

# Humoral and Cell-Mediated Adaptive Immune Responses Are Required for Protection against *Burkholderia pseudomallei* Challenge and Bacterial Clearance Postinfection

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***Burkholderia pseudomallei*, the causative agent of melioidosis, is a gram-negative bacillus endemic to areas of southeast Asia and northern Australia. Presently, there is no licensed vaccine for *B. pseudomallei* and the organism is refractive to antibiotic therapy. The bacterium is known to survive and multiply inside both phagocytic and nonphagocytic host cells and may be able to spread directly from cell to cell. Current vaccine delivery systems are unlikely to induce the correct immune effectors to stimulate a protective response to the organism. In this study, we have developed a procedure to utilize dendritic cells as a vaccine delivery vector to induce cell-mediated immune responses to *B. pseudomallei*. Dendritic cells were produced by culturing murine bone marrow progenitor cells in medium containing granulocyte-macrophage colony-stimulating factor and tumor necrosis factor alpha. Purified dendritic cells were pulsed with heat-killed whole-cell *B. pseudomallei* and used to immunize syngeneic mice. Strong cellular immune responses were elicited by this immunization method, although antibody responses were low. Booster immunizations of either a second dose of dendritic cells or heat-killed *B. pseudomallei* were administered to increase the immune response. Immunized animals were challenged with fully virulent *B. pseudomallei*, and protection was demonstrated in those with strong humoral and cell-mediated immunity. These results indicate the importance of both cell-mediated and humoral immune mechanisms in protection against intracellular pathogens.**

*Burkholderia pseudomallei* is a gram-negative, motile, facultative anaerobic bacillus (17) that is responsible for a broad spectrum of illnesses observed in both humans and animals (8, 17) and is a common cause of human pneumonia and fatal bacteremias in areas of endemicity (6). Incidence is particularly high in southeast Asia and northern Australia (6–9), although the disease is becoming increasingly prevalent in China (32) and the Indian subcontinent (7).

Manifestations of the disease caused by *B. pseudomallei*, melioidosis, vary from an asymptomatic state to chronic illness to acute overwhelming septicemia (30, 31). Increasing recognition of melioidosis as a significant cause of morbidity and mortality, together with complications in treatment, has led to a number of studies investigating immunoprophylaxis for the disease (31). While presently no vaccine exists to protect against melioidosis, a number of approaches have shown promise, including immunoglobulin therapy (3, 5) and conjugate vaccines (reviewed in reference 4). Other approaches include the use of recombinant vaccines based on both *B. pseudomallei* (1) and other bacteria (10) and the attenuated mutants of *B. pseudomallei*, which have been used with some success (1).

To better achieve the goal of these programs of research, there is a need to understand the optimum immune response required for protection. Such understanding would facilitate rational vaccine design with an aim at producing the appropriate immune response required. A common failing of many

current vaccine approaches is their lack of ability to stimulate cell-mediated immunity (CMI). Recent studies have demonstrated the importance of gamma interferon production within the first 24 h of infection in conferring resistance to acute lethal *B. pseudomallei* infection (26). In addition, the intracellular nature of *B. pseudomallei* and its ability to survive and multiply in the intracellular environment highlight the need to generate CMI responses to combat infection.

During host immune responses to infection, dendritic cells (DC) play a key role in the generation of adaptive immune responses (2). DC are the most potent stimulators of naïve T-cell responses and reside in most tissues and organs (16, 23) at major portals of microbial entry (24). Here, they perform a sentinel-like function, continuously sampling their external environment (24) for foreign antigens.

Under physiological conditions, DC exist in an immature state primed for antigen uptake and processing. Pathogen recognition (via receptors such as the toll-like receptor family), together with the production of proinflammatory signals, initiates cell maturation, which transforms the DC into efficient T-cell stimulators (16). During maturation, DC up-regulate their expression of chemokine receptors (25), as well as molecules essential for the activation of T cells, such as major histocompatibility complex (MHC) molecules and the costimulatory molecules CD80 and CD86 (2). The DC then leave the peripheral tissues and migrate to the draining lymph nodes (16), final maturation occurring under the control of specific T cells through the interaction of CD40 and CD40L (14).

In this study, we employed DC as a delivery vector to generate CMI responses to *B. pseudomallei*. We also compared the immune responses generated following immunization with *B. pseudomallei*-pulsed DC or soluble *B. pseudomallei* in an at-

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tempt to develop an effective vaccination regimen for protection against *B. pseudomallei* infection.

## MATERIALS AND METHODS

**Experimental animals.** BALB/c mice were obtained from Charles River Ltd. and maintained under specific-pathogen-free conditions with free access to food and water. All procedures carried out were in accordance with the requirements of the Animal (Scientific Procedures) Act of 1986.

**Isolation and culture of dendritic cells from murine bone marrow.** A method was derived from established methods (19, 23, 27, 29), described briefly as follows. Bone marrow was extracted from murine rear tibiae and fibulas and cultured at a concentration of  $2 \times 10^6$  cells  $\text{ml}^{-1}$  in six-well tissue culture plates. Standard culture medium was comprised of RPMI 1640 (Sigma, United Kingdom) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, United Kingdom), 1% penicillin-streptomycin-glutamine (Sigma, United Kingdom), and 50  $\mu\text{M}$  2-mercaptoethanol. The culture medium was supplemented with 20 ng  $\text{ml}^{-1}$  granulocyte-macrophage colony-stimulating factor (R&D Systems) and 10 ng  $\text{ml}^{-1}$  tumor necrosis factor alpha (R&D Systems). Cells were cultured for 96 h at 37°C in the presence of 5%  $\text{CO}_2$  in a fully humidified atmosphere, after which time they were removed from the culture plates by gentle scraping. After being washed, the cell suspension was layered onto 13.7% (wt/vol) metrizamide (Sigma, United Kingdom) and the DC were purified using centrifugation (23a).

**Flow cytometry.** Cells were counted, washed, and then resuspended in phosphate-buffered saline (PBS) supplemented with 2.5% heat-inactivated fetal bovine serum at a concentration of  $10^6$  cells per 100  $\mu\text{l}$ . The appropriate fluorochrome-labeled antibodies at the correct concentrations for each fluorochrome were added (all antibodies were obtained from Pharmingen, BD Biosciences, United Kingdom) and the cells were incubated at 4°C for 30 min. Next, 4% paraformaldehyde was then added to prevent any further binding. Samples were then fixed at 4°C overnight before analysis on a Becton Dickinson FACScan flow cytometer using Cell Quest Pro analysis software.

**Growth of *B. pseudomallei*.** *B. pseudomallei* NCTC 4845 was grown in nutrient broth in a static culture for 18 h at 37°C. Viable counts were obtained following use of a culture by culturing aliquots at 37°C overnight on nutrient agar plates.

**Heat inactivation of bacteria.** Bacterial cells were harvested by centrifugation and washed three times in PBS before resuspending them in 1/10 the original volume of PBS. The bacterial cell suspension was then incubated in a water bath at 80°C for 3 h with occasional shaking. After inactivation, the suspension was checked for viability by inoculating 10-ml volumes of nutrient broth with 0.5-ml aliquots of the suspension and incubating the broth at 37°C for 7 days. Nutrient agar plates were then inoculated with the total volume of the broth cultures to check for bacterial growth and incubated for a further 7 days. If no growth occurred on the agar plates, the bacterial suspension was considered inactivated.

**Primary lymphocyte proliferation assay.** DC were cultured using the previously described protocol and, after washing, were adjusted to a concentration of  $2 \times 10^6$  cells  $\text{ml}^{-1}$  in culture medium, together with 20 ng  $\text{ml}^{-1}$  granulocyte-macrophage colony-stimulating factor and 10 ng  $\text{ml}^{-1}$  tumor necrosis factor alpha. The cells were then incubated with heat-killed *B. pseudomallei* ( $10^4$  CFU  $\text{ml}^{-1}$ ) at 37°C in a fully humidified environment in the presence of 5%  $\text{CO}_2$  for 18 h.

Spleens were isolated from naïve mice killed by cervical dislocation, and they were gently passed through a 70- $\mu\text{m}$ -pore-size nylon sieve. Erythrocytes were lysed using 0.85% ammonium chloride and the remaining cells then washed to prevent lysis of leukocytes. A viable cell count was performed and the cells were resuspended to a concentration of  $5 \times 10^6$  cells  $\text{ml}^{-1}$ .

Splenocyte suspensions were then added to the wells of 96-well tissue culture plates in 100- $\mu\text{l}$  aliquots. Replicates of five wells were used for each of the test groups and controls, with 1  $\mu\text{g}$   $\text{ml}^{-1}$  concanavalin A (Sigma, United Kingdom) being used as a positive control and culture medium as a negative control.

The *B. pseudomallei*-pulsed DC were washed to remove any extracellular antigen, resuspended to the required concentration in culture medium, and aliquoted in 100- $\mu\text{l}$  volumes into the appropriate wells on the proliferation assay plates. The plates were then incubated at 37°C in 5%  $\text{CO}_2$  for 24 to 144 h.

Following the required incubation period, 37 MBq of [ $^3\text{H}$ ]thymidine was added to each test well on the proliferation plates and the plates were reincubated for a further 24 h. Cells were then harvested onto 96-well filter plates (PerkinElmer Life Sciences) using an automated cell harvester and the plates were allowed to dry at room temperature overnight. Once the plates were dry, 20  $\mu\text{l}$  of scintillation fluid (PerkinElmer Life Sciences) was added to each well and the plate sealed before being read on a scintillation counter (PerkinElmer Life Sciences).

**Secondary lymphocyte proliferation assay.** Spleens from immunized mice were used to assess T-cell recall responses to soluble *B. pseudomallei*. Spleen cells ( $5 \times 10^5$ ) from immunized mice were incubated with heat-killed *B. pseudomallei*, which was added to the splenocyte cultures in 100- $\mu\text{l}$  aliquots at a concentration of  $10^4$  CFU  $\text{ml}^{-1}$ , and the cultures were incubated for 72 h in 5%  $\text{CO}_2$  at 37°C.

**Enzyme-linked immunosorbent assay for serum antibody.** Serum antibody titers to *B. pseudomallei* were assayed by enzyme-linked immunosorbent assay as previously described (21). Concentrations of antigen-specific immunoglobulin G were determined using Ascent software (Thermo LabSystems).

**Immunization. (i) DC immunizations.** DC were cultured as described above. Once isolated and purified from culture, DC were resuspended to  $2 \times 10^6$  cells  $\text{ml}^{-1}$  and pulsed with heat-killed *B. pseudomallei* at  $10^4$  CFU  $\text{ml}^{-1}$  for 18 h at 37°C in a fully humidified 5%  $\text{CO}_2$  environment. The cells were then washed to remove any extracellular antigen and resuspended in sterile PBS to a concentration of  $1 \times 10^6$  cells per 100  $\mu\text{l}$  for the intradermal (ID) immunizations and  $1 \times 10^6$  cells per 50  $\mu\text{l}$  for the intranasal (IN) immunizations.

**(ii) *B. pseudomallei* immunizations.** Heat-killed *B. pseudomallei* was prepared in sterile PBS at  $5 \times 10^3$  CFU per 100  $\mu\text{l}$  for ID immunization without adjuvant, which provided an antigen concentration equivalent to that received by  $1 \times 10^6$  DC. Inocula for IN immunization were prepared at  $5 \times 10^3$  CFU per 50  $\mu\text{l}$ . *B. pseudomallei* administered with adjuvant was again prepared to an equivalent concentration using the monophosphoryl lipid A-trehalose dicorynomycolate adjuvant system (Sigma, United Kingdom) and delivered via the intramuscular route.

For all immunization combinations, a regimen of priming at day 0 and boosting at day 28 was used.

**Challenge with *B. pseudomallei*.** *B. pseudomallei* NCTC 4845 was grown in overnight culture as described previously and diluted to give an estimated challenge dose of  $10^4$  CFU per mouse. Actual challenge doses were determined by overnight culture of inoculum samples at 37°C on nutrient agar plates. Groups of five female BALB/c mice (10 to 12 weeks old) were challenged by the intraperitoneal route and closely observed for 35 days postchallenge, at which point any survivors were culled. The growth of and challenge with *B. pseudomallei* was performed under Advisory Committee on Dangerous Pathogens category III conditions.

**Assessment of bacterial load.** Following culling of the challenge survivors, the brain, liver, spleen, and blood were assessed for bacterial load. Organs were passed through 70- $\mu\text{m}$ -pore-size nylon sieves into sterile PBS, and blood was obtained by cardiac puncture. Samples were inoculated onto nutrient agar plates and incubated overnight at 37°C. Plates were then examined for the presence or absence of *B. pseudomallei* and the number of CFU were recorded.

**Statistical analysis.** Statistical analyses were performed using Student's sample *t* test for all in vitro experiments. Analysis of the challenge data was performed using PRISM GraphPad survival analysis software and *P* values were calculated using the log rank test for a trend.

## RESULTS

**Isolation and culture of dendritic cells.** DC produced from this culture system were phenotypically myeloid DC, which were MHC class II $^{2+}$ , CD80 $^{+}$ , CD86 $^{+}$ , CD8 $\alpha^{+}$ , and CD11b $^{+}$ . Yields ranged from  $7 \times 10^6$  to  $10 \times 10^6$  per mouse used.

**Dendritic cell maturation.** The importance of DC maturation in the generation of T-cell responses led us to investigate this response upon exposure to heat-killed *B. pseudomallei*. Flow cytometry was used to assess MHC class II, CD80, CD86, and CD54 expression on the DC surface (Fig. 1) in response to *B. pseudomallei* exposure. Expression of all markers tested was seen to increase 12 h postexposure, demonstrating that maturation of the dendritic cells was occurring following exposure to heat-killed *B. pseudomallei*. Comparison to lipopolysaccharide (LPS)-induced maturation (data not shown) showed that maturation induced by *B. pseudomallei* was faster (12 h compared to 24 to 48 h for LPS-induced maturation) and associated with greater increases in marker expression.

**Processing and presentation of immunogenic peptides.** Optimum proliferation was observed when DC were pulsed with

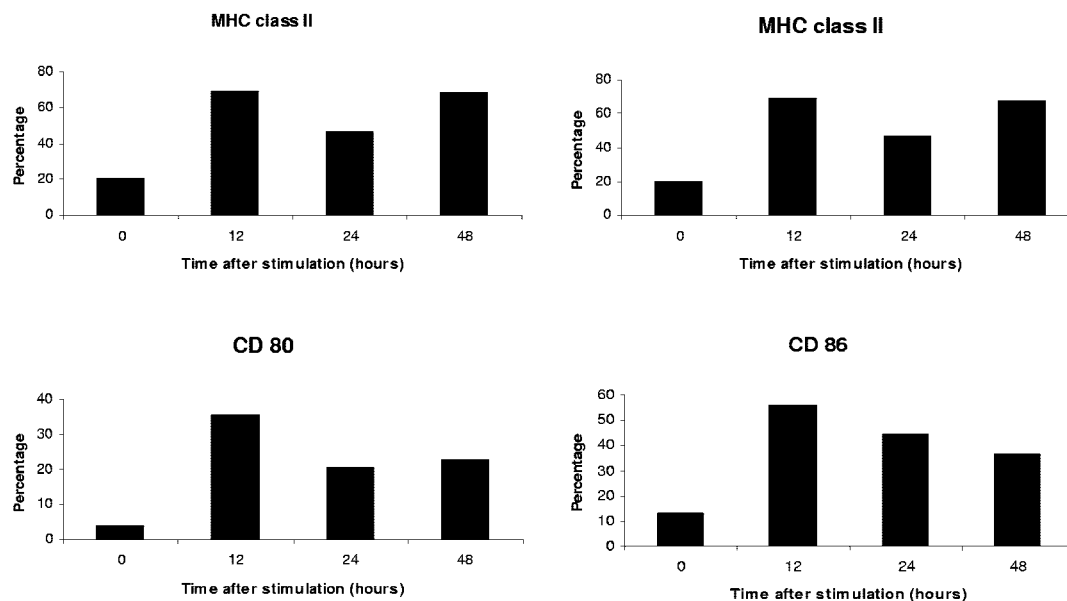


FIG. 1. Activation of DC following exposure to heat-killed *B. pseudomallei*. Expression of all markers tested was seen to increase 12 h postexposure, demonstrating that maturation of the dendritic cells was occurring following exposure to heat-killed *B. pseudomallei*. Comparison to LPS-induced maturation (data not shown) showed that maturation induced by *B. pseudomallei* was faster (12 h compared to 24 to 48 h for LPS-induced maturation) and associated with greater increases in marker expression.

$10^4$  CFU of heat-killed *B. pseudomallei* per  $2 \times 10^6$  cells and coincubated with the splenocyte suspension at a ratio of 1 to 10 for 72 h (optimization data not shown). Proliferation greater than that for the negative control was seen in all test groups ( $P < 0.05$ ). Proliferation following stimulation with *B. pseudomallei*-pulsed DC was significantly greater ( $P < 0.01$ ) than that following stimulation with heat-killed *B. pseudomallei* (Fig. 2).

**Immunization with dendritic cells.** To assess the in vivo effect of immunization with pulsed DC, antibody and cellular

responses stimulated following the administration of combinations of pulsed DC and/or *B. pseudomallei* with or without adjuvant were assayed. A regimen of prime at day 0 and booster at day 28 was chosen, and both ID and IN immunization routes were investigated. Figure 3 shows the antibody and CMI responses obtained following ID immunization with *B. pseudomallei*-pulsed DC or *B. pseudomallei*. Immunization with DC was found to stimulate significantly greater CMI responses ( $P < 0.001$ ) than immunization with *B. pseudomallei*

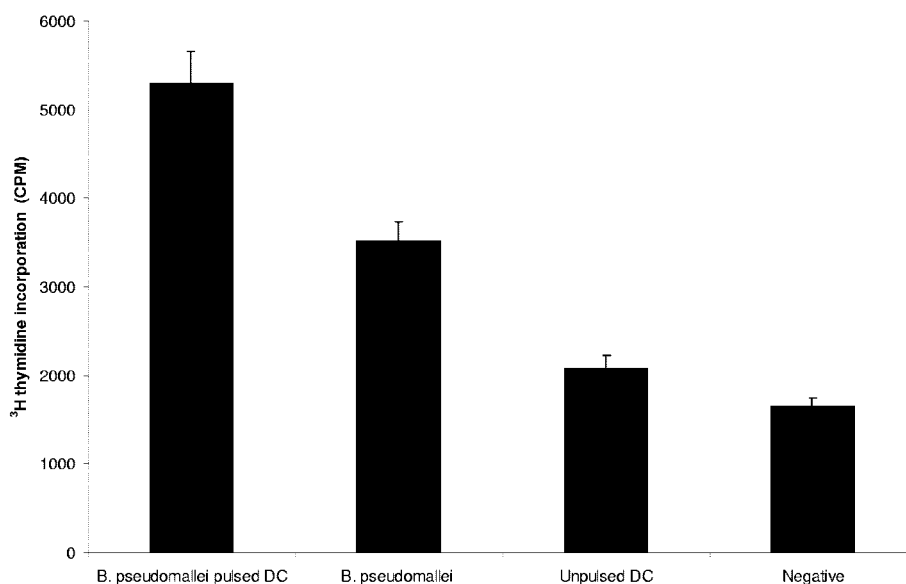


FIG. 2. Naïve lymphocyte proliferation after 72 h of stimulation with *B. pseudomallei*-pulsed DC or heat-killed *B. pseudomallei*. Proliferation greater than that in the negative control was seen in all groups ( $P < 0.05$ ). The greatest proliferation was seen following stimulation with *B. pseudomallei*-pulsed DC ( $P < 0.01$ ) compared to proliferation with *B. pseudomallei*. Error bars signify standard errors of the means (standard errors calculated from the means of five replicates assayed on pooled splenocytes from two animals). CPM, counts per minute.

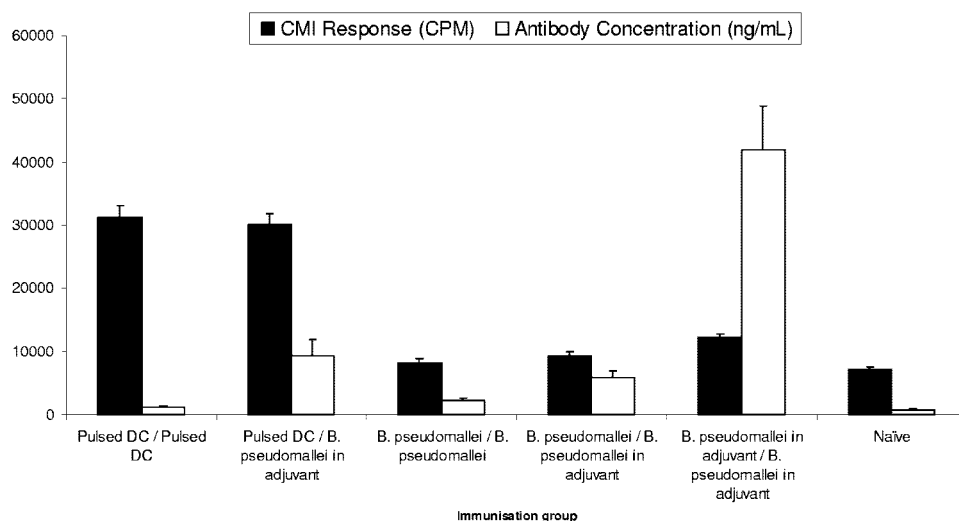


FIG. 3. Antibody ( $\text{ng ml}^{-1}$ ) and CMI ( $^3\text{H}$ )thymidine incorporation in counts per minute [CPM] responses following ID immunization with different *B. pseudomallei*-pulsed DC/*B. pseudomallei* combinations. Doses were administered at days 0 and 28, and *B. pseudomallei* in adjuvant was administered intramuscularly. Immunization with DC produced significantly greater CMI responses ( $P < 0.001$ ) than immunization with *B. pseudomallei* with or without adjuvant. Conversely, immunization with *B. pseudomallei* in adjuvant stimulated significantly higher antibody production ( $P < 0.05$ ) than DC immunization. For CMI data, error bars signify standard errors of the means (standard errors calculated from the means of five replicates assayed on pooled splenocytes from two animals). For antibody data, error bars signify standard errors of the means (standard errors calculated from the means of five animals each assayed in duplicate).

with or without adjuvant. Conversely, immunization with *B. pseudomallei* in adjuvant stimulated significantly higher antibody production ( $P < 0.05$ ) than DC immunization.

For the IN groups (Fig. 4), immunization with pulsed DC again produced a significantly greater CMI response than immunization with *B. pseudomallei* ( $P < 0.001$ ). Poor antibody responses were observed for all groups following intranasal immunization.

The substitution of the second DC dose at day 28 for a dose

of *B. pseudomallei* in adjuvant was also investigated for changes in the CMI and antibody responses. For the ID immunized groups (Fig. 3), no significant difference was observed in the CMI response when the day 28 dose was *B. pseudomallei* in adjuvant. A significant increase in antibody production was, however, noted for the pulsed DC group following substitution of the day 28 DC dose for *B. pseudomallei* in adjuvant ( $P < 0.05$ ). No difference in CMI response or antibody production was observed in any of the IN immunized groups (Fig. 4).

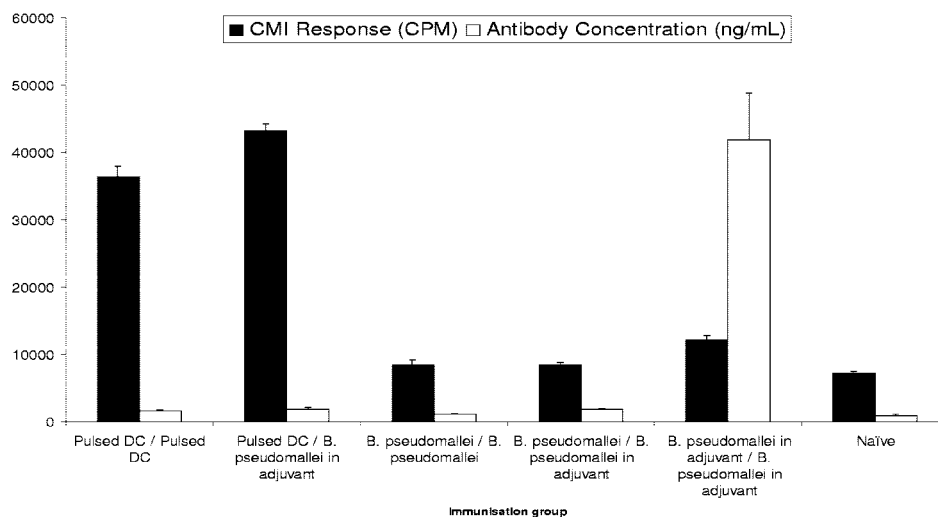


FIG. 4. Antibody ( $\text{ng ml}^{-1}$ ) and CMI ( $^3\text{H}$ )thymidine incorporation in counts per minute [CPM] responses following IN immunization with different *B. pseudomallei*-pulsed DC/soluble *B. pseudomallei* combinations. Immunization with DC again produced a significantly greater CMI response than immunization with soluble *B. pseudomallei* ( $P < 0.001$ ). Poor antibody responses were observed for all groups. The group immunized with two doses of *B. pseudomallei* in adjuvant was immunized via the intramuscular route and was included for comparison purposes. For CMI data, error bars signify standard errors of the means (standard errors calculated from the means of five replicates assayed on pooled splenocytes from two animals). For antibody data, error bars signify standard errors of the means (standard errors calculated from the means of five animals each assayed in duplicate).

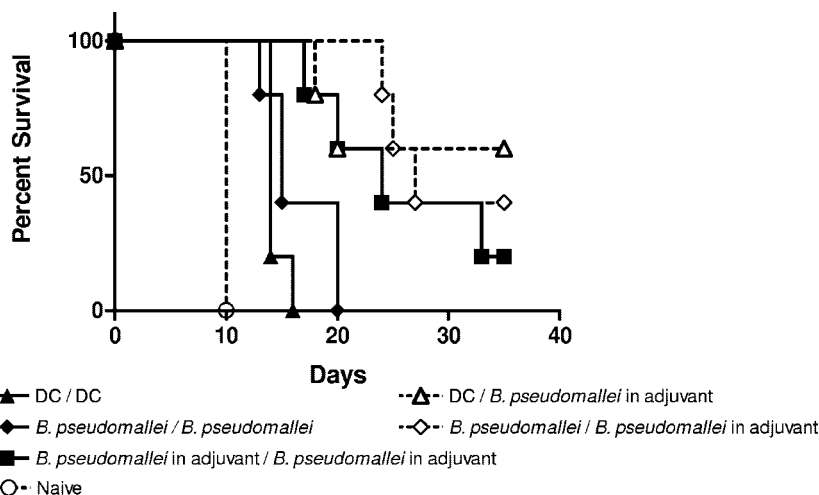


FIG. 5. Survival curves for each of the challenge groups. All naïve mice died by day 10. Mice immunized with two pulsed DC doses together with those immunized with two doses of *B. pseudomallei* in PBS died by day 20. Groups immunized with *B. pseudomallei* displayed a 20 to 40% ( $P < 0.005$ ) survival rate, while those immunized with pulsed DC and boosted with *B. pseudomallei* in adjuvant had a 60% ( $P < 0.005$ ) survival rate. In addition, where the second dose was substituted for soluble *B. pseudomallei* in adjuvant, a significant increase in survival rate was noted ( $P < 0.005$ ) compared to the equivalent group without this substitution.

**Protection against virulent *B. pseudomallei* challenge.** Mice immunized with the different pulsed DC/*B. pseudomallei* combinations were challenged with approximately  $5.3 \times 10^4$  CFU (50% lethal dose of 163 CFU) of virulent *B. pseudomallei* strain NCTC 4845 by the intraperitoneal route (Fig. 5). Animals were closely observed for 35 days following challenge, during which time any animals showing signs of severe illness were humanely culled. The naïve mice had all died by day 10 postchallenge. All mice immunized with two pulsed DC doses, together with those immunized with two doses of *B. pseudomallei* in PBS, died by day 20. Groups immunized with *B. pseudomallei* in adjuvant or *B. pseudomallei*-pulsed DC/soluble *B. pseudomallei* in adjuvant displayed 20 to 40% ( $P < 0.005$ ) and 60% ( $P < 0.005$ ) survival rates, respectively, compared to the naïve controls. In addition, for each of the groups where the second DC dose was replaced with *B. pseudomallei* in adjuvant, a significant increase in survival rate was noted ( $P < 0.005$ ) compared to the equivalent group without this substitution.

Assessment of bacterial burden in the challenge survivors (Table 1) revealed that both survivors from the group immunized with *B. pseudomallei*/*B. pseudomallei* in adjuvant had significant splenomegaly, and in the mouse where *B. pseudomallei* was cultured from more than one tissue sample, splenic and hepatic abscesses were present. The presence of hepatosplenomegaly and multiple abscesses suggested that while these animals had survived the primary stage of the infection, chronic infectious states were present and relapse would be anticipated, leading to death of the animals.

In the remaining immunization groups, *B. pseudomallei*-pulsed DC/*B. pseudomallei* in adjuvant and the double dose of *B. pseudomallei* in adjuvant, evidence of *B. pseudomallei* in the tissues was greatly reduced. One mouse from each group had no *B. pseudomallei* present in any of the tissue samples cultured. While the other survivors from the DC-dosed group did have evidence of *B. pseudomallei* in their spleen samples, this

was limited and the inability to culture organisms from one survivor suggests that effective bacterial clearance was in progress and that, had the experiment been extended, complete clearance may have been achieved.

**DISCUSSION**

*B. pseudomallei* is a significant cause of morbidity and mortality in areas of endemicity (6–9, 17) and is becoming increasingly resistant (6, 8, 28) to antibiotic therapy. The lack of potential therapeutics for *B. pseudomallei* infection has opened the stage for prophylactic vaccine approaches. A common failing of many of the current vaccine approaches, however, is their lack of ability to stimulate CMI, highlighting the need for a vaccination strategy that drives the development of CMI.

TABLE 1. Number of *B. pseudomallei* CFU counted in brain, liver, spleen, and blood samples from challenge survivors after culture of organ samples<sup>a</sup>

Immunization group	No. of CFU counted			
	Brain	Liver	Spleen	Blood
Pulsed DC/ <i>B. pseudomallei</i> in adjuvant	0	0	<10	0
	0	0	0	0
	0	0	0	0
<i>B. pseudomallei</i> / <i>B. pseudomallei</i> in adjuvant	0	>10	>10	0
	0	<10	0	0
<i>B. pseudomallei</i> in adjuvant/ <i>B. pseudomallei</i> in adjuvant	0	0	0	0

<sup>a</sup> Survivors from the group immunized with *B. pseudomallei*/*B. pseudomallei* in adjuvant ( $n = 2$ ) had *B. pseudomallei* present in at least one of the tissue samples tested. In the immunization groups with DC/*B. pseudomallei* in adjuvant ( $n = 3$ ) and *B. pseudomallei* in adjuvant/*B. pseudomallei* in adjuvant ( $n = 1$ ), evidence of bacterial clearance was apparent. One mouse from each of these groups had no *B. pseudomallei* present in any of the tissue samples cultured.  $n$ , number of mice still alive 35 days postchallenge.

Dendritic cells are a rapidly developing vaccine tool that have been utilized in both cancer and infectious disease vaccine research (15). The attractiveness of DC as a delivery system lies in their innate ability to generate adaptive immune responses. Our data support their suitability for this purpose but also shed light on the role of type 1 and type 2 immune responses in protection against *B. pseudomallei* challenge.

The isolation and culture of a wide range of DC subtypes are now becoming common practice, and here, we have demonstrated the ability to generate high-yield, high-purity cultures of DC in a relatively short time scale. Further, we have demonstrated the ability of these DC to respond to exposure to killed *B. pseudomallei*, undergo maturation, and then present *B. pseudomallei*-associated immunogenic epitopes to naïve T cells. From our proliferation data, it is evident that the administration of antigen-pulsed DC generates superior T-cell responses ( $P < 0.01$ ) compared to the delivery of antigen. Investigation of the immune responses generated in response to DC immunization showed DC to be a highly efficient vector for the generation of CMI memory responses. DC immunization generated significantly greater CMI memory responses ( $P < 0.001$ ) than *B. pseudomallei* delivered with and without adjuvant via both the ID and IN immunization routes. We did not observe any antibody production as a result of DC immunization, however, and thus modified our vaccination regimen to successfully increase the anti-*B. pseudomallei* antibody titer ( $P < 0.05$ ) above that seen in the groups given two doses of antigen-pulsed DC and naïve groups.

Testing of the protective efficacy of these immunization approaches demonstrated that where antibody responses significantly greater than those for the naïve controls were observed, survival rates were increased to 20 to 40% ( $P < 0.005$ ) for immunization with *B. pseudomallei* and 60% ( $P < 0.005$ ) for immunization including antigen-pulsed DC.

Assessment of bacterial burden at day 35 postchallenge demonstrated that the groups with the greatest cellular and humoral immunity were successfully clearing the infection. This was in stark contrast to those survivors that had failed to mount such an effective response and were heavily burdened by infection, suggesting that relapse would likely have occurred.

The resistance of *B. pseudomallei* to complement means that effective clearance during the extracellular phase of infection is wholly dependent on opsonization. On the basis of our data, we would hypothesize that a role for antibody during the primary phase of infection is necessary, as, despite the presence of a substantial CMI response in the group immunized with two doses of DC, no animals survived challenge. The inclusion of *B. pseudomallei* in the adjuvant boosting dose which stimulated antibody production resulted in a significant increase in survival rate to 60% ( $P < 0.005$ ). This theory is also corroborated by passive-protection studies with polyclonal sera and monoclonal antibodies, which highlight the importance of antibody in protection against *B. pseudomallei* infection (3, 5, 21).

The ability of *B. pseudomallei* to invade and replicate in a number of cell types including professional phagocytic cells has been previously demonstrated (13, 31). Although mechanisms for intracellular survival are yet to be elucidated (13, 20), we have shown the importance of an effective CMI response in achieving bacterial clearance following survival of the primary

infective phase. We have also demonstrated the suitability of DC as a delivery vector capable of generating effective CMI responses against intracellular bacteria corroborating previous research (11, 12, 15, 18).

Our data demonstrate the importance not only of cell-mediated immune mechanisms in defense against infection with *B. pseudomallei* but also an important role for antibody, thus providing a basis for rational vaccine design. We would suggest that optimum levels of protection will be achieved only when a vaccination strategy which can evoke both cell-mediated and humoral mechanisms is optimized. Dendritic cells have shown considerable potential as candidates for the induction of cellular immune responses, but as our data suggest, alternative mechanisms are needed to induce vital antibody production. There are also a number of drawbacks to using DC to deliver vaccine antigens. We would, however, propose that DC are excellent tools for the exploration of protective immune responses and for testing potential candidate vaccines. Future studies will address the development of optimally balanced immune responses to provide robust protection against intracellular bacteria.

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