD88 dependent pathway. Similarly, CpG motifs (TLR 9 agonist

(Hemmi et al.; Krieg et al.)) have been shown to protect mice from a variety of bacterial pathogens including *B. pseudomallei* (Duong et al., 2013; Elkins et al.; Rozak et al.; Waag et al.). Moreover, TLR 3 agonist lipopolysaccharide (LPS) has been studied as a potential *B. pseudomallei* vaccine candidate in various forms (Nelson et al.; Ngugi et al.). In our study, the two surviving mice, one sterile, from the Resiq/MP group displays a similar response as the CpG study, wherein stimulation of TLR 7/8 by resiquimod results in protection. Ac-DEX It is possible that Ac-DEX encapsulated resiquimod, aided by the shortened vaccination schedule utilized here, stimulated the innate immune response and cleared the *B. pseudomallei* infection.

Ac-DEX encapsulated *B. pseudomallei* antigens elicit a mixed and robust immune response. Given the modest protection we observed above, we sought to characterize the immune response elicited by Ac-DEX encapsulated *B. pseudomallei* antigens in the most promising combinations from above. However, the protection observed in the previous trial could be attributed to the truncated vaccination schedule resulting in an activated immune system at the time of challenge. To mitigate this potentially confounding factor, we attempted a more traditional vaccination schedule to allow the vaccinated mice to further develop an appropriate immune response. We have previously demonstrated that Ac-DEX encapsulating subunit antigens, when delivered in combination with encapsulated resiquimod, elicits a robust combined Th1 and Th2 response (Schully et al.). To examine the *in vivo* host response to the *B. pseudomallei* Ac-DEX vaccine preparations, we vaccinated BALB/c mice with vaccine preparations described in Table 1. As shown in Figure 5, two vaccinations separated by 14 days were administered and blood was collected prior to each vaccination and 14 days after the second vaccination. PBS immunized mice served as the only control because previous studies demonstrated that empty Ac-DEX particles produce results similar to the PBS control (Bachelder et al., 2010b; Peine et al., 2013; Schully et al.). The sera was

analyzed to measure mouse antigen specific IgG and subclass (IgG1 and IgG2a) titers by ELISA and splenocytes were stimulated *ex vivo* to examine their antigen-specific response. Additionally, a cohort of mice were challenged and monitored for 28 days.

The humoral response to Ac-DEX encapsulated *B. pseudomallei* antigens.

Substantial *B. pseudomallei* lysate-specific IgG titers (Figure 6A) were present in the sera of all three vaccinated groups 14 days after the second vaccination. All of these titers increased significantly ($p<0.005$) relative to the unvaccinated controls (PBS), however, there was no significant difference between the experimental groups. There were no significant titers in sera collected prior to the second vaccination (data not shown).

The antibody response to *B. pseudomallei* lysate antigens demonstrated a mixture of IgG1 and IgG2a (Figure 6B) indicates a balanced Th1/Th2 response. Levels of IgG1 is generally indicative of a predominately Th2 response since IL-4, a Th2-associated cytokine, is needed for B cell isotype class switching to IgG1. Conversely, levels of IgG2a is indicative of a predominately Th1 response consisting of heightened levels of IFN- γ . The higher levels of IgG2a was expected because resiquimod is a potent activator of B cells (Tomai et al.), and we previously observed a similar mixed response with Ac-DEX MPs formulations involving resiquimod (Schully et al.). The Fc portions of IgG2a antibodies interact with complement components and high affinity Fc receptors resulting in the stimulation of antibody-dependent cell mediated cytotoxicity and enhanced phagocytosis by macrophages (Snapper and Paul). Early intervention by macrophages (Conejero et al.) and strong cell-mediated cytotoxic responses (Tippayawat et al.) are considered essential to control of *B. pseudomallei* infection.

The antigen recall response to Ac-DEX encapsulated *B. pseudomallei* antigens.

In addition to the *in vivo* antibody titer characterization, we also performed *ex vivo* analysis of the immune response by an antigen-specific splenocyte recall assay. An additional five mice were immunized and euthanized without challenge. Their splenocytes were harvested 14 days after the second vaccination and stimulated with *B. pseudomallei* lysate to assess the cytokine response to antigen.

As shown in Figure 7, stimulating the splenocytes resulted in a significantly increased Th1- and Th2-associated cytokine expression. Of all the vaccine combinations, Lysate/MP + Resiq/MP elicited the strongest overall response, significantly increasing in the production of IL-4 ($p<0.01$), IL-5 ($p<0.001$), IL-12 ($p<0.001$), IFN- γ ($p<0.001$), IL-17A ($p<0.001$), TNF- α ($p<0.001$) and GM-CSF ($p<0.001$) relative to the PBS control groups. Mice vaccinated with Lysate + Resiq/MP produced a similar, albeit weaker cytokine expression profile which showed significant increases in IL-4 ($p<0.05$), IL-5 ($p<0.05$), IFN- γ ($p<0.001$), TNF- α ($p<0.01$), and GM-CSF ($p<0.01$) relative to the PBS control. Animals vaccinated with Resiq/MP + Lysate + Alum produced significant increases in IL-4 ($p<0.05$), IL-5 ($p<0.05$), IL-12 ($p<0.01$), and TNF- α ($p<0.01$), relative to the PBS control groups. The Resiq/MP + Lysate + Alum formulation was the only group that displayed a significant increase in IL-6 relative to the PBS control. Neither IL-2 nor IL-10 were significantly up-regulated in any of the treatment groups in the antigen recall assay (data not shown).

The mixed Th1/Th2 response we observed in both proximal and peripheral tissues were likely a consequence of our choice of resiquimod as the adjuvant in our Ac-DEX vaccine formulations and are consistent with our observations in previous studies (Schully et al.). Adjuvants potentiate antigen immunogenicity by prolonging antigen availability at the injection site, incorporating danger signals to recruit and activate

phagocytes, thereby enhancing vaccine efficacy. Unfortunately for vaccine manufacturers, there are only a limited number of adjuvants that are FDA-approved for clinical use. Since aluminum salts primarily promote a Th2 response (Lambrecht et al.), other adjuvants may be needed to enhance the Th1 response necessary for protection against intracellular pathogens such as *B. pseudomallei* (Silva and Dow). Resiquimod is a synthetic TLR 7/8 agonist that induces dendritic cell maturation and secretion of proinflammatory cytokines to activate the inflammatory and downstream cellular and humoral immune responses, typically biased towards Th1 development (Schully et al., 2013). All combinatorial formulations (lysate + resiquimod) increased the concentration of *B. pseudomallei*-specific IgG including the IgG1 and IgG2a subclasses when compared to the PBS control group. These particular subclasses are useful for determining the comparative skewing towards Th1- or Th2-predominant *in vivo* immune response. IgG1 is generally indicative of Th2 cell development promoted by high levels of IL-4, whereas IgG2a is generally indicative of Th1 cell development characterized by high levels of IFN- γ and IL-12 (Paul, 1994). Interestingly, animals exposed to Lysate + Alum + Resiq/MP had slightly higher antigen-specific IgG2a levels than either of the other combinatorial groups. This finding suggests that combining resiquimod and alum enhanced *in vivo* IFN- γ expression. We observed a similar effect in mice immunized with rPA + Alum + Resiq/MP in a previous study characterizing how these adjuvants and MPs influenced the immune response to vaccination in a murine model of anthrax (Schully et al.).

Vaccination with Lysate/MP + Resiq/MP induced the strongest overall cytokine responses, expressing higher levels of Th1 (IL-12, IFN- γ , TNF- α) and Th2 (IL-4, IL-5) cytokines than any other group, including the unencapsulated Lysate + Resiq/MP group. This observation is particularly important in this disease model because IFN- γ and IL-12

are considered essential for early control of *B. pseudomallei* infection (Haque et al.; Haque et al.; Koo and Gan; Santanirand et al.; Silva and Dow). Ac-DEX encapsulated lysate also provoked the highest levels of GM-CSF, a cell growth factor that promotes both Th1 and Th2 cell the growth and development of, as well as macrophages and CTL, among others. The disparities in inflammatory cytokine expression observed between encapsulated and unencapsulated cell lysates suggests that Ac-DEX MP encapsulation potentiates the inflammatory response to lysate, and enhances the production of critical cytokines. Further, while unencapsulated lysate elicited a high IgG1:IgG2a ratio (Th2-polarized), encapsulated lysate elicited a lower, more balanced IgG1:IgG2a ratio (Figure 6). When considered alongside the high-levels of both Th1 and Th2 cytokine expression induced by Lysate/MP, it would suggest that Ac-DEX encapsulation may promote a robust, balanced Th1/Th2 response.

The increased IL-17a levels observed in Lysate/MP + Resiq/MP -vaccinated mice is consistent with the response produced by rPA/MP + Resiq/MP -vaccinated mice in our previous anthrax studies (Schully et al.). IL-17a was recently demonstrated as important for protection in lung infection models of intractable pathogens including *Mycobacterium tuberculosis* (Gopal et al.) through the induction of an innate immune response. Thus, the presence of an increased IL-17a production in response to *B. pseudomallei* lysate *in vitro* suggests that the combination of Lysate/MP and Resiquimod/MP may be inducing a similar protective response.

Animals vaccinated with Resiq/MP + Lysate + Alum had significant increases in IL-4, IL-5, IL-12, and TNF- α in the antigen recall assay compared with the PBS control group. This group also showed a significant increase in IL-6 production, something that was not observed in the other combinatorial preparations. We have seen this increase in IL-6 in our previous murine model of anthrax where we examined rPA + Alum +

Resiq/MP (Schully et al., 2013), suggesting that the addition of alum to the Resiq/MP enhanced the acute phase response.

Although the levels of IgG2a suggests that vaccinated mice also had enhanced IFN- γ levels, the concentration of IFN- γ measured in the supernatants from the antigen-specific recall assay was not significantly higher than the PBS control. We did not add an adjuvant in the antigen recall assay, so this may help to explain the discrepancy between the enhanced IgG2a (and therefore *in vivo* IFN- γ levels) in the sera from our Resiq/MP + Lysate + Alum vaccinated mice compared with the concentration of IFN- γ in the supernatants from the antigen recall assay.

The cellular response to Ac-DEX encapsulated *B. pseudomallei* antigens. A robust cell-mediated immune response is of key importance to developing protective immunity to *B. pseudomallei* in both humans (Barnes et al.; Healey et al.; Ketheesan et al.; Ramsay et al.) and mice (Haque et al.; Healey et al.). We assessed indicators of CD8⁺ T cell and natural killer (NK) cell cytotoxic killing activity by quantifying cell surface degranulation markers. Lysosomal associated membrane proteins (LAMPs) line the membranes of cytolytic granules. Upregulated LAMP-1 (CD107a) has been described as a marker of cytotoxicity on stimulated cells and its induction occurs in parallel with cytokine secretion and target cell lysis. Thus, CD107a surface expression can be used as a marker of cytotoxic CD8⁺ T cell (CD8⁺ CD3⁺) and NK cell (CD8⁺ CD3⁻) degranulation (Betts et al.; Betts and Koup).

The cytotoxic killing activity elicited by each vaccine formulation can be determined from measurements of degranulation marker (CD107a) upregulated in NK cell and CD8⁺ T cell populations after restimulation. Splenocytes from each vaccine formulation group were subdivided and incubated with either PBS (unstimulated controls) or lysate protein (re-stimulated experimental groups). Flow cytometry was used

to measure the CD8⁺ CD3⁺ splenocyte population that expressed CD107a in lysate-stimulated and unstimulated cells. The absolute change in CD107a expression produced by antigen re-stimulation was calculated by subtracting the value produced by unstimulated cells (baseline) from the value produced by lysate-stimulated cells.

The difference (net change) between stimulated and unstimulated CD8⁺CD3⁺ CD107a⁺ (CTL) population percentages is depicted in Figure 8A, while Figure 8B presents those for the CD8⁺CD3⁻ CD107a⁺ (NK) population. Lysate + Resiq/MP group-derived CD8⁺CD3⁺ and CD8⁺CD3⁻ cells were not significantly different with lysate restimulation, suggesting this formulation provokes a weak cell-mediated response (Fig. 8). This is not unexpected, as un-encapsulated antigens are processed as extracellular antigen and provoke predominantly a humoral response, as was observed in the present study (Figure 4). The Lysate/MP + Resiq/MP group-derived CD8⁺CD3⁺ and CD8⁺CD3⁻ populations both increased CD107a expression when stimulated with lysate, suggesting that Ac-DEX encapsulation potentiated the cell-mediated response (Figure 8). Resiq/MP + Lysate + Alum group-derived CD8⁺CD3⁺ and CD8⁺CD3⁻ cells both responded to lysate re-stimulation more strongly than all other groups, upregulating CD107a expression at significantly higher levels than all other groups (Figure 8). Collectively, these results suggest the cellular response to *B. pseudomallei* lysate is enhanced both by Ac-DEX encapsulation and alum, prompting increased CD8⁺ T cell and NK cell cytotoxicity on subsequent exposure.

NK cells are believed to aid in the early control of *B. pseudomallei* infection by activating macrophages. While the role of CD8⁺ T cells in immunity to other persistent or chronic pathogens has been extensively characterized (Coffman and Carty; Edelson and Unanue; Flynn and Chan; Mosmann et al.), this information is limited with respect to *B. pseudomallei*. However, there is a strong correlation between resolution of clinical

meliodosis and T cell response (Barnes et al.; Healey et al.; Ketheesan et al.; Ramsay et al.; Tipayawat et al., 2011). After vaccinating T cell depleted mice with an auxotrophic mutant, Haque *et al.* demonstrated protective responses to *B. pseudomallei* that were CD4⁺, but not CD8⁺ cell, dependent (Haque et al.). Despite the uncertainty in the exact role of cell-mediated immunity, it is very likely that CD8⁺ T cells will play a role (Bondi and Goldberg, 2008; Haque et al., 2006b), and antigens and adjuvants encapsulated in Ac-DEX MPs have demonstrated the ability to generate a CD8⁺ T cell response.

Protective efficacy of Ac-DEX-encapsulated *B. pseudomallei* lysate.

In an effort to determine if Ac-DEX encapsulating *B. pseudomallei* antigens and resiquimod could provide protection against a lethal challenge with *B. pseudomallei*, BALB/c mice were vaccinated two times, 14 days apart, by subcutaneous injection with the vaccine preparations described in Table 1. Immunized mice were allowed to rest for two weeks prior to being challenged by i.p. injection of $\sim 1.5 \times 10^6$ cfu *B. pseudomallei* 1026b. Challenged mice were observed for 28 days post challenge. At the study endpoint, surviving mice were euthanized as described and their blood, lungs, livers, and spleens were harvested for *B. pseudomallei* colonization assessment.

As shown in Figure 9, at least 90% of vaccinated mice survived for at least 14 days in the Lysate/MP + Resiq/MP and Resiq/MP + Lysate + Alum groups. As compared to the PBS control mice, which rapidly succumbed to the disease, the protection we observed with the encapsulated groups was significant ($p = 0.0030$, 0.0010 , and 0.0016 for the Lysate + Resiq/MP, Lysate/MP + Resiq/MP, and Resiq/MP Lysate + Alum groups, respectively). Beyond 14 days, however, progressive death was observed in all vaccinated groups. Ultimately, 20% of mice vaccinated with Resiq/MP + Lysate + Alum

and 10% of mice vaccinated with Lysate/MP + Resiq/MP survived. However, upon necropsy at the study endpoint (28 days post- challenge), the surviving animals were also found to be colonized with heavy bacterial burdens (not shown). Therefore, increasing the length of the vaccination schedule marginally improved survival for each of the groups that contained an Ac-DEX encapsulated components, compared to those vaccinated on a more acute schedule.

While the improvement was not determined to be statistically significant, the trend is encouraging. With the increased vaccination schedule there was a single surviving mouse in the PBS control group, which displayed an interesting phenomenon. In this individual, a swollen area appeared on the tail, approximately 18 mm from the base, which progressively increased in size over the course of the observation period. The remainder of the tail eventually became necrotic. Upon necropsy, the swollen portion (~8 mm) was homogenized in PBS and plated on selective media. This portion of the tail was found to contain $\sim 1 \times 10^7$ cfu of *B. pseudomallei* per gram of tissue. Interestingly, this mouse had a normal weight (22 g). Also, while no *B. pseudomallei* was detectable in the blood or lung tissue, 218 cfu and 1×10^4 cfu *B. pseudomallei* were present in liver and spleen, respectively. These data indicate that the animal was infected but the infection was somehow sequestered to the tail. A literature search did not indicate that this is a common phenomenon but other investigators have witnessed similar occurrences (Welkos et al.).

Conclusions

The goal of this study was to evaluate novel, biodegradable Ac-DEX polymeric MP as a potential carrier for a *B. pseudomallei* subunit vaccine. Protection against *B. pseudomallei* will likely require humoral as well as cellular mediated immunity. We

observed a strong CD3⁺CD107⁺ cellular immune response and mixed IgG1 and IgG2a humoral response by vaccinating mice with Ac-DEX encapsulations of *B. pseudomallei* lysate proteins and resiquimod. Additionally, we observed delayed time to death following challenge for both the rapid vaccine and the protective vaccine. Ac-DEX encapsulation took a complex and uncharacterized antigen like whole cell lysate and produced 100% protection for two weeks in combinatorial vaccines that included lysate antigen and resiquimod. We were encouraged by the observation of sterility in the blood, liver and spleen within a small population of the mice and cytokine responses with antigen recall would seem to indicate our adjuvant formulations stimulate a protective immune response. We would anticipate that a more effective antigen, once identified and formulated into Ac-DEX MPs, would produce increased survival and potentially sterilizing immunity from *B. pseudomallei* infection. Finally, neither NK cells nor CTL responded to un-encapsulated lysate. This is presumably because the lysate had no means of entering the cytosol, was processed through the MHC2 pathway rather than MHC1 and thus humoral immunity was stimulated over cell mediated immunity. We therefore speculate that the most effective *B. pseudomallei* vaccine formulation would consist of Ac-DEX encapsulated antigen and adjuvant, possibly within the same MP.

Acknowledgements:

This work was funded in part by DARPA W911NF-10-1-0264 as well as DTRA and Ohio State University institutional funds.

673 **Disclaimers**

674 The views expressed in this article are those of the author and do not necessarily reflect
675 the official policy or position of the Department of the Navy, Department of Defense, nor
676 the U. S. Government.

677 **References**

678 Andresen, T.L., Jensen, S.S., Jorgensen, K., 2005. Advanced strategies in liposomal cancer
679 therapy: problems and prospects of active and tumor specific drug release. *Prog Lipid Res* 44,
680 68-97.
681 Atkins, T., Prior, R.G., Mack, K., Russell, P., Nelson, M., Oyston, P.C., Dougan, G., Titball, R.W.,
682 2002. A mutant of *Burkholderia pseudomallei*, auxotrophic in the branched chain amino acid
683 biosynthetic pathway, is attenuated and protective in a murine model of melioidosis. *Infect*
684 *Immun* 70, 5290-5294.
685 Audran, R., Peter, K., Dannull, J., Men, Y., Scandella, E., Groettrup, M., Gander, B., Corradin, G.,
686 2003. Encapsulation of peptides in biodegradable microspheres prolongs their MHC class-I
687 presentation by dendritic cells and macrophages in vitro. *Vaccine* 21, 1250-1255.
688 Bachelder, E.M., Beaudette, T.T., Broaders, K.E., Dashe, J., Frechet, J.M., 2008. Acetal-
689 derivatized dextran: an acid-responsive biodegradable material for therapeutic applications.
690 *Journal of the American Chemical Society* 130, 10494-10495.
691 Bachelder, E.M., Beaudette, T.T., Broaders, K.E., Frechet, J.M., Albrecht, M.T., Mateczun, A.J.,
692 Ainslie, K.M., Pesce, J.T., Keane-Myers, A.M., 2010a. In vitro analysis of acetalated dextran
693 microparticles as a potent delivery platform for vaccine adjuvants. *Molecular pharmaceutics* 7,
694 826-835.
695 Bachelder, E.M., Beaudette, T.T., Broaders, K.E., Frechet, J.M.J., Albrecht, M.T., Mateczun, A.J.,
696 Ainslie, K.M., Pesce, J.T., Keane-Myers, A.M., 2010b. In Vitro Analysis of Acetalated Dextran
697 Microparticles as a Potent Delivery Platform for Vaccine Adjuvants. *Mol. Pharm.* 7, 826-835.
698 Barnes, J.L., Warner, J., Melrose, W., Durrheim, D., Speare, R., Reeder, J.C., Ketheesan, N., 2004.
699 Adaptive immunity in melioidosis: a possible role for T cells in determining outcome of infection
700 with *Burkholderia pseudomallei*. *Clinical immunology* 113, 22-28.
701 Betts, M.R., Brenchley, J.M., Price, D.A., De Rosa, S.C., Douek, D.C., Roederer, M., Koup, R.A.,
702 2003. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric
703 assay for degranulation. *Journal of immunological methods* 281, 65-78.
704 Betts, M.R., Koup, R.A., 2004. Detection of T-cell degranulation: CD107a and b. *Methods in cell*
705 *biology* 75, 497-512.
706 Bondi, S.K., Goldberg, J.B., 2008. Strategies toward vaccines against *Burkholderia mallei* and
707 *Burkholderia pseudomallei*. *Expert review of vaccines* 7, 1357-1365.
708 Breitbach, K., Kohler, J., Steinmetz, I., 2008. Induction of protective immunity against
709 *Burkholderia pseudomallei* using attenuated mutants with defects in the intracellular life cycle.
710 *Trans R Soc Trop Med Hyg* 102 Suppl 1, S89-94.
711 Broaders, K.E., Cohen, J.A., Beaudette, T.T., Bachelder, E.M., Frechet, J.M., 2009. Acetalated
712 dextran is a chemically and biologically tunable material for particulate immunotherapy.
713 *Proceedings of the National Academy of Sciences of the United States of America* 106, 5497-
714 5502.

715 Coffman, R.L., Carty, J., 1986. A T cell activity that enhances polyclonal IgE production and its
 716 inhibition by interferon-gamma. *J Immunol* 136, 949-954.
 717 Conejero, L., Patel, N., de Reynal, M., Oberdorf, S., Prior, J., Felgner, P.L., Titball, R.W., Salguero,
 718 F.J., Bancroft, G.J., 2011. Low-dose exposure of C57BL/6 mice to burkholderia pseudomallei
 719 mimics chronic human melioidosis. *The American journal of pathology* 179, 270-280.
 720 Currie, B.J., Fisher, D.A., Anstey, N.M., Jacups, S.P., 2000. Melioidosis: acute and chronic disease,
 721 relapse and re-activation. *Trans R Soc Trop Med Hyg* 94, 301-304.
 722 Dance, D., 1990. Melioidosis. *Reviews in Medical Microbiology* 1, 143-150.
 723 Dance, D.A., 2000. Melioidosis as an emerging global problem. *Acta tropica* 74, 115-119.
 724 Dannenberg, A.M., Jr., Scott, E.M., 1958a. Melioidosis: pathogenesis and immunity in mice and
 725 hamsters. I. Studies with virulent strains of *Malleomyces pseudomallei*. *The Journal of*
 726 *experimental medicine* 107, 153-166.
 727 Dannenberg, A.M., Jr., Scott, E.M., 1958b. Melioidosis: pathogenesis and immunity in mice and
 728 hamsters. II. Studies with avirulent strains of *Malleomyces pseudomallei*. *The American journal*
 729 *of pathology* 34, 1099-1121.
 730 Ding, A.G., Schwendeman, S.P., 2008. Acidic microclimate pH distribution in PLGA microspheres
 731 monitored by confocal laser scanning microscopy. *Pharm Res* 25, 2041-2052.
 732 Druar, C., Yu, F., Barnes, J.L., Okinaka, R.T., Chantratita, N., Beg, S., Stratilo, C.W., Olive, A.J.,
 733 Soltes, G., Russell, M.L., Limmathurotsakul, D., Norton, R.E., Ni, S.X., Picking, W.D., Jackson, P.J.,
 734 Stewart, D.I., Tsvetnitsky, V., Picking, W.L., Cherwonogrodzky, J.W., Ketheesan, N., Peacock, S.J.,
 735 Wiersma, E.J., 2008. Evaluating *Burkholderia pseudomallei* Bip proteins as vaccines and Bip
 736 antibodies as detection agents. *FEMS Immunol Med Microbiol* 52, 78-87.
 737 Duong, A.D., Sharma, S., Peine, K.J., Gupta, G., Satoskar, A.R., Bachelder, E.M., Wyslouzil, B.E.,
 738 Ainslie, K.M., 2013. Electrospray encapsulation of toll-like receptor agonist resiquimod in
 739 polymer microparticles for the treatment of visceral leishmaniasis. *Mol Pharm* 10, 1045-1055.
 740 Edelson, B.T., Unanue, E.R., 2000. Immunity to *Listeria* infection. *Current opinion in immunology*
 741 12, 425-431.
 742 Elkins, K.L., Rhinehart-Jones, T.R., Stibitz, S., Conover, J.S., Klinman, D.M., 1999. Bacterial DNA
 743 containing CpG motifs stimulates lymphocyte-dependent protection of mice against lethal
 744 infection with intracellular bacteria. *Journal of immunology* 162, 2291-2298.
 745 Flynn, J.L., Chan, J., 2001. Immunology of tuberculosis. *Annu Rev Immunol* 19, 93-129.
 746 Gautam, S., Kumar, R., Singh, N., Singh, A.K., Rai, M., Sacks, D., Sundar, S., Nylen, S., CD8 T cell
 747 exhaustion in human visceral leishmaniasis. *J Infect Dis* 209, 290-299.
 748 Gopal, R., Rangel-Moreno, J., Slight, S., Lin, Y., Nawar, H.F., Fallert Junecko, B.A., Reinhart, T.A.,
 749 Kolls, J., Randall, T.D., Connell, T.D., Khader, S.A., 2013. Interleukin-17-dependent CXCL13
 750 mediates mucosal vaccine-induced immunity against tuberculosis. *Mucosal immunology* 6, 972-
 751 984.
 752 Hadinoto, K., Sundaresan, A., Cheow, W.S., 2013. Lipid-polymer hybrid nanoparticles as a new
 753 generation therapeutic delivery platform: A review. *European journal of pharmaceuticals and*
 754 *biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische*
 755 *Verfahrenstechnik e.V.*
 756 Hamdy, S., Haddadi, A., Hung, R.W., Lavasanifar, A., 2011. Targeting dendritic cells with nano-
 757 particulate PLGA cancer vaccine formulations. *Adv Drug Deliv Rev* 63, 943-955.
 758 Haque, A., Chu, K., Easton, A., Stevens, M.P., Galyov, E.E., Atkins, T., Titball, R., Bancroft, G.J.,
 759 2006a. A live experimental vaccine against *Burkholderia pseudomallei* elicits CD4+ T cell-
 760 mediated immunity, priming T cells specific for 2 type III secretion system proteins. *J Infect Dis*
 761 194, 1241-1248.

762 Haque, A., Easton, A., Smith, D., O'Garra, A., Van Rooijen, N., Lertmemongkolchai, G., Titball,
 763 R.W., Bancroft, G.J., 2006b. Role of T cells in innate and adaptive immunity against murine
 764 *Burkholderia pseudomallei* infection. *J Infect Dis* 193, 370-379.
 765 Healey, G.D., Elvin, S.J., Morton, M., Williamson, E.D., 2005. Humoral and cell-mediated adaptive
 766 immune responses are required for protection against *Burkholderia pseudomallei* challenge and
 767 bacterial clearance postinfection. *Infect Immun* 73, 5945-5951.
 768 Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K.,
 769 Wagner, H., Takeda, K., Akira, S., 2000. A Toll-like receptor recognizes bacterial DNA. *Nature*
 770 408, 740-745.
 771 Jiang, W., Schwendeman, S.P., 2008. Stabilization of tetanus toxoid encapsulated in PLGA
 772 microspheres. *Mol Pharm* 5, 808-817.
 773 Joshi, V.B., Geary, S.M., Salem, A.K., 2013. Biodegradable particles as vaccine delivery systems:
 774 size matters. *The AAPS journal* 15, 85-94.
 775 Kanthamneni, N., Sharma, S., Meenach, S.A., Billet, B., Zhao, J.C., Bachelder, E.M., Ainslie, K.M.,
 776 2012. Enhanced stability of horseradish peroxidase encapsulated in acetalated dextran
 777 microparticles stored outside cold chain conditions. *International journal of pharmaceutics* 431,
 778 101-110.
 779 Kauffman, K.J., Kanthamneni, N., Meenach, S.A., Pierson, B.C., Bachelder, E.M., Ainslie, K.M.,
 780 2012. Optimization of rapamycin-loaded acetalated dextran microparticles for
 781 immunosuppression. *International journal of pharmaceutics* 422, 356-363.
 782 Ketheesan, N., Barnes, J.L., Ulett, G.C., VanGessel, H.J., Norton, R.E., Hirst, R.G., LaBrooy, J.T.,
 783 2002. Demonstration of a cell-mediated immune response in melioidosis. *J Infect Dis* 186, 286-
 784 289.
 785 Koo, G.C., Gan, Y.H., 2006. The innate interferon gamma response of BALB/c and C57BL/6 mice
 786 to in vitro *Burkholderia pseudomallei* infection. *BMC immunology* 7, 19.
 787 Krieg, A.M., Yi, A.K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretzky, G.A.,
 788 Klinman, D.M., 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374,
 789 546-549.
 790 Lambrecht, B.N., Kool, M., Willart, M.A., Hammad, H., 2009. Mechanism of action of clinically
 791 approved adjuvants. *Current opinion in immunology* 21, 23-29.
 792 Lauw, F.N., Simpson, A.J., Hack, C.E., Prins, J.M., Wolbink, A.M., van Deventer, S.J., Chaowagul,
 793 W., White, N.J., van Der Poll, T., 2000. Soluble granzymes are released during human
 794 endotoxemia and in patients with severe infection due to gram-negative bacteria. *J Infect Dis*
 795 182, 206-213.
 796 Li, L., Schwendeman, S.P., 2005. Mapping neutral microclimate pH in PLGA microspheres.
 797 *Journal of controlled release : official journal of the Controlled Release Society* 101, 163-173.
 798 Liljeqvist, S., Stahl, S., 1999. Production of recombinant subunit vaccines: protein immunogens,
 799 live delivery systems and nucleic acid vaccines. *Journal of biotechnology* 73, 1-33.
 800 Mahapatro, A., Singh, D.K., 2011. Biodegradable nanoparticles are excellent vehicle for site
 801 directed in-vivo delivery of drugs and vaccines. *Journal of nanobiotechnology* 9, 55.
 802 Meenach, S.A., Kim, Y.J., Kauffman, K.J., Kanthamneni, N., Bachelder, E.M., Ainslie, K.M., 2012.
 803 Synthesis, optimization, and characterization of camptothecin-loaded acetalated dextran porous
 804 microparticles for pulmonary delivery. *Molecular pharmaceutics* 9, 290-298.
 805 Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., Coffman, R.L., 1986. Two types of
 806 murine helper T cell clone. I. Definition according to profiles of lymphokine activities and
 807 secreted proteins. *J Immunol* 136, 2348-2357.

Nelson, M., Prior, J.L., Lever, M.S., Jones, H.E., Atkins, T.P., Titball, R.W., 2004. Evaluation of lipopolysaccharide and capsular polysaccharide as subunit vaccines against experimental melioidosis. *J Med Microbiol* 53, 1177-1182.

Ngugi, S.A., Ventura, V.V., Qazi, O., Harding, S.V., Kitto, G.B., Estes, D.M., Dell, A., Titball, R.W., Atkins, T.P., Brown, K.A., Hitchen, P.G., Prior, J.L., 2010. Lipopolysaccharide from *Burkholderia thailandensis* E264 provides protection in a murine model of melioidosis. *Vaccine* 28, 7551-7555.

Nieves, W., Asakrah, S., Qazi, O., Brown, K.A., Kurtz, J., Aucoin, D.P., McLachlan, J.B., Roy, C.J., Morici, L.A., 2011. A naturally derived outer-membrane vesicle vaccine protects against lethal pulmonary *Burkholderia pseudomallei* infection. *Vaccine* 29, 8381-8389.

Nieves, W., Petersen, H., Judy, B.M., Blumentritt, C.A., Russell-Lodrigue, K., Roy, C.J., Torres, A.G., Morici, L.A., 2014. A *Burkholderia pseudomallei* outer membrane vesicle vaccine provides protection against lethal sepsis. *Clinical and vaccine immunology : CVI* 21, 747-754.

Orange, J.S., Ballas, Z.K., 2006. Natural killer cells in human health and disease. *Clin Immunol* 118, 1-10.

Patel, N., Conejero, L., De Reynal, M., Easton, A., Bancroft, G.J., Titball, R.W., 2011. Development of vaccines against *burkholderia pseudomallei*. *Frontiers in microbiology* 2, 198.

Paul, W.E., 1994. *Fundamental Immunology*, 4th ed. Raven Press, New York.

Peacock, S.J., Limmathurotsakul, D., Lubell, Y., Koh, G.C., White, L.J., Day, N.P., Titball, R.W., 2012. Melioidosis vaccines: a systematic review and appraisal of the potential to exploit biodefense vaccines for public health purposes. *PLoS Negl Trop Dis* 6, e1488.

Peine, K.J., Bachelder, E.M., Vangundy, Z., Papenfuss, T., Brackman, D.J., Gallovic, M.D., Schully, K., Pesce, J., Keane-Myers, A., Ainslie, K.M., 2013. Efficient Delivery of the Toll-like Receptor Agonists Polyinosinic:Polycytidylic Acid and CpG to Macrophages by Acetalated Dextran Microparticles. *Mol Pharm*.

Peine, K.J., Gupta, G., Brackman, D.J., Papenfuss, T.L., Ainslie, K.M., Satoskar, A.R., Bachelder, E.M., 2014. Liposomal resiquimod for the treatment of *Leishmania donovani* infection. *The Journal of antimicrobial chemotherapy* 69, 168-175.

Petersen, H., Nieves, W., Russell-Lodrigue, K., Roy, C.J., Morici, L.A., 2014. Evaluation of a Outer Membrane Vesicle Vaccine in Nonhuman Primates. *Procedia in vaccinology* 8, 38-42.

Pilatz, S., Breitbach, K., Hein, N., Fehlhaber, B., Schulze, J., Brenneke, B., Eberl, L., Steinmetz, I., 2006. Identification of *Burkholderia pseudomallei* genes required for the intracellular life cycle and in vivo virulence. *Infect Immun* 74, 3576-3586.

Pirofski, L.A., Casadevall, A., 1998. Use of licensed vaccines for active immunization of the immunocompromised host. *Clin Microbiol Rev* 11, 1-26.

Ramsay, S.C., Ketheesan, N., Norton, R., Watson, A.M., LaBrooy, J., 2002. Peripheral blood lymphocyte subsets in acute human melioidosis. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* 21, 566-568.

Registry, N.S.A., 2013. HHS AND USDA SELECT AGENTS AND TOXINS, in: APHIS/CDC (Ed.).

Rozak, D.A., Gelhaus, H.C., Smith, M., Zadeh, M., Huzella, L., Waag, D., Adamovicz, J.J., 2010. CpG oligodeoxynucleotides protect mice from *Burkholderia pseudomallei* but not *Francisella tularensis* Schu S4 aerosols. *Journal of immune based therapies and vaccines* 8, 2.

Santanirand, P., Harley, V.S., Dance, D.A., Drasar, B.S., Bancroft, G.J., 1999. Obligatory role of gamma interferon for host survival in a murine model of infection with *Burkholderia pseudomallei*. *Infection and immunity* 67, 3593-3600.

Sarkar-Tyson, M., Titball, R.W., 2010. Progress toward development of vaccines against melioidosis: A review. *Clin Ther* 32, 1437-1445.

856 Schully, K.L., Sharma, S., Peine, K.J., Pesce, J., Elberson, M.A., Fonseca, M.E., Prouty, A.M., Bell,
 857 M.G., Borteh, H., Gallovic, M., Bachelder, E.M., Keane-Myers, A., Ainslie, K.M., 2013. Rapid
 858 Vaccination Using an Acetalated Dextran Microparticulate Subunit Vaccine Confers Protection
 859 Against Triplicate Challenge by *Bacillus Anthracis*. *Pharm Res*.
 860 Scott, A.E., Burtneck, M.N., Stokes, M.G., Whelan, A.O., Williamson, E.D., Atkins, T.P., Prior, J.L.,
 861 Brett, P.J., 2014. *Burkholderia pseudomallei* capsular polysaccharide conjugates provide
 862 protection against acute melioidosis. *Infect Immun* 82, 3206-3213.
 863 Shen, H., Ackerman, A.L., Cody, V., Giodini, A., Hinson, E.R., Cresswell, P., Edelson, R.L.,
 864 Saltzman, W.M., Hanlon, D.J., 2006. Enhanced and prolonged cross-presentation following
 865 endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles.
 866 *Immunology* 117, 78-88.
 867 Silva, E.B., Dow, S.W., 2013. Development of *Burkholderia mallei* and *pseudomallei* vaccines.
 868 *Frontiers in cellular and infection microbiology* 3, 10.
 869 Smith, C., Beagley, L., Khanna, R., 2009. Acquisition of polyfunctionality by Epstein-Barr virus-
 870 specific CD8+ T cells correlates with increased resistance to galectin-1-mediated suppression. *J*
 871 *Virology* 83, 6192-6198.
 872 Snapper, C.M., Paul, W.E., 1987. Interferon-gamma and B cell stimulatory factor-1 reciprocally
 873 regulate Ig isotype production. *Science* 236, 944-947.
 874 Stevens, M.P., Haque, A., Atkins, T., Hill, J., Wood, M.W., Easton, A., Nelson, M., Underwood-
 875 Fowler, C., Titball, R.W., Bancroft, G.J., Galyov, E.E., 2004. Attenuated virulence and protective
 876 efficacy of a *Burkholderia pseudomallei* bsa type III secretion mutant in murine models of
 877 melioidosis. *Microbiology* 150, 2669-2676.
 878 Tippayawat, P., Pinsiri, M., Rinchai, D., Riyapa, D., Romphruk, A., Gan, Y.H., Houghton, R.L.,
 879 Felgner, P.L., Titball, R.W., Stevens, M.P., Galyov, E.E., Bancroft, G.J., Lertmemongkolkhai, G.,
 880 2011. *Burkholderia pseudomallei* proteins presented by monocyte-derived dendritic cells
 881 stimulate human memory T cells in vitro. *Infect Immun* 79, 305-313.
 882 Titball, R.W., 2008. Vaccines against intracellular bacterial pathogens. *Drug discovery today* 13,
 883 596-600.
 884 Tomai, M.A., Imbertson, L.M., Stanczak, T.L., Tygrett, L.T., Waldschmidt, T.J., 2000. The immune
 885 response modifiers imiquimod and R-848 are potent activators of B lymphocytes. *Cell Immunol*
 886 203, 55-65.
 887 Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., Weigle, M., 1972.
 888 Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the
 889 picomole range. *Science* 178, 871-872.
 890 Vietri, N.J., DeShazer, D., 2007. Melioidosis. *Medical Aspects of Biological Warfare*, 147-166.
 891 Waag, D.M., McCluskie, M.J., Zhang, N., Krieg, A.M., 2006. A CpG oligonucleotide can protect
 892 mice from a low aerosol challenge dose of *Burkholderia mallei*. *Infect Immun* 74, 1944-1948.
 893 Waeckerle-Men, Y., Allmen, E.U., Gander, B., Scandella, E., Schlosser, E., Schmidtke, G., Merkle,
 894 H.P., Groettrup, M., 2006. Encapsulation of proteins and peptides into biodegradable poly(D,L-
 895 lactide-co-glycolide) microspheres prolongs and enhances antigen presentation by human
 896 dendritic cells. *Vaccine* 24, 1847-1857.
 897 Welkos, S.L., Klimko, C.P., Kern, S.J., Bearss, J.J., Bozue, J.A., Bernhards, R.C., Trevino, S.R., Waag,
 898 D.M., Amemiya, K., Worsham, P.L., Cote, C.K., 2015. Characterization of *Burkholderia*
 899 *pseudomallei* Strains Using a Murine Intraperitoneal Infection Model and In Vitro Macrophage
 900 Assays. *PLoS One* 10, e0124667.
 901 Whitlock, G.C., Deeraksa, A., Qazi, O., Judy, B.M., Taylor, K., Propst, K.L., Duffy, A.J., Johnson, K.,
 902 Kitto, G.B., Brown, K.A., Dow, S.W., Torres, A.G., Estes, D.M., 2010. Protective response to
 903 subunit vaccination against intranasal and challenge. *Procedia in vaccinology* 2.

904 Wu, J.J., Huang, D.B., Tying, S.K., 2004. Resiquimod: a new immune response modifier with
905 potential as a vaccine adjuvant for Th1 immune responses. Antiviral Res 64, 79-83.

906

907

908 **Figures:**

909 **Figure 1.** Representative scanning electron micrographs of Ac-DEx microparticles
910 encapsulating (A) resiquimod and (B) *B. pseudomallei* lysate. The scale bar size is
911 indicated in the inset.
912
913

Figure 2. Schematic representation of first study protocol: Vaccination schedule, challenge route and dose were executed as requested by the funding source, Defense Advanced Research Projects Agency (DARPA). Mice were vaccinated subcutaneously two times as indicated with a syringe. On day 14, mice were challenged with intraperitoneal injection of *B. pseudomallei* strain 1026b ($\sim 1 \times 10^6$ cfu per mouse) and mice were observed for an additional 26 days.

Figure 3. Survival of mice following rapid vaccination protocol with Ac-DEX *B. pseudomallei* vaccine. BALB/c mice (n=25) were vaccinated two times, seven days apart (-7 and -14 days relative to x-axis), with vaccine formulations in the groups detailed in Table 1. They were challenged seven days after the final vaccination with *B. pseudomallei* 1026b by intraperitoneal injection. Groups which were significantly different from controls are indicated with asterisks and detailed in the text.

Figure 4. *B. pseudomallei* colonization of survivor organs. Mice that survived to the study endpoint were weighed and evaluated for signs of illness prior to being euthanized. Blood (△) was serially diluted in PBS and the spleens (○) and livers (□) were harvested, weighed and homogenized in PBS. Individuals displaying clinical signs of illness (i.e., decreased body weight, squinted eyes and ruffled fur) are designated with S (for sick) in the table while healthy individuals (i.e., smooth coats, wide eyes and healthy weights) are designated with an H. The number of healthy or sick survivors from each group is shown above each designation. Body weight is presented in grams while bacterial burden is presented as colony forming units (cfu) per gram of tissue or ml of whole blood. Where more than one individual is present, the body weight is presented as the average of the individuals. Serial dilutions were performed to confirm between 10^0 and 10^7 cfu, and the upper and lower limits of detection are indicated with a horizontal dashed line. Naïve mice were age-matched and unchallenged and utilized to set the criteria for “healthy” mice at the study endpoint.

Figure 5. Schematic representation of second study protocol: Mice were bled by tail nick at the time points indicated with a blood droplet. Mice were vaccinated subcutaneously 14 days apart. Fourteen days following the second vaccination, mice were challenged by intraperitoneal injection of *Burkholderia pseudomallei* strain 1026b ($\sim 1 \times 10^6$ cfu per mouse) or euthanized for immune response studies. Challenged mice were observed for an additional 28 days.

Figure 6. *In vivo* antibody response to Ac-DEX Vaccine Preparations. (A) IgG class and (B) subclass (IgG1 and IgG2a) in response to vaccinations were determined by ELISA. Titers presented as mean $\pm$ SEM, are the reciprocal of the dilution factor which produced an OD₄₀₅ greater than a cutoff value determined for each assay as described in the methods. **Denotes that IgG titers were significant ($p < 0.005$) when compared to the PBS control ($n = 10$).

Figure 7. Antigen-specific Cytokine Response to Ac-DEX Vaccine Preparations. Splenocytes (n = 5) were isolated from vaccinated mice 14 days after the second vaccination, cultured with antigen for 48 hours, and then analyzed for cytokine expression. Statistical significance as compared to the unvaccinated (PBS) control is indicated with asterisks (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Figure 8. Cellular Immune response to Ac-DEX Vaccine Preparations. Splenocytes were harvested from mice vaccinated and cultured with *B. pseudomallei* lysate or PBS and stained for surface markers. Degranulation was assessed by measuring LAMP1 (CD107a) expression on cytotoxic CD8⁺ T cells and NK cells. (A) presents the difference (absolute change) between the CD8⁺ CD3⁺ CD107⁺ population percentages observed in stimulated vs. unstimulated splenocytes. Untreated control (PBS) values were subtracted from *B. pseudomallei* lysate treated groups to yield values presented in figure (absolute change). (B) presents the difference (absolute change) between the CD8⁺ CD3⁻ CD107⁺ population percentages observed in stimulated vs. unstimulated splenocytes. Statistical significance between the control (PBS) and *B. pseudomallei* lysate stimulated splenocytes is indicated by asterisks (* $p < 0.05$, *** $p < 0.0001$).

Figure 9. Ac-DEX-encapsulated lysate proteins extended time to death following Lethal Challenge with *B. pseudomallei* 1026b. BALB/c mice (n= 10) were vaccinated two times, 14 days apart with *B. pseudomallei* lysate + resiquimod/MP (■), lysate/MP + resiquimod/ MP (●) or lysate + Alum + resiquimod MP (◆) and challenged by intraperitoneal injection of *B. pseudomallei*. Mice were observed for 28 days following challenge. Statistical significance as compared to the PBS controls is indicated in the figure.

992 **Tables**

Description	Protein (µg/dose)	Alum (mg/dose)	Resiquimod (µg/dose)	Total Particles (mg/dose)
PBS	NA	NA	NA	NA
Empty MP	NA	NA	NA	1.8
Lysate Protein Adsorbed on Alum (Lysate + Alum)	10	4	NA	NA
Resiquimod/MP	NA	NA	8	1.8
Lysate Protein + Resiquimod MP (Lysate + Resiq/MP)	10	NA	8	1.8
Lysate/MP	10	NA	NA	0.8
Lysate MP + Resiq/MP	10	NA	8	2.6
Lysate Protein Adsorbed on Alum + Resiquimod/MP (Resiq/ MP + Lysate + Alum)	10	4	8	1.8

993 **Table 1: Vaccine Preparations.** BALB/c mice were vaccinated using free or
994 encapsulated *B. pseudomallei* lysate protein and adjuvant in the combinations described
995 above. The dose of each component and the total mass of Ac-DEX microparticles (MP)
996 is provided.
997

Encapsulate	Nominal Loading (wt %)	Encapsulation Efficiency (%)	Quantitative Loading (µg compound/mg Ac-DEX)	Mean Particle Diameter (nm)
Lysate Protein	5.0	26.7	13.4	631 ± 124
Resiquimod	3.0	14.0	4.2	272 ± 25

Table 2: Characterization of *B. pseudomallei* vaccine microparticles. Nominal loading represents the amount of protein/resiquimod initially added during the formulation process. The encapsulation efficiency refers the percentage of the nominal load which remained in the particles after fabrication. The quantitative loading represents the weight percent (µg compound/mg Ac-DEX) of protein/resiquimod in the formulated particle. The mean diameter of the particles was obtained using dynamic light scattering. The range in particle size is reported as the standard deviation of five readings.