Protection against Heterologous *Burkholderia pseudomallei* Strains by Dendritic Cell Immunization

Stephen J. Elvin,^1*^ Gareth D. Healey,^1^ Angie Westwood,^1^ Stella C. Knight,^2^ James E. Eyles,^1^ and E. Diane Williamson^1^  

**Biomedical Sciences, Dstl Porton Down, Salisbury SP4 0JQ, United Kingdom,** and **University of London Imperial College of Science, Technology and Medicine, Antigen Presentation Research Group, Northwick Park Campus, Watford Road, Harrow, Middx HA1 3UJ, United Kingdom**^2^  

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*B. pseudomallei*, the causative agent of melioidosis, is a gram-negative bacterium which can cause either chronic infections or acute lethal sepsis in infected individuals. The disease is endemic in Southeast Asia and northern Australia, but little is known about the mechanisms of protective immunity to the bacterium. In this study, we have developed a procedure to utilize dendritic cells in combination with CpG oligodeoxynucleotides as a vaccine delivery vector to induce protective immune responses to various strains of *B. pseudomallei*. Our results show that strong cell-mediated immune responses were generated, while antibody responses, although low, were detectable. Upon virulent challenge with *B. pseudomallei* strain K96243, NCTC 4845, or 576, animals immunized with dendritic cells that were pulsed with heat-killed K96243 and matured in the presence of CpG 1826 showed significant levels of protection. These results show that a vaccine strategy that actively targets dendritic cells can evoke protective immune responses.

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*B. pseudomallei* is a motile, nonspore-forming, gram-negative bacillus (12). It is the causative agent of melioidosis and is endemic to areas of Southeast Asia and northern Australia. The manifestation of melioidosis can differ greatly from an asymptomatic pulmonary infiltration to an acute localized suppurative infection, an acute pulmonary infection, or an acute septicemic infection, or it may become a chronic suppurative infection (5, 16). In some cases, the bacteria remain latent and cause clinical manifestations after a prolonged quiescence, with cases being reported as many as 62 years after the initial infection (24, 25, 28). The organism is able to disseminate throughout the body from localized sites of infection, and it is able to invade nonphagocytic cells and to also survive and replicate in phagocytes (15, 34). The exact mechanisms of intracellular survival are as yet undetermined (37), although escape from lysosomal endosomes is known to occur (35). Presently, there are no vaccines for *B. pseudomallei* and the organism is refractive to antibiotic therapy (7).

The mechanisms of resistance to *B. pseudomallei* infection are not well characterized, although cell-mediated immunity would appear to be important, given that the organism is able to survive and replicate within cells (2, 27). There is also evidence that *B. pseudomallei* can interfere with the inducible nitric oxide synthase system of macrophages to avoid killing (37) and that the organism is resistant to complement lysis (33). Efficient interactions between infected host cells and antigen-specific T cells are required for pathogen elimination in intracellular infections, but little is known about the role of adaptive cell-mediated immunity responses in melioidosis (2). It is possible that humoral responses are less important in protection against *B. pseudomallei* due to the intracellular lifestyle of the organism (10).

The ability of *B. pseudomallei* to infect by the airborne route and its severe course of infection, coupled to the widespread distribution of the organism, have raised concerns that it could be used as a biological warfare or bioterrorism agent (3). Therefore, there is a requirement to understand the pathogenesis of the organism and to develop vaccines, therapeutics, and diagnostics to combat the perceived threat.

Dendritic cells (DCs) are specialized antigen-presenting cells that have a central role in initiating T-cell responses. Immature DCs engulf pathogens, initiating a process of maturation which includes their migration to lymphoid organs and culminates in the enhanced expression of major histocompatibility complex class II-peptide complexes and various costimulatory molecules (8, 20, 26). They convey information regarding the nature of the microbial stimulus to T cells and direct the development of polarized T-cell responses along either the type 1 or type 2 pathway (29). In this study, we have shown that cultured dendritic cells that are pulsed with heat-killed *B. pseudomallei* can be used to immunize animals and evoke both cell-mediated and humoral immune responses in the recipients. Additionally, CpG oligodeoxynucleotides (ODN) were used to enhance DC maturation in vitro or as an adjuvant when administered to animals at the time that they were immunized with antigen-pulsed DCs, as it has been demonstrated previously that CpG can enhance DC maturation and activation (38). In vitro and in vivo exposure of DCs to CpG causes the upregulation of major histocompatibility complex class II and expression of the costimulatory molecules CD40, CD80, and CD86 (14). Cohorts of animals that were immunized with dendritic cells were taken forward for virulent challenge with different strains of *B. pseudomallei*, and protection against parenteral challenge was demonstrated.

* Corresponding author. Mailing address: Dstl Porton Down, Salisbury SP4 0JQ, United Kingdom. Phone: 44 (0)1980 614785. Fax: 44 (0)1980 613741. E-mail: SJElvin@dstl.gov.uk.
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 were carried out in accordance with the requirements of the Animals (Scientific Procedures) Act 1986.

Growth of B. pseudomallei and heat inactivation of bacteria. B. pseudomallei K96243 (11) was grown in Luria broth for 18 h at 37°C in a shaking incubator. A viable count was obtained by counting aliquots of the broth culture at 37°C overnight on L agar plates.

For the heat killing of bacteria, broth cultures were harvested by centrifugation and washed three times in phosphate-buffered saline (PBS) before being resuspended in 1/10 of the original volume of PBS. The bacterial cell suspension was then incubated in a water bath at 70°C for 3 h, with occasional shaking. After the inactivation, the suspension was checked for viability by inoculating four 10-ml volumes of L broth with 0.5-ml aliquots of the heat-killed suspension and incubating at 37°C for 7 days. The remainder of the PBS suspension was held at 4°C during the sterility check procedure. L agar plates were then inoculated with the total volume of the broth cultures and incubated for a further 7 days to check for bacterial growth from the broth cultures. If no growth occurred on the agar plates, the bacterial suspension in PBS was considered inactivated.

Isolation and culture of dendritic cells from murine bone marrow. Procedures were modified and developed from published techniques to optimize the yield and viability of DCs from murine bone marrow (13, 22, 32, 36). Briefly, bone marrow was extracted from murine rear tibiae and fibulae and cultured at a concentration of 2 × 10⁶ cells ml⁻¹ in medium comprising of RPMI-1640 (Sigma, United Kingdom) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, United Kingdom) 65 µg ml⁻¹ penicillin-streptomycin-glutamine (Sigma, United Kingdom) and 50 µM 2-mercaptoethanol. The culture medium was supplemented with 20 ng ml⁻¹ granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems) and 10 ng ml⁻¹ tumor necrosis factor alpha (TNF-α) (R&D Systems). Cells were cultured for 96 h at 37°C in the presence of 5% CO₂ in a fully humidified atmosphere, after which time they were removed from the culture plates by gentle scraping. After washing, the cell suspension was layered onto 13.7% metrizamide (wt/vol, Sigma, United Kingdom) and the DCs were purified using centrifugation.

Immunization. DCs were cultured in the presence of GM-CSF and TNF-α as described above. Once isolated and purified from culture, DCs were resuspended to 2 × 10⁷ cells ml⁻¹ and pulsed with heat-killed B. pseudomallei K96243 at 10⁴ CFU ml⁻¹ for 18 h at 37°C in a humidified 5% CO₂ environment. CpG 1826 (Coley Pharmaceuticals, Wellesley, Massachusetts) was added to some DC cultures at a concentration of 6 µg ml⁻¹ when the cells were pulsed with antigen. The cells were then washed three times to remove any extracellular antigen and resuspended in sterile PBS to a concentration of 1 × 10⁵ cells per 100 µl for immunization by the intradermal route on day 0 and day 21 of the immunization schedule. When used as an adjuvant concurrently with DC immunization, CpG 1826 was delivered at a dose of 75 µg per mouse in sterile PBS by the intravenous route.

Primary response of naïve splenocytes. Antigen-pulsed DCs were added to syngeneic splenic lymphocytes to measure primary responses (19). DCs were cultured as described above and, after washing, adjusted to a concentration of 2 × 10⁶ cells ml⁻¹ in culture medium containing 20 ng ml⁻¹ GM-CSF and 10 ng ml⁻¹ TNF-α. The cells were then incubated with heat-killed B. pseudomallei (10⁴ CFU ml⁻¹) at 37°C in a fully humidified environment in 5% CO₂ for 18 h.

Naïve mice were humanely killed by cervical dislocation, and their spleens were removed and passed through a 70-µm nylon sieve. Red blood cells were removed from the spleen cell suspension by using lysing buffer (Sigma), and the remaining splenocytes were washed, counted, and resuspended to a concentration of 10⁶ cells ml⁻¹.

Aliquots (100 µl) of the splenocyte suspension were then added to 96-well tissue culture plates. Replicates of five wells were used for each of the test groups and controls, with 1 µg ml⁻¹ concanavalin A (Sigma, United Kingdom) being used as a positive control and culture medium being used as a negative control. B. pseudomallei-pulsed DCs were washed three times to remove any extracellular antigen, resuspended to the required concentration in culture medium, and added to the splenocyte-containing wells of the assay plates in 100-µl aliquots. The plates were then incubated at 37°C in 5% CO₂ for 96 h.

Following the incubation period, 37 MBq of [³H]thymidine was added to each test well on the proliferation plates and the plates were reincubated for a further 6 h. Cells were then harvested onto 96-well filter plates (PerkinElmer Life Sciences) by using an automated cell harvester, and the plates were allowed to dry at room temperature overnight. Once dry, 20 µl of scintillation fluid (PerkinElmer Life Sciences) was added to each well, and the plate was sealed before being read on a scintillation counter (PerkinElmer Life Sciences).

Recall response of primed splenocytes. Splenocytes of mice that were immunized with heat-killed, B. pseudomallei K96243-pulsed DCs were used to assess T-cell recall responses to soluble B. pseudomallei strain K96243, NCTC 4845, or 576. Splenocyte cultures (5 × 10⁶ cells ml⁻¹ in 100 µl aliquots) from immunized mice were incubated with heat-killed B. pseudomallei, which was added to the splenocyte cultures in 100-µl aliquots at a concentration of 1 × 10⁶ CFU ml⁻¹. The cultures were incubated for 72 h in 5% CO₂ at 37°C before the addition of [³H]thymidine as described above.

Enzyme-linked immunosorbent assay for serum antibody. Serum antibody titers to B. pseudomallei were assayed by enzyme-linked immunosorbent assay, as previously described (17), by using heat-killed B. pseudomallei K96243 as the capture antigen. Concentrations of antigen-specific immunoglobulin G were determined by using Ascent software (Thermo Labsystems), and the data were presented as geometric mean titers with standard deviations (SDs).

EliSpot assays for cytokine production. Secretion of interleukin-4 (IL-4) and gamma interferon (IFN-γ) by spleen cells from naive and immunized mice was examined by EliSpot assay (BD Biosciences, ELISPOT kits). On day 35 following the primary immunization, animals were culled by cervical dislocation and spleens were removed and forced through disposable 70-µm cell strainers (BD Biosciences) to obtain single-cell suspensions. Following centrifugation to pellet the cells, red blood cells were removed by using lysing buffer (Sigma). The remaining cells were washed, counted, and seeded onto EliSpot plates (4 × 10⁶ cells/ml double diluted to 5 × 10⁵ cells/ml in medium containing heat-killed B. pseudomallei K96243 at a final concentration of 1 × 10⁶ CFU ml⁻¹. Four replicates were plated for each of five samples per treatment group. Concanavalin A (Sigma) at a final concentration of 1 µg ml⁻¹ was used as a positive control. EliSpot plates were incubated overnight at 37°C in the presence of 5% CO₂ in a humidified incubator. Assay development was performed according to the kit manufacturer’s instructions. The data are presented as mean values with SDs.

Challenge with B. pseudomallei. The growth of and challenge with B. pseudomallei were performed under containment level III conditions of the Advisory Committee on Dangerous Pathogens. B. pseudomallei NCIC-4 (17), and K96243 (11) were grown in overnight culture as previously described and diluted to give an estimated challenge dose of 10⁶ CFU per mouse. Actual challenge doses were determined by overnight culture of inoculum samples at 37°C on L agar plates. Groups of 10 BALB/c mice were challenged by the intraperitoneal route on day 35 following primary immunization and observed for 42 days postchallenge, at which point any survivors were culled. The challenge survivors were assessed for bacterial load by culture of spleens and blood. Organs were passed through 70-µm nylon sieves into sterile PBS, and blood, which was obtained by cardiac puncture, was diluted 1:10 in sterile PBS. Samples were inoculated onto L agar plates and incubated overnight at 37°C. Plates were then examined for the presence or absence of B. pseudomallei.

Statistical analysis. Statistical analyses were performed using the Student paired t test for all in vitro experiments. Analysis of the challenge data was performed using PRISIM graph pad survival analysis software, and P values were calculated by using the log rank test for trend.

RESULTS

Proliferation assays. Primary proliferation assays revealed that ex vivo antigen-pulsed DCs were capable of inducing proliferation in naive mouse spleen cells (Fig. 1). Secondary proliferation assays that were performed with spleen cells from mice immunized with antigen-pulsed DCs in combination with CpG 1826 showed a significant increase in proliferation when the CpG was used either as a conditioning agent for the DC culture or as an adjuvant with injected DCs. The effect was greatest when B. pseudomallei K96243 was used as the challenge antigen. However, there was still a significant increase in proliferation relative to the in vivo-primed, unstimulated controls when other strains of B. pseudomallei were used (Fig. 2), indicating that the dendritic cells had extracted common epitopes from the different strains.

EliSpot assays. EliSpot assays for IL-4 and IFN-γ were performed at day 35, following primary immunization. Spleen cells...
from animals that were immunized with DCs pulsed with heat-killed *B. pseudomallei* K96243 in the presence or absence of CpG 1826 or those immunized with DCs and CpG 1826 as an adjuvant were incubated with heat-killed *B. pseudomallei*, and the number of cytokine-producing cells was determined. Spleen cells from animals that were immunized with DCs matured in the presence of CpG 1826 produced significantly (*P* < 0.05) more IFN-γ-positive cells than did those of DCs matured with antigen alone or those matured with antigen and control (non-CpG) ODN (Fig. 3).

**FIG. 1.** Primary proliferative responses to antigen-pulsed DCs. Shown is the proliferation of splenocytes from naïve mice that were stimulated in vitro with DCs pulsed with *B. pseudomallei* strain K96243, NCTC 4845, or 576. The proliferation of cells stimulated by antigen-pulsed DCs was greater than the proliferation of unstimulated splenocytes or DC alone, irrespective of which strain of heat-killed *B. pseudomallei* was used in the assays. Each bar represents the mean of the counts ± SD from cultures of cells from five individuals. Con A, concanavalin A.

**FIG. 2.** Secondary responses to *B. pseudomallei* in immunized animals. Shown is the proliferation of spleen cells from mice that were immunized with *B. pseudomallei* K96243-pulsed DCs, matured in the presence or absence of CpG 1826 or administered with CpG 1826 as an adjuvant, in response to various strains of *B. pseudomallei*. The data show significantly enhanced proliferation in vitro of in vivo-primed cells to different strains of *B. pseudomallei*, compared with unstimulated in vivo-primed cells in vitro. Each bar represents the mean of counts ± SD of cells from five individuals. Statistically significant differences between immunized and control samples are indicated by asterisks: *, *P* value of <0.001; **, *P* value of <0.0001. Con A, concanavalin A.
Enzyme-linked immunosorbent assay for serum antibody. An analysis of sera from immunized mice for *B. pseudomallei*-specific immunoglobulin revealed low titers of antibody (Fig. 4) to *B. pseudomallei* K96243. Sera from the animals given DCs matured in the presence of CpG 1826 and those given CpG 1826 as an adjuvant with an immunizing dose of DCs had significantly higher titers than those of animals given DCs alone (*P* < 0.01). The presence of antibody that was reactive with *B. pseudomallei* strains 576 and NCTC 4845 was not assayed, in view of the low titers that developed to the immunizing strain.

Protection against challenge and bacterial clearance. Animals that were immunized with DCs pulsed with *B. pseudomallei* K96243 either with or without CpG 1826 treatment and animals that were given antigen-pulsed DCs with CpG 1826 coinjected as an adjuvant were challenged at day 35 following primary immunization. The exact challenge doses were determined as $3.8 \times 10^4$ CFU for strain K96243, $5.1 \times 10^4$ CFU for strain 576, and $4.3 \times 10^4$ CFU for strain NCTC 4845.

The animals that were immunized with DCs matured in the presence of CpG 1826 showed the highest levels of protection against all three challenge strains of the organism. The use of CpG 1826 as an adjuvant with the DC immunization also resulted in high levels of protection (60 to 70%). The groups of control non-CpG ODN-treated DCs and DCs alone showed very poor levels of protection, with no better than three of eight mice surviving in the K96243-challenged group (Table 1). These results demonstrated that DC immunization was capable of inducing protective responses in immunized animals, but the addition of CpG 1826 greatly increased the levels of protection achievable.

At the end of the postchallenge observation period (42 days), splenocytes and blood derived from challenge survivors were cultured at 37°C for 48 h on L agar (100-ml aliquots in duplicate for each sample). No bacterial growth was detected in any of the samples, indicating that bacterial clearance had been achieved in challenge survivors.
DISCUSSION

The disease melioidosis exhibits a wide range of clinical manifestations in both humans and animals. The mechanisms by which the different forms of the disease develop are poorly understood, although differences in the virulence of different strains and a variety of host factors are important in pathogenesis and disease progression (21, 27, 31). The present study revealed that antigen-pulsed dendritic cells were able to extract protective epitopes from heat-killed bacteria and present them to cells of the adaptive immune system in such a way as to induce protective responses against not only the immunizing strain but also the heterologous strains of *B. pseudomallei*.

The use of antigen-pulsed DCs can result in the stimulation of primary proliferative responses in vitro in syngeneic T cells, while adding free antigen into the responder cells allows only the detection of secondary responses (19, 23). The ability of DCs pulsed with heat-killed *B. pseudomallei* to induce proliferation of naïve mouse spleen cells was demonstrated here. A proliferative response was also obtained with the antigens that were added into the cultures of cells from animals which had been preimmunized with antigen-pulsed DCs. The magnitude of this secondary proliferative response was higher than that seen in the primary stimulation. These data show that DC immunization has the potential to induce antigen-specific memory immune responses in recipient animals. Additionally, significantly increased numbers of gamma interferon-producing cells in animals immunized with CpG 1826-treated, heat-killed, *B. pseudomallei*-pulsed DCs correlate with data from other studies involving the use of CpG ODN in which enhanced IFN-γ production has been seen (4, 9, 18, 30). Elevated levels of IFN-γ correlate with protection against challenge in murine models of melioidosis (31), and the use of CpG 1826-matured, *B. pseudomallei*-pulsed DCs to enhance IFN-γ production in the present study also resulted in significantly enhanced protection.

The low titers of antigen-specific immunoglobulin G in the sera of vaccinees may have been due to the small amounts of antigen that were delivered by DC immunization, as it is known that relatively large amounts of antigen are required in order for a robust antibody response to be mounted (39). The use of CpG 1826 either in DC maturation or as an adjuvant at the point of DC immunization increased antibody titers significantly relative to the non-CpG-treated group, as has been seen in other studies (6). This increased antibody production could have contributed to the robust protection seen in the CpG 1826-treated groups, as antibody may have an important role in defense against melioidosis, with high circulating titers of antigen-specific monoclonal antibody being shown to provide protection against challenge (17). The importance of antibody is likely to be limited to the early stages of an infection, however, before the bacteria are able to gain access to the intracellular niche in which they are known to thrive. Once the bacteria have established an intracellular infection, cell-mediated immune mechanisms are required for efficient eradication of the pathogen.

The DC immunization strategy employed in this study was able to evoke cell-mediated immune mechanisms, as evidenced by increased IFN-γ production and proliferation of spleen cells in response to antigen stimulation in vitro, and these effectors were likely to be contributing to the high levels of challenge survival described here.

When animals were immunized with DCs matured in the presence of CpG 1826 and then challenged with *B. pseudomallei* K96243, 9 of 10 were survivors, while when CpG 1826 was used as an adjuvant, 7 of 10 were survivors. These survival rates are significantly better than those seen when control non-CpG ODN was used to mature the DCs (3 of 8 were survivors) and when DC vaccination alone was used (2 of 10 were survivors) (*P* < 0.01 and *P* < 0.001, respectively). Challenging similarly immunized animals with *B. pseudomallei* strain NCTC 4845 revealed a similar pattern of resistance. With CpG 1826-matured DC immunization, 7 of 10 animals were survivors, which was significantly better (*P* < 0.02) than the survival rate from DC immunization alone. Challenge with strain 576 again gave a higher rate of survival in animals that were immunized with CpG 1826-treated DCs, with significantly improved protection compared with that of naïve controls (*P* < 0.0001).

The ability of the DC immunization strategy to evoke protective immune responses to heterologous strains of *B. pseudomallei* is encouraging, as different strains found in human infections have different characteristics, enabling induction of different forms of disease and lengthy latency periods. A vaccination strategy is required that will be able to protect against this wide variety of strains and induce long-lasting immunity. The use of cultured DCs as a candidate human vaccine is simply not viable, as each individual would require a personalized syngeneic vaccine. A vaccine that can stimulate DC in situ to evoke the protective immune responses that are demonstrated by this work is feasible, e.g., by means of a formulation carrying recombinant *B. pseudomallei* antigens and Toll-like receptor ligands. Binding of the Toll-like receptor ligands would activate the DCs, initiating the process of antigen uptake, processing, and presentation required for the generation of a protective immune response. This study has shown that DC immunization can protect against challenge with a virulent pathogen and points to the development of strategies utilizing the immune-stimulating potential of dendritic cells to evoke long-lasting protective immune responses.

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