IAI Accepts, published online ahead of print on 7 October 2013
Infect. Immun. doi:10.1128/IAI.00915-13

Correlates of Immune Protection Following Cutaneous Immunization with an Attenuated *Burkholderia pseudomallei* Vaccine

Ediane B. Silva[1], Andrew Goodyear[1], Marjorie D. Sutherland[1], Nicole L. Podnecky[1], Mercedes Gonzalez-Juarrero[1], Herbert P. Schweizer[1] and Steven W. Dow[1#]

[1]Rocky Mountain Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research and Department of Microbiology, Immunology, and Pathology, Colorado State University, Ft. Collins, CO, USA.

Running title: Immune Protection Mechanisms with *Burkholderia* Vaccine

Key words: antibody; cytokine; T cell; bacterium; vaccine

[#] Corresponding author: Department of Microbiology, Immunology, and Pathology, Colorado State University, 1619 Campus Delivery, Ft. Collins, CO 80523. Phone: (970) 297-4014. Fax: (970) 297-1275. E-mail: sdow@colostate.edu

ABSTRACT. Infections with the Gram-negative bacterium *Burkholderia pseudomallei* (meliodosis) are associated with high mortality and there is currently no approved vaccine to prevent the development of meliodosis in humans. Infected patients also do not develop protective immunity to re-infection and some individuals will develop chronic, subclinical infections with *B. pseudomallei*. At present our understanding of what constitutes effective protective immunity against *B. pseudomallei* infection remains incomplete. Therefore, we conducted a study to elucidate immune correlates of vaccine-induced protective immunity against acute *B. pseudomallei* infection. BALB/c and C57BL/6 mice were immunized subcutaneously with a highly attenuated, Select Agent excluded *purM* deletion mutant of *B. pseudomallei* (strain Bp82), and then subjected to intranasal challenge with virulent *B. pseudomallei* strain 1026b. Immunization with Bp82 generated significant protection from challenge with *B. pseudomallei*, and protection was associated with a significant reduction in bacterial burden in lungs, liver, and spleen of immunized mice. Humoral immunity was critically important for vaccine-induced protection, as mice lacking B cells were not protected by immunization and serum from Bp82-vaccinated mice could transfer partial protection to non-vaccinated animals. In contrast, vaccine-induced protective immunity was found to be independent of both CD4 and CD8 T cells. Tracking studies demonstrated uptake of the Bp82 vaccine strain predominately by neutrophils in vaccine draining lymph nodes, and by smaller numbers of DCs and monocytes. We concluded that protection following cutaneous immunization with a live, attenuated *Burkholderia* vaccine strain was dependent primarily on generation of effective humoral immune responses.

Keywords: antibody; cytokine; T cell; bacterium; vaccine

Introduction

Melioidosis is a serious disease of humans caused by the Gram-negative facultative intracellular bacterium *B. pseudomallei* (1-3). The disease is endemic in South-East Asia, Northern Australia, and parts of South and Central America (1, 4-6). *B. pseudomallei* is being isolated increasingly in other parts of the world as well, likely due to greater awareness and surveillance for the organism (7-8). Infection with *B. pseudomallei* can be contracted via several routes, including subcutaneous inoculation, inhalation and likely ingestion (9-10). The route of *B. pseudomallei* infection is correlated to the severity of infection, with inhalational infection generally associated with more rapid disease course. Bacteremic infection is common with *B. pseudomallei* and sepsis in melioidosis patients is associated with high mortality rates (11-13). In patients that develop chronic infection, the disease may manifest as disseminated abscesses in multiple sites including the spleen, liver, joints, and CNS (14-18). *B. pseudomallei* is inherently resistant to multiple classes of antibiotics, most notably aminoglycosides and some beta-lactam drugs (19), due to the expression of efflux pumps and PenA β-lactamase (20-23). A delay in diagnosis is often associated with treatment failures in patients with acute infection (24-27).

Currently there is no approved vaccine for protection of humans against *B. pseudomallei* infection. A number of candidate vaccines have been developed and tested in animal models of melioidosis, and the state of melioidosis vaccines has been reviewed recently (28). Briefly, the most effective immunity to date has been achieved by use of live, attenuated *B. pseudomallei* vaccines, including strains lacking *ilv*l (29-30), *serC* (31), *aro*B (32), *pur*N, *pur*M, BPSS1509, *lip*B and *pab*B (33), *bipD* (34) and by *aro*C (35) mutants.

Subunit vaccines have also been developed for immunization against *B. pseudomallei*, with the most promising protection to date being achieved with purified proteins such as BipD (34) BipB, BipC (36), with recombinant proteins including Hcp1, Hcp2, Hcp3 and Hcp6 (37), with lipopolysaccharide (38), with purified flagellin (39), with LolC, PotF and OppA-Non membrane protein (40), with outer membrane vesicles (41) and with recombinant Omp85 protein (42). However, the use of single antigen subunit vaccines for *Burkholderia* infection is unlikely to generate broad, protective immunity against this very genetically diverse and unstable organism (35, 43). Effective immunization with subunit vaccines administered by the subcutaneous route has also not been reported.

While the risk of reversion to virulence is a primary concern with the use of live, attenuated bacterial vaccines, the ability to induce rapidly broad protective immunity is a plus for this type of vaccine. Our group previously developed a highly attenuated strain of *B. pseudomallei* 1026b (strain Bp82) which was recently excluded from Select Agent regulations, which with Institutional Biosafety Committee approval can be used under BSL-2 conditions (44). This Δ*purM* strain of *B. pseudomallei* was extensively tested in several different highly immune compromised animal strains and reversion to virulence or persistence of the organism was not found (44). In addition, a *purM* deletion mutant of the K96243 strain of *B. pseudomallei* was also highly attenuated and safe in animal studies (though this strain is not yet excluded from Select Agent regulations) (44). The ability of these Select Agent-excluded mutant strains of *B. pseudomallei* to induce protective immunity from meliodosis has not been previously investigated in animal models. Nor has it been determined whether protection could be achieved by subcutaneous (s.c.) vaccine administration, which is practical route of immunization than the

intranasal or intraperitoneal routes used in most prior studies of attenuated *Burkholderia* vaccines.

Therefore, in the present study we intended to determine whether the Bp82 strain of *B. pseudomallei* was capable of inducing protective immunity following cutaneous immunization. We also sought to elucidate immune mechanisms by which Bp82 immunization could induce protective immunity, and to also understand how the Bp82 vaccine antigens were processed by antigen presenting cells in lymph nodes. Our findings indicate that the Bp82 vaccine is immunogenic following s.c. immunization and capable of inducing significant protection against acute inhaled *B. pseudomallei* challenge. Protective immunity was provided primarily by humoral immune responses. Therefore, these new insights into protective immune responses generated by live attenuated vaccines such as Bp82 should help guide the development of newer meliodosis vaccines and clinical evaluation of vaccine efficacy using immune correlates.

MATERIALS AND METHODS

Mice. Specific pathogen-free 6-8 week old female BALB/c and C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). In addition, mutant mouse strains (on the C57Bl/6 background), including α/β $TCR^{-/-}$, $uMT^{-/-}$, $CD4^{-/-}$ and $CD8^{-/-}$ were also purchased from Jackson. Mice were used studies when they were between 8 and 12 weeks of age. Animals were housed in micro-isolator cages under pathogen-free conditions. All studies were conducted in ABSL-3 facilities at the Rocky Mountain Regional Biosafety Laboratory and were approved by the Institutional Animal Care and Use Committee at Colorado State University.

Bacteria and culture conditions. *B. pseudomallei* strain 1026b (Bp1026b) is a clinical isolate from a patient with septicemic melioidosis in Thailand (45-46). Culture stocks were grown overnight in Luria Bertani broth (LB) (BD Biosciences, San Jose, CA) at 37ºC with shaking. Frozen stocks of *B. pseudomallei* were prepared by adding 15% glycerol (Fisher BioReagent, Pittsburgh, PA) to the overnight culture and dividing the sample into 1 ml aliquots. Aliquots were stored at -80ºC and were titered prior to use. The construction and characterization of 1026b Δ*purM* strain Bp82 has been previously described (44). A 114-bp fragment was deleted from the *purM* coding sequence, causing attenuation of the strain for melioidosis animal infection model (44). Because Bp82 is a purine auxotroph, culture was done in LB medium supplemented with 0.6 mM adenine for optimal bacterial growth. For animal experiments, each strain was thawed just before use, and the bacteria were diluted to obtain the desired numbers of cells using sterile phosphate buffered saline (PBS) (Sigma-Alderich, St. Louis, MO).

Mouse vaccination. Mice were restrained in a tail bleed apparatus allowing access to the hind leg without the need for anesthesia. Mice were vaccinated s.c. in the hind leg with $5x10^6$ CFU Bp82, in 100 µl PBS. Mice received a booster immunization, 10 days after the first immunization.

In vitro stimulation assay for T cell immunity. Mice were euthanized and single-cell suspensions from spleens were generated via mechanical disruption. Cells were filtered through a 70 µm nylon mesh screen (BD Biosciences, San Jose, CA) and treated with NH_4Cl to remove red blood cells. Spleen cells were plated in 24-well plates at a concentration of $2x10^6$ cell/ml and re-stimulated with antigens for 72 h. These antigens included heat killed Bp82 ($2x10^7$ cells/ml), Bp82 lysates (5 µg/ml), or recombinant GroEL (12.5 µg/ml) (kindly provided by Dr. Katherine Brown, University of Texas Austin). Cell culture supernatants were collected and analyzed for IFN-γ and IL-17 production via ELISA, using kits performed according to manufacturer's instructions (R&D Systems, Minneapolis, MN).

Determination of antibody titers. Nunc Maxisorp 96 well plates (Thermo Fisher Scientific, San Jose, CA) were coated with heat killed Bp82 ($5x10^7$ bacteria/ml) in carbonate/bicarbonate buffer pH 9.6 and incubated at 4ºC overnight. Plates were washed with PBS and Tween 20 (0.05%) and blocked with PBS-Tween containing 5% non-fat dry milk for 2 h at room temperature. Sera were serially diluted (10-fold dilutions) in blocking buffer and incubated for 90 min at room temperature. All secondary antibodies were conjugated to horseradish peroxidase including rat anti-mouse IgM and IgG (BD Biosciences, San Jose, CA), goat anti-mouse IgG2a

(Abcam, Cambridge, MA), and rat anti-mouse IgG1 (BD Biosciences, San Jose, CA). These antibodies were diluted 1:2000 in blocking buffer were incubated on the plate for 1 h at room temperature. 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma-Aldrich, St. Louis, MO) substrate was used to develop the plates. The reaction was stopped by the addition of 50 µl of 1N HCL. The absorbance at 450 nm was determined using a Thermo Multiskan EX spectrophotometer (Thermo Scientific, Rockford, IL).

Serum transfer studies. To obtain immune serum, naïve BALB/c mice were vaccinated with Bp82 ($5x10^6$ CFU) s.c. and boosted 10 days later. Two weeks after the boost, mice were anesthetized via i.p. injection with 100 mg/kg ketamine plus 10 mg/kg xylazine diluted in sterile water, and blood was collected via terminal cardiac puncture. Blood was allowed to clot for 30 minutes at 4°C, then centrifuged to prepare serum, which was collected and stored at -80°C prior to use. Non-immune serum was also collected from unvaccinated mice in a similar manner. Anti-*Burkholderia* IgG titers were quantified via ELISA prior to transfer as previously described (47-48). Recipient mice received 250 µl of immune serum or naïve (control) serum given i.p. one day prior to challenge.

***B. pseudomallei* i.n. challenge model.** For intranasal (i.n.) challenge, mice were anesthetized as described above. Bacteria were thawed just prior to use and diluted in sterile PBS (pH 7.4) for inoculation. Mice were challenged with approximately $5\times LD_{50}$ *B. pseudomallei* ($5x10^3$ CFU for BALB/c mice or $1.2x10^4$ CFU for C57BL/6 mice), in a total volume of 20 µl administered in

sequential droplets on alternating nares. Actual infectious doses delivered to the mice were determined by plating the inoculum.

Determination of bacterial burden in organs. Lungs, liver, and spleen were harvested at 72 h post-infection in the acute challenge model and at 30 and 60 days post-infection for the chronic challenge model and placed in 4 ml of sterile PBS. Organs were homogenized using a Seward Stomacher (Seward, Bohemia, NY). Homogenates (300 µl) were removed, placed in a 96 well plate, and serially diluted in sterile in PBS, using 10-fold dilutions. Bacterial counts were determined by plating serial 10-fold dilutions on LB agar, placed at 37ºC, and CFUs were enumerated after 48 h of incubation.

Tracking Bp82 uptake in vaccine draining lymph nodes. To facilitate tracking the fate of Bp82 in vaccine draining lymph nodes following immunization, Bp82 was engineered to express GFP (Bp82-gfp). Briefly, strain Bp82 (44) was labeled tagged with an enhanced green fluorescent protein (eGFP) whose expression is driven by the constitutive *B. thailandensis* ribosomal S12 gene promoterP_{S12} (49) in single copy from the pUC18T-mini-Tn*7*T-P_{S12}-eGFP plasmid using previously described methods (49). The stable chromosomal insertion of the site-specific mini-Tn*7* Kanamycin (Kan) resistant element was performed by tri-parental conjugation of Bp82 with RHO3/pTNS3 and RHO3/pUC18T-mini-Tn*7*T-P_{S12}-eGFP, and transformants were selected on LB medium containing 1000 µg/ml kanamycin and 0.6 mM adenine. Isolates were screened by PCR to identify single insertions in the *glmS2*-associated *att*Tn*7* site. An unmarked strain was obtained by Flp-mediated excision of the Kan resistance marker usingpFLPe2. Bp82-

gfp was injected in left footpad ($1.2x10^8$ CFU/mouse) in 50 µl PBS and popliteal lymph nodes were harvested 10 h after injection and processed as previously described (50). Briefly, lymph nodes were harvested and placed in 5 ml of Hank's Balanced Salt Solution (HBSS) and digested in Collagenase D (Roche, San Francisco, CA) for 30 min at 37ºC. Following digestion, the tissue was triturated using a glass pipette and filtered through a 70 µm nylon mesh screen (BD Biosciences, San Jose, CA).

Flow cytometry. Cells from spleen or lymph nodes were suspended in FACS buffer (PBS with 2% FBS (Atlas Biologicals, Fort Collins, CO) and 0.05% sodium azide (Fisher Scientific, Philadelphia, PA) and stained as previously described (51). To block non-specific binding, cells were incubated at room temperature for 15 min in FACS block consisting of normal mouse serum (Jackson ImmunoResearch, West Grove, PA), human IgG (Jackson ImmunoResearch, West Grove, PA), along with unlabeled anti-mouse CD16/32 (clone 93) (eBioscience, San Diego, CA) prior to staining. After blocking, cells were stained with the following antibodies: anti-Ly6-G (PE, clone HK1.4), anti-Ly6-C (PE CY7, clone RB6-8C5), anti-CD11c (Biotin, clone N418), anti-CD11b (APC Alexa 750, clone M1/70), anti-CD169 (Alexa Flour 647, clone MOMA-1). The antibodies were purchased from eBioscience (San Diego, CA), BD Pharmingen (San Diego, CA) or AbD Serotec (Raleigh, NC). Cells were immunostained at room temperature for 30 min with antibodies diluted in FACS buffer. After the primary antibody incubation cells were washed in FACS buffer and incubated with streptavidin PerCP for 20 minutes at room temperature. Cell acquisition was done using a Gallios (Beckman Coulter, Brea, CA). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

209

210 **Immunohistochemistry.** Lungs and spleen were collected 48 h post-infection and fixed in 4%
211 paraformaldehyde for 48h. Tissues were paraffin embedded and cut into 5-7 µm sections and
212 deparaffinized using EZ-DeWax solution (Biogenex Lab, San Ramon, CA) and transferred into
213 PBS. Sections were blocked with blocking eraser (Biocare Medical, Concord, CA) for 5 min at
214 room temperature and then incubated with appropriately diluted rabbit polyclonal anti-*B.*
215 *pseudomallei* antibody (provided by Dr. D. Waag from USAMRIID), and the slides were
216 incubated overnight at room temperature. Slides were washed three times with PBS followed by
217 incubation with anti-rabbit Cy3-conjugated secondary antibody (Millipore, Billerica, MA) for 1
218 h at room temperature. Slides were washed and counterstained with DAPI (0.5 µg/ml) for 2 min
219 and cover slipped with Prolong Gold mounting media (Invitrogen, Carlsbad, CA). Sections were
220 examined using a Zeiss 510 confocal microscope and analyzed using Volocity software.

221

222 **Statistical Analyses.** Statistical analyses were performed using GraphPad Prism5 software (La
223 Jolla, CA). A non-parametric t-test was used to analyze statistical differences between 2 groups.
224 Comparisons between multiple groups were done using one-way ANOVA, followed by Tukey's
225 multiple means comparison test. Survival differences were compared using Kaplan-Meier
226 survival curves, followed by log rank test. Statistical significance was defined as $p < 0.05$.

227

228 **RESULTS**

229

230 **Immunization with Bp82 protects against lethal *B. pseudomallei* challenge.** To determine
231 whether s.c. immunization with Bp82 was capable of generating protective immunity against
232 lethal inhaled challenge with *B. pseudomallei*, BALB/c and C57BL/6 mice were immunized
233 twice with Bp82 (5 X 10^6 CFU per immunization), then subjected to lethal i.n. challenge with *B.*
234 *pseudomallei* strain 1026b. The immunizing dose of Bp82 selected for these studies was
235 comparable to doses used in previous studies of live attenuated Burkholderia vaccines.
236 Following challenge with *B. pseudomallei*, mice were monitored closely and euthanized when
237 they met pre-determined endpoints for signs of illness. We observed that unvaccinated mice
238 (both C57Bl/6 and BALB/c) reached morbidity endpoints within 3 day of challenge and were
239 humanely euthanized. In contrast, there was 100% survival of vaccinated C57BL/6 mice (**Fig.**
240 **1A**) and 80% of survival of vaccinated BALB/c, for at least 30 days after challenge (**Fig. 1B**)
241 (p<0.05 compared to unvaccinated mice). At 60 days post challenge, Bp82 vaccination conferred
242 100% protection to C57BL/6 mice (**Fig. 1A**), whereas 60% of BALB/c mice were still alive and
243 apparently healthy (**Fig. 1B**).

244 **Vaccination with Bp82 reduces bacterial burden.** The evidence that Bp82 is not detect in
245 mice organ after 48h of inoculation, was previously considered by (44) which challenged mice
246 by i.n. route with 6x10^3 CFU of Bp82 or *B. pseudomallei* 1026b. After 48h of infection, the
247 authors find out that Bp82 was below the limit of detection in the lung, liver and spleen.
248 Therefore studies were done next to assess the effects of Bp82 vaccination on the *B. pseudomalei*
249 1026b bacterial burden in key organs shortly after challenge. We observed that the bacterial

250 burden in the lung and the spleen 72 h after *B. pseudomallei* challenge was significantly reduced
251 in Bp82-vaccinated animals compared to unvaccinated animals (**Fig. 2A**). In addition, bacteria
252 were not detected in the livers of Bp82 vaccinated animals compared to the high bacterial burden
253 in the livers of unvaccinated mice (**Fig. 2B**). At 30 and 60 days after challenge, Bp82 vaccinated
254 mice still has detectable *B. pseudomallei* on spleen and small amount at lung and liver (data not
255 shown).

256

257 **Induction of antibody responses following Bp82 immunization.** Serum from vaccinated mice
258 was analyzed 10 days after the last immunization for induction of antibodies against Bp82 intact
259 organisms. In the serum of vaccinated animals, significantly increased titers ($p < 0.05$) of
260 antibodies against intact Bp82 were detected, compared to unvaccinated control animals (**Fig. 3**).
261 Vaccinated animals mounted strong IgG responses (**Fig. 3A**), as well as IgM responses (**Fig. 3B**)
262 against heat killed Bp82. In addition, there was significant induction of IgG responses of the
263 IgG1 and IgG2a isotypes (**Fig. 3C and D**, $p<0.05$). Immunized animals also had significantly
264 increased titers against antigens present in Bp82 lysates (data not shown). Immunization with
265 Bp82 also induced antibody responses against certain immunogenic proteins of *B. pseudomallei*,
266 including GroEL (data not shown), but not other antigens such as BimA (data not shown).

267

268 **Induction of cellular immune responses following Bp82 immunization.** The ability of the
269 Bp82 vaccine to induce specific T cell responses was evaluated next. Spleens were harvested
270 from immunized and non-immunized mice 2 weeks after s.c. Bp82 vaccination. Single cell
271 suspensions of spleen cells were placed in triplicate wells of 96-well plates as noted in Methods

and incubated with heat-killed Bp82 or lysed Bp82 or recombinant GroEL protein. Incubation of spleen cells from immunized mice with heat-killed Bp82 generated production of significant increases in IFN-γ and IL-17 production (**Fig. 4A and B**). However, incubation with Bp82 did not induce production of IL-10 from spleen cells (data not shown). Lysates of Bp82, or recombinant GroEL however did not induce significant cytokine production. Thus, immunization with the Bp82 vaccine induced cellular immune responses that appeared to be directly primarily towards surface determinants on Bp82.

Role of humoral immunity in vaccine-induced protection against *B. pseudomallei*. In previous studies where immune mechanisms of protection have been examined, humoral immunity was found to be a primary mediator of vaccine-induced immune protection against acute *B. pseudomallei* challenge (52-55). Therefore, the role of antibody-mediated protection was investigated in the Bp82 vaccine model. First, serum transfer experiments were conducted, by transferring serum from Bp82 vaccinated animals to naive animals, which were then subjected to *B. pseudomallei* challenge. Immune serum transfer generated partial protection to *B. pseudomallei* challenge, with 38% of immune serum recipient mice protected from lethal challenge, compared to 0% of mice receiving non-immune serum (**Fig. 5A**). In addition, B cell deficient mice ($uMT^{-/-}$) were also vaccinated with Bp82 and challenged. In this study, only 50% of the $uMT^{-/-}$ were protected from challenge, compared to all of the vaccinated WT animals (**Fig. 5B**). These results indicated therefore that humoral immunity played an important, but incomplete, role in Bp82 vaccine-induced protection from *B. pseudomallei* infection.

Role of T cells in Bp82 vaccine-induced protection. Studies were done next to elucidate the role of T cells in vaccine-induced immunity to *Burkholderia* infection. Mice lacking CD4 T cells ($CD4^{-/-}$) or CD8 T cells ($CD8^{-/-}$) on the C57Bl/6 background were vaccinated s.c. with the Bp82 vaccine and subjected to *B. pseudomallei* challenge. Survival times were compared to those of WT animals (**Fig. 6**). At 30 days after challenge there was no statistical difference in survival times between $CD8^{-/-}$ or $CD4^{-/-}$ vaccinated animals compared to vaccinated WT mice (**Fig. 6A and B**). All animals from $CD4^{-/-}$ and $TCR\alpha/\beta^{-/-}$ groups survived to at least 60 days after challenge and did not manifest signs of infection, whereas unvaccinated C57Bl/6 mice were euthanized due to progressive infection 3 days after challenge. Mice lacking all conventional T cells ($TCR\alpha/\beta^{-/-}$) were also immunized with the Bp82 vaccine and subjected to challenge. These animals were also significantly protected from infection (**Fig. 6C**). These results indicated that protection from inhaled *B. pseudomallei* challenge induced by the Bp82 vaccine was largely independent of T cells and mediated almost entirely by B cells and antibody production. Since $CD4^{-/-}$ mice are unable to produce IgG efficiently, these results also suggest that it is likely that T cell independent antibodies such as IgM antibodies may have played a major role in vaccine induced protection.

Delivery of Bp82 vaccine to draining lymph nodes (LN). Finally, studies were conducted to assess the uptake and trafficking of the Bp82 vaccine to vaccine-draining lymph nodes (LN). We recently demonstrated that following immunization with conventional adjuvanted vaccines, a significant and rapid influx of inflammatory monocytes into vaccine draining lymph nodes occurs (56). Therefore, studies were conducted to determine whether the Bp82 vaccine elicited a similar leukocyte response in LNs, and to also identify potential antigen presenting cells (APC)

317 responsible for trafficking of Bp82 from the cutaneous inoculation site to the LN. Therefore, a
318 *gfp*-expressing construct of Bp82 was used to track the early movement of Bp82 vaccine bacteria
319 from the skin site to the nearest draining LN. For these studies, the vaccine was administered s.c.
320 in the footpad and the popliteal LN response was monitored using flow cytometry.

321 We found that following s.c. administration of the Bp82 vaccine, there was a marked
322 influx of $CD11b^{+}Ly6G^{+}Ly6C^{-}$ neutrophils into the nearest draining LN, as well as a much
323 smaller influx of $CD11b^{+}Ly6G^{-}Ly6C^{+}$ monocytes (**Fig. 7A and B**). We also found that
324 approximately 80% of all Bp82-*gfp* bacteria found in the LN after vaccination were contained
325 within neutrophils (**Fig. 7C**). In addition, bacteria in the LN were also found within Langerhans
326 DC (10%), $CD169^{+}$ subcapsular macrophages (3%) and inflammatory monocytes (3%), with
327 very few non-cell associated bacterial being detected.

328 To compare the relative efficiency of uptake of GFP-Bp82 by antigen presenting cells in
329 the LN, the percentages of each cell population containing Bp82 was calculated. We calculated
330 that 22.12% of all neutrophils in the LN, 1.87% of all DC in the LN, 16.17% of subcapsular
331 macrophages in the LN, and 11.62% of all inflammatory monocytes in the LN contained Bp82-
332 gfp. Thus, neutrophils appeared to be the most efficient antigen presenting cells at taking up and
333 transporting Bp82 bacteria to the draining LN, and the majority of Bp82 in the LN were found
334 within neutrophils.

335

DISCUSSION

A number of different live attenuated and subunit vaccines have been evaluated for their ability to generate protective immunity against *B. pseudomallei* (29-30, 32-36, 38-42, 55). However, there is still relatively little known regarding the most effective methods of generating protection against melioidosis, or the immune mechanisms of protection. In the present study, we demonstrated long-term (ie, > 60 days) protection against acute *B. pseudomallei* challenge following cutaneous immunization with a live, attenuated strain of *B. pseudomallei*. Importantly, our study utilized a more conventional route of immunization (ie, cutaneous immunization) than previous studies, which have typically relied on intranasal or intra-peritoneal routes of immunization. In addition, we found that the Bp82 vaccine did not require an adjuvant for activity (data not shown). Another important consideration is the fact that the Bp82 vaccine strain used in this study has been extensively evaluated for attenuation and safety. Thus, issues of reversion to virulence are potentially less of a concern with the *purM* deletion mutant of *B. pseudomallei*.

There remain substantial gaps in our knowledge regarding mechanisms of immune protection from melioidosis. The best protective immunity to date has been achieved using live attenuated *Burkholderia* vaccines, but the mechanisms of protection are not completely understood. There is some evidence that protective immunity is dependent on induction of innate immune responses by live bacterial vaccines (43, 61). Also there is a speculation that live attenuated vaccines generate better immunity due to prolonged antigen persistence in the host (33).

Protection generated by the Bp82 vaccine was found to be mediated almost entirely by antibodies, as revealed by serum transfer studies and by studies in B cell and T cell deficient mice. These findings are consistent with those of prior studies, where protection from inhaled *Burkholderia* challenge was found to be mediated largely by humoral immune mechanisms. However, these are the first studies to demonstrate that protection acquired by s.c. immunization is also antibody mediated, and that routes of mucosal administration were not require to induce effective levels of immune protection against inhalational challenge.

Interestingly, our studies also revealed a minor role for T cells in vaccine mediated protection. For example, while $CD4^{-/-}$ and $CD8^{-/-}$ animals did not have a defect in immune protection, mice that lacking both CD4 and CD8 T cells (i.e. $TCR\alpha/\beta^{-/-}$ mice) did have a small though not statistically significant loss of immune protection following Bp82 immunization (**Fig. 6C**). While the Bp82 vaccine induced both IgG and IgM antibodies, the fact that $CD4^{-/-}$ mice were fully protected following immunization suggests an important potential role for IgM antibodies in mediating vaccine protection, as $CD4^{-/-}$ mice are generally unable to effectively produce antibodies of the IgG subclass (57).

Our findings indicate that administration of a live attenuated vaccine by the s.c. route is effective in generating systemic protection from bacterial challenge by a mucosal route. Though relatively high titers of anti-*Burkholderia* antibodies were detected in serum of vaccinated animals, it is not clear exactly where bacterial control by vaccine antibodies occurred. It is plausible to suggest that vaccination may protect from lethality mainly by blocking bacterial dissemination from the lungs to other sites (eg, spleen, liver), rather than by neutralizing bacteria directly in the lungs. The protective antibody immune response in *Burkholderia* infection has

been shown previously to be specific to the LPS of *B. pseudomallei*, which promotes opsonic phagocytic killing (58-59).

The response of antigen presenting cells in LNs to immunization with the live, attenuated Bp82 vaccine was found to be quite different from the response to conventional adjuvanted vaccines, with the primary difference being the much more pronounced neutrophilic response in the case of mice immunized with the Bp82 vaccine. It is unlikely that neutrophils are able to present Bp82 antigens directly to T cells, but much more likely that neutrophils containing vaccine bacteria hand the antigens off to professional antigen presenting cells in the LNs, including DC, subcapsular macrophages, or monocytes. Indeed, it been recently observed for a live BCG vaccine that the vaccine bacteria are taken up primarily by neutrophils, but then later handed off to other antigen presenting cells (eg, DC) in the LN and peritoneal cavity (60). With the Bp82 vaccine, most of the bacteria were delivered to the LN in neutrophils, but the most avidly phagocytic antigen presenting cells for Bp82 in the LN were found to be $CD169^{+}$ macrophages and inflammatory monocytes. Thus, it is likely that initial uptake of live attenuated vaccines such as Bp82 may be mediated by neutrophils, while the bacterial antigens may ultimately be delivered to macrophages and monocytes within the LN for presentation to T cells.

In summary, we have found that s.c. immunization with a highly attenuated strain of *B. pseudomallei* can generate significant protection against inhalational challenge with virulent *B. pseudomallei*. These studies suggest therefore that it may be possible to develop conventionally delivered vaccines (ie, vaccines administered by the s.c. or i.m. route) capable of generating effective humoral immune protection against meliodosis. While subunit vaccines are generally preferred over live attenuated bacterial vaccines, it may be possible as an interim measure for meliodosis protection to use an attenuated vaccine, particularly if vaccine administration is safe

and easily accomplished. Our studies in mice indicated that vaccine site reactions did not occur (data not shown), suggesting that the whole cell Bp82 vaccine did not induce significant local inflammation. Thus, there is reason for optimism that an effective subunit or non-living bacterial cell vaccine for meliodosis that is safe and easily administered can be developed.

ACKNOWLEDGEMENTS. The authors would like to thank Dr. Todd French, University of California Los Angeles, for providing the pUC18T-mini-Tn*7*T-P_{S12}-eGFP plasmid. These studies were supported by NIH NIAID grant U54 AI065357-05 to SD and HS.

REFERENCES

1. **Cheng AC, Currie BJ.** 2005. Melioidosis: epidemiology, pathophysiology, and management. Clin Microbiol Rev **18:**383-416.
2. **Wiersinga WJ, van der Poll T, White NJ, Day NP, Peacock SJ.** 2006. Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*. Nat Rev Microbiol **4:**272-282.
3. **Wiersinga WJ, Currie BJ, Peacock SJ.** 2012. Melioidosis. N Engl J Med **367:**1035-1044.
4. **Dance DAB.** 2000. Melioidosis as an emerging global problem. Acta Tropica **74:**115-119.
5. **Dance DA.** 1991. Melioidosis: the tip of the iceberg? Clin Microbiol Rev **4:**52-60.
6. **Currie BJ, Dance DA, Cheng AC.** 2008. The global distribution of *Burkholderia pseudomallei* and melioidosis: an update. Trans R Soc Trop Med Hyg **102 Suppl 1:**S1-4.
7. **Batchelor BI, Paul J, Trakulsomboon S, Mgongo M, Dance DA.** 1994. Melioidosis survey in Kenya. Trans R Soc Trop Med Hyg **88:**181.
8. **Yabuuchi E, Arakawa M.** 1993. *Burkholderia pseudomallei* and melioidosis: be aware in temperate area. Microbiol Immunol **37:**823-836.
9. **Wiersinga WJ, van der Poll T.** 2009. *Burkholderia pseudomallei* tropism and the melioidosis road map. J Infect Dis **199:**1720-1722.
10. **Barnes JL, Ketheesan N.** 2005. Route of infection in melioidosis. Emerg Infect Dis **11:**638-639.
11. **White NJ.** 2003. Melioidosis. Lancet **361:**1715-1722.
12. **Tiangpitayakorn C, Songsivilai S, Piyasangthong N, Dharakul T.** 1997. Speed of detection of *Burkholderia pseudomallei* in blood cultures and its correlation with the clinical outcome. Am J Trop Med Hyg **57:**96-99.
13. **Chaowagul W, White NJ, Dance DA, Wattanagoon Y, Naigowit P, Davis TM, Looareesuwan S, Pitakwatchara N.** 1989. Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand. J Infect Dis **159:**890-899.
14. **Rode JW, Webling DD.** 1981. Melioidosis in the Northern Territory of Australia. Med J Aust **1:**181-184.
15. **Thin RN, Brown M, Stewart JB, Garrett CJ.** 1970. Melioidosis: a report of ten cases. Q J Med **39:**115-127.
16. **Ngauy V, Lemeshev Y, Sadkowski L, Crawford G.** 2005. Cutaneous melioidosis in a man who was taken as a prisoner of war by the Japanese during World War II. J Clin Microbiol **43:**970-972.
17. **Vatcharapreechasakul T, Suputtamongkol Y, Dance DA, Chaowagul W, White NJ.** 1992. *Pseudomonas pseudomallei* liver abscesses: a clinical, laboratory, and ultrasonographic study. Clin Infect Dis **14:**412-417.
18. **Wong KT, Puthucheary SD, Vadivelu J.** 1995. The histopathology of human melioidosis. Histopathology **26:**51-55.
19. **Schweizer HP.** 2012. Mechanisms of antibiotic resistance in *Burkholderia pseudomallei*: implications for treatment of melioidosis. Future Microbiol **7:**1389-1399.
20. **Rholl DA, Papp-Wallace KM, Tomaras AP, Vasil ML, Bonomo RA, Schweizer HP.** 2011. Molecular Investigations of PenA-mediated beta-lactam Resistance in *Burkholderia pseudomallei*. Front Microbiol **2:**139.
21. **Mima T, Schweizer HP.** 2010. The BpeAB-OprB efflux pump of *Burkholderia pseudomallei* 1026b does not play a role in quorum sensing, virulence factor production, or extrusion of aminoglycosides but is a broad-spectrum drug efflux system. Antimicrob Agents Chemother **54:**3113-3120.

22. **Moore RA, DeShazer D, Reckseidler S, Weissman A, Woods DE.** 1999. Efflux-mediated aminoglycoside and macrolide resistance in *Burkholderia pseudomallei*. Antimicrob Agents Chemother **43:**465-470.
23. **Trunck LA, Propst KL, Wuthiekanun V, Tuanyok A, Beckstrom-Sternberg SM, Beckstrom-Sternberg JS, Peacock SJ, Keim P, Dow SW, Schweizer HP.** 2009. Molecular basis of rare aminoglycoside susceptibility and pathogenesis of *Burkholderia pseudomallei* clinical isolates from Thailand. PLoS Negl Trop Dis **3:**e519.
24. **Carruthers MM.** 1981. Recrudescent melioidosis mimicking lung abscess. Am Rev Respir Dis **124:**756-758.
25. **Chan CK, Hyland RH, Leers WD, Hutcheon MA, Chang D.** 1984. Pleuropulmonary melioidosis in a Cambodian refugee. Can Med Assoc J **131:**1365-1367.
26. **Pit S, Chea FK, Jamal F.** 1988. Melioidosis with brain abscess. Postgrad Med J **64:**140-142.
27. **Wilson JW, Ashdown LR, Richards MJ, Sutherland AD, Cade JF.** 1987. Subacute pulmonary melioidosis in a temperate climate. Med J Aust **147:**95-96.
28. **Silva EB, Dow SW.** 2013. Development of *Burkholderia mallei* and *pseudomallei* vaccines. Front Cell Infect Microbiol **3:**10.
29. **Atkins T, Prior R, Mack K, Russell P, Nelson M, Prior J, Ellis J, Oyston PC, Dougan G, Titball RW.** 2002. Characterisation of an acapsular mutant of *Burkholderia pseudomallei* identified by signature tagged mutagenesis. J Med Microbiol **51:**539-547.
30. **Haque A, Chu K, Easton A, Stevens MP, Galyov EE, Atkins T, Titball R, Bancroft GJ.** 2006. A live experimental vaccine against *Burkholderia pseudomallei* elicits CD4+ T cell-mediated immunity, priming T cells specific for 2 type III secretion system proteins. J Infect Dis **194:**1241-1248.
31. **Rodrigues F, Sarkar-Tyson M, Harding SV, Sim SH, Chua HH, Lin CH, Han X, Karuturi RK, Sung K, Yu K, Chen W, Atkins TP, Titball RW, Tan P.** 2006. Global map of growth-regulated gene expression in *Burkholderia pseudomallei*, the causative agent of melioidosis. J Bacteriol **188:**8178-8188.
32. **Cuccui J, Easton A, Chu KK, Bancroft GJ, Oyston PC, Titball RW, Wren BW.** 2007. Development of signature-tagged mutagenesis in *Burkholderia pseudomallei* to identify genes important in survival and pathogenesis. Infect Immun **75:**1186-1195.
33. **Breitbach K, Kohler J, Steinmetz I.** 2008. Induction of protective immunity against *Burkholderia pseudomallei* using attenuated mutants with defects in the intracellular life cycle. Trans R Soc Trop Med Hyg **102 Suppl 1:**S89-94.
34. **Stevens MP, Haque A, Atkins T, Hill J, Wood MW, Easton A, Nelson M, Underwood-Fowler C, Titball RW, Bancroft GJ, Galyov EE.** 2004. Attenuated virulence and protective efficacy of a *Burkholderia pseudomallei bsa* type III secretion mutant in murine models of melioidosis. Microbiology **150:**2669-2676.
35. **Srilunchang T, Proungvitaya T, Wongratanacheewin S, Strugnell R, Homchampa P.** 2009. Construction and characterization of an unmarked *aroC* deletion mutant of *Burkholderia pseudomallei* strain A2. Southeast Asian J Trop Med Public Health **40:**123-130.
36. **Druar C, Yu F, Barnes JL, Okinaka RT, Chantratita N, Beg S, Stratilo CW, Olive AJ, Soltes G, Russell ML, Limmathurotsakul D, Norton RE, Ni SX, Picking WD, Jackson PJ, Stewart DI, Tsvetnitsky V, Picking WL, Cherwonogrodzky JW, Ketheesan N, Peacock SJ, Wiersma EJ.** 2008. Evaluating *Burkholderia pseudomallei* Bip proteins as vaccines and Bip antibodies as detection agents. FEMS Immunol Med Microbiol **52:**78-87.
37. **Burtnick MN, Brett PJ, Harding SV, Ngugi SA, Ribot WJ, Chantratita N, Scorpio A, Milne TS, Dean RE, Fritz DL, Peacock SJ, Prior JL, Atkins TP, Deshazer D.** 2011. The cluster 1 type VI secretion system is a major virulence determinant in *Burkholderia pseudomallei*. Infect Immun **79:**1512-1525.

38. **Nelson M, Prior JL, Lever MS, Jones HE, Atkins TP, Titball RW.** 2004. Evaluation of lipopolysaccharide and capsular polysaccharide as subunit vaccines against experimental melioidosis. J Med Microbiol **53:**1177-1182.

39. **Brett PJ, Mah DC, Woods DE.** 1994. Isolation and characterization of *Pseudomonas pseudomallei* flagellin proteins. Infect Immun **62:**1914-1919.

40. **Harland DN, Chu K, Haque A, Nelson M, Walker NJ, Sarkar-Tyson M, Atkins TP, Moore B, Brown KA, Bancroft G, Titball RW, Atkins HS.** 2007. Identification of a LolC homologue in *Burkholderia pseudomallei*, a novel protective antigen for melioidosis. Infect Immun **75:**4173-4180.

41. **Nieves W, Asakrah S, Qazi O, Brown KA, Kurtz J, Aucoin DP, McLachlan JB, Roy CJ, Morici LA.** 2011. A naturally derived outer-membrane vesicle vaccine protects against lethal pulmonary *Burkholderia pseudomallei* infection. Vaccine **29:**8381-8389.

42. **Su YC, Wan KL, Mohamed R, Nathan S.** 2010. Immunization with the recombinant Burkholderia pseudomallei outer membrane protein Omp85 induces protective immunity in mice. Vaccine **28:**5005-5011.

43. **Atkins T, Prior RG, Mack K, Russell P, Nelson M, Oyston PC, Dougan G, Titball RW.** 2002. A mutant of *Burkholderia pseudomallei*, auxotrophic in the branched chain amino acid biosynthetic pathway, is attenuated and protective in a murine model of melioidosis. Infect Immun **70:**5290-5294.

44. **Propst KL, Mima T, Choi KH, Dow SW, Schweizer HP.** 2010. A *Burkholderia pseudomallei* deltapurM mutant is avirulent in immunocompetent and immunodeficient animals: candidate strain for exclusion from select-agent lists. Infect Immun **78:**3136-3143.

45. **DeShazer D, Brett PJ, Carlyon R, Woods DE.** 1997. Mutagenesis of *Burkholderia pseudomallei* with Tn5-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. J Bacteriol **179:**2116-2125.

46. **Hayden HS, Lim R, Brittnacher MJ, Sims EH, Ramage ER, Fong C, Wu Z, Crist E, Chang J, Zhou Y, Radey M, Rohmer L, Haugen E, Gillett W, Wuthiekanun V, Peacock SJ, Kaul R, Miller SI, Manoil C, Jacobs MA.** 2012. Evolution of *Burkholderia pseudomallei* in recurrent melioidosis. PLoS One **7:**e36507.

47. **Frey A, Di Canzio J, Zurakowski D.** 1998. A statistically defined endpoint titer determination method for immunoassays. J Immunol Methods **221:**35-41.

48. **Sutherland MD, Goodyear AW, Troyer RM, Chandler JC, Dow SW, Belisle JT.** 2012. Post-exposure immunization against *Francisella tularensis* membrane proteins augments protective efficacy of gentamicin in a mouse model of pneumonic tularemia. Vaccine **30:**4977-4982.

49. **Choi KH, Mima T, Casart Y, Rholl D, Kumar A, Beacham IR, Schweizer HP.** 2008. Genetic tools for select-agent-compliant manipulation of *Burkholderia pseudomallei*. Appl Environ Microbiol **74:**1064-1075.

50. **Mitchell LA, Hansen RJ, Beaupre AJ, Gustafson DL, Dow SW.** 2013. Optimized dosing of a CCR2 antagonist for amplification of vaccine immunity. Int Immunopharmacol **15:**357-363.

51. **Bosio CM, Goodyear AW, Dow SW.** 2005. Early interaction of *Yersinia pestis* with APCs in the lung. J Immunol **175:**6750-6756.

52. **Liu B, Koo GC, Yap EH, Chua KL, Gan YH.** 2002. Model of differential susceptibility to mucosal *Burkholderia pseudomallei* infection. Infect Immun **70:**504-511.

53. **Bottex C, Gauthier YP, Hagen RM, Finke EJ, Splettstosser WD, Thibault FM, Neubauer H, Vidal DR.** 2005. Attempted passive prophylaxis with a monoclonal anti-*Burkholderia pseudomallei* exopolysaccharide antibody in a murine model of melioidosis. Immunopharmacol Immunotoxicol **27:**565-583.

54. **Bryan LE, Wong S, Woods DE, Dance DA, Chaowagul W.** 1994. Passive protection of diabetic rats with antisera specific for the polysaccharide portion of the lipopolysaccharide isolated from *Pseudomonas pseudomallei*. Can J Infect Dis **5:**170-178.

55. **Jones SM, Ellis JF, Russell P, Griffin KF, Oyston PC.** 2002. Passive protection against *Burkholderia pseudomallei* infection in mice by monoclonal antibodies against capsular polysaccharide, lipopolysaccharide or proteins. J Med Microbiol **51:**1055-1062.

56. **Mitchell LA, Henderson AJ, Dow SW.** 2012. Suppression of vaccine immunity by inflammatory monocytes. J Immunol **189:**5612-5621.

57. **Nash AA, Jayasuriya A, Phelan J, Cobbold SP, Waldmann H, Prospero T.** 1987. Different roles for L3T4+ and Lyt 2+ T cell subsets in the control of an acute herpes simplex virus infection of the skin and nervous system. J Gen Virol **68 (Pt 3):**825-833.

58. **Ho M, Schollaardt T, Smith MD, Perry MB, Brett PJ, Chaowagul W, Bryan LE.** 1997. Specificity and functional activity of anti-*Burkholderia pseudomallei* polysaccharide antibodies. Infect Immun **65:**3648-3653.

59. **Zhang S, Feng SH, Li B, Kim HY, Rodriguez J, Tsai S, Lo SC.** 2011. In Vitro and In Vivo studies of monoclonal antibodies with prominent bactericidal activity against *Burkholderia pseudomallei* and *Burkholderia mallei*. CVI **18:**825-834.

60. **Abadie V, Badell E, Douillard P, Ensergueix D, Leenen PJ, Tanguy M, Fiette L, Saeland S, Gicquel B, Winter N.** 2005. Neutrophils rapidly migrate via lymphatics after *Mycobacterium bovis* BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. Blood **106:**1843-1850.

61. **Lazar Adler NR, Govan B, Cullinane M, Harper M, Adler B, Boyce JD.** 2009. The molecular and cellular basis of pathogenesis in melioidosis: how does *Burkholderia pseudomallei* cause disease? FEMS Microbiol Rev **33:**1079-1099.

Figure Legends.

Figure 1: *Subcutaneous immunization with Bp82 protects mice from an acutely lethal B. pseudomallei infection*. C57BL/6 and BALB/c mice (N = 5), were immunized twice 10 days apart with 5 × 10^6 CFU of Bp82 suspended in PBS. All animals were then challenged intranasally with ~5×LD_{50} (1.2 x 10^4 (C57BL/6) or 5 x 10^3 (BALB/c) CFU/mouse) *B. pseudomallei* strain 1026b. Animals were euthanized upon reaching a pre-determined euthanasia endpoint. Kaplan-Meier survival curves were generated for C57Bl/6 mice for 60 d survival (A) and for 60 d survival for BALB/c mice (B). Statistical differences in survival were determined by log-rank test. Data are pooled from two combined experiments.

Figure 2: *Immunization reduces bacterial burden in vaccinated mice at 72 h after challenge*. Bacterial burden in lung, spleen and liver tissues was determined in naïve and vaccinated mice (n = 5) 72 h after intranasal challenge with *B. pseudomallei* strain 1026b. Statistical comparisons between vaccinated and naïve groups were done using non-parametric t-test. * denotes $p < 0.05$. Similar results were obtained in one additional experiment.

Figure 3*: Humoral immune responses in Bp82 vaccinated mice*. Serum was prepared from BALB/c and C57BL/6 mice (N=5) vaccinated twice s.c. with Bp82 and titers to heat-killed Bp82 determined by endpoint dilution ELISA. Titers of total IgG (A), IgM (B), IgG1 (C), IgG2a (D) were expressed as the reciprocal of the endpoint dilution. Significant differences (*$P < 0.05$, **$P < 0.005$, ***$P<0.0001$) were determined by nonparametric Kruskal-Wallis Test followed by

Dunnett's multiple comparison test. Data represent pooled data from two independent experiments.

Figure 4. *Cellular immune responses to Bp82 immunization.* BALB/c mice (N = 5) were immunized twice s.c. with Bp82. Fourteen days after the second immunization, spleen cells from naïve or vaccinated animals were re-stimulated *in vitro* with heat-killed Bp82 ($2x10^7$ CFU/ml), lysate from Bp82 (5µg/ml), recombinant GroEL (12.5 µg/ml), or medium alone for 3 days. Supernatants were assayed by ELISA for IFN-γ (A) and IL-17 (B) production. (* $P<0.05$ using Mann-Whitney U test. Data represent pooled data from two independent experiments.

Figure 5. *Role of antibody in Bp82 vaccine protection*. In (A), serum from immunized and naïve animals was transferred to naïve BALB/c recipient animals (N = 5 per group), which were then subjected to lethal i.n. challenge with *B. pseudomallei* 24 h later. Survival percentages were significantly increased in the animals that received immune serum, compared to animals that received non-immune serum. In (B), $uMT^{-/-}$ and WT C57Bl/6 mice (N = 5) were immunized twice s.c. with Bp-82 and then challenged intranasally with *B. pseudomallei* 1026b and survival times determined. Survival was significantly reduced in $uMT^{-/-}$ mice compared to WT animals. Data represent pooled data from two independent experiments.

Figure 6. *Role of T cells in vaccine-induced protection against B. pseudomallei.* $CD8^{-/-}$(A), $CD4^{-/-}$ (B) and $TCRa/b^{-/-}$ (C) and WT C57Bl/6 mice were immunized twice with Bp82. Naïve and

619 immunized animals were then challenged intranasally with 1.2 x 10^4 cfu *B. pseudomallei* 1026b
620 and survival percentages determined. Survival percentages for $CD8^{-/-}$, $CD4^{-/-}$ and $TCRa/b^{-/-}$ mice
621 compared to WT mice were not significantly different. Survival differences were determined
622 using Kaplan-Meier survival curves and log-rank analysis. Data are representative of two
623 independent experiments.

624

625 **Figure 7:** *Lymph node cellular response to Bp82 vaccination*. BALB/c mice (n=5 per group)
626 were injected in one rear limb foot pad with 1.2x10^8 cfu Bp82-*gfp.* Ten hours after inoculation,
627 cells from the ipsilateral popliteal LN were collected and immunostained. Cells from the
628 contralateral popiteal LN served as the control. In (A), the appearance of GFP^+ $CD11b^+$ cells in
629 the draining LN was determined by flow cytometry at 10h after injection. The GFP^+ cells were
630 further subdivided into PMN ($CD11b^+Ly6G^+Ly6C^-$) and monocytes ($CD11b^+Ly6G^-Ly6C^+$). In
631 (C), the distribution of Bp82-*gfp* bacteria in relevant LN APC populations was calculated,
632 including neutrophils (PMN), dendritic cells (DC), CD169+ subcapsular macrophages, and
633 monocytes. Data are representative of two independent experimets. Statisitcal differences
634 between Bp82 injected and uninjected contralateral LN lymph node were determined by
635 student's two-tailed test. (**P <0.05). For comparasions between 4 groups (Panel C), ANOVA
636 was used, followed by Tukey post-hoc test.

637