Specific Activation of Dendritic Cells Enhances Clearance of *Bacillus anthracis* following Infection

Iain J. T. Thompson¹, Elizabeth R. Mann², Margaret G. Stokes¹, Nicholas R. English², Stella C. Knight², Diane Williamson¹*

¹Biomedical Sciences Department, Defence Science and Technology Laboratory, Porton Down, Salisbury, Wiltshire, SP4 0JQ, United Kingdom, ²Antigen Presentation Research Group, Imperial College London, Northwick Park and St. Mark’s Campus, Watford Road, Harrow, HA1 3UJ, United Kingdom

**Abstract**

Dendritic cells are potent activators of the immune system and have a key role in linking innate and adaptive immune responses. In the current study we have used *ex vivo* pulsed bone marrow dendritic cells (BMDC) in a novel adoptive transfer strategy to protect against challenge with *Bacillus anthracis*, in a murine model. Pre-pulsing murine BMDC with either recombinant Protective Antigen (PA) or CpG significantly upregulated expression of the activation markers CD40, CD80, CD86 and MHC-II. Passive transfusion of mice with pulsed BMDC, concurrently with active immunisation with rPA in alum, significantly enhanced (p<0.001) PA-specific splenocyte responses seven days post-immunisation. Parallel studies using *ex vivo* DCS expanded from human peripheral blood and activated under the same conditions as the murine DC, demonstrated that human DCs had a PA dose-related significant increase in the markers CD40, CD80 and CCR7 and that the increases in CD40 and CD80 were maintained when the other activating components, CpG and HK *B. anthracis* were added to the rPA in culture. Mice vaccinated on a single occasion intra-muscularly with rPA and alum and concurrently transfused intra-dermally with pulsed BMDC, demonstrated 100% survival following lethal *B. anthracis* challenge and had significantly enhanced (p<0.05) bacterial clearance within 2 days, compared with mice vaccinated with rPA and alum alone.

**Introduction**

Dendritic cells (DCs) are potent activators of the adaptive immune system [1] and are critical for host defence against pathogens. Naïve DCs perform a surveillance function, constantly sampling their environment, taking up antigen by phagocytosis and with increased efficiency by receptor-mediated endocytosis [2]. Upon maturation, DCs alter their phenotype and home to lymph nodes where they initiate and polarise an adaptive immune response by presentation of peptides on MHC-II molecules to CD4⁺ T cells or by cross-priming on MHC-I molecules to CD8⁺ T cells [3]. Their ability to induce antigen-specific T cell and antibody responses and their ability to be easily cultured *in vitro* has enabled DCs to be trialled as cellular vaccines, both for infectious diseases and in cancer, resulting in either tumour or disease principally by inducing high titre antibody to PA, which neutralises anthrax toxins in the early stages of infection [8], [9], [10]. Despite the ability to induce a high titre antibody response, vaccines comprising alum as an adjuvant are generally poor inducers of cell-mediated immune (CMI) responses and enhanced CMI responses through DC vaccination may improve vaccine protective efficacy [11].

In this study we have explored the synergistic effects of passively administering specifically-activated DC at the same time as pulmonary and gastrointestinal anthrax. The sporulating nature of *B. anthracis* confers survival advantages in the environment and enables infection and persistence *in vivo*. Spores can exploit host cells to persist in niches and evade host immune responses capable of bacterial clearance, before germinating into vegetative cells. *B. anthracis* possesses two virulence plasmids: pXO1, encoding the proteins protective antigen (PA), lethal factor (LF) and edema factor (EF) which can form the binary toxins Lethal Toxin (LT) and Edema Toxin (ET) [6]; and pXO2, encoding the poly-D-glutamic acid capsule [6] required for immune evasion and intracellular survival [7].

Vaccines are an important strategy to protect at-risk individuals. Licensed anthrax vaccines such as Anthrax Vaccine Precipitate (AVP) and Anthrax Vaccine Adsorbed (AVA), together with next generation anthrax vaccines such as rPA and alum, require several priming doses followed by annual boosters. They protect against disease principally by inducing high titre antibody to PA, which neutralises anthrax toxins in the early stages of infection [8], [9], [10]. Despite the ability to induce a high titre antibody response, vaccines comprising alum as an adjuvant are generally poor inducers of cell-mediated immune (CMI) responses and enhanced CMI responses through DC vaccination may improve vaccine protective efficacy [11].
actively immunising mice with rPA and alum, to determine whether this will significantly reduce the time required to develop protective immunity against anthrax. In addition to survival, a key endpoint of this study was to determine whether this approach significantly enhanced bacterial clearance from the spleen in vaccinated and challenged mice and whether human DC would respond to activation in the same manner qualitatively and quantitatively as murine DC.

Results

Activation of DCs

The activation status of DCs was investigated prior to their use in transfection. DCs were stimulated ex vivo, with vaccine antigens (rPA or heat-killed (HK) B. anthracis spores) or adjuvant (CpG) and the upregulation of costimulatory markers was assessed by flow cytometry. Pulsing of murine BMDC with rPA significantly upregulated (p<0.001) the expression of CD40, CD80, CD86 and MHCI, as effectively as pulsing with CpG. In contrast, HK B. anthracis caused the significant upregulation (p<0.05) of CD40 only on DC (Figure 1a).

In parallel with the studies on murine BMDC, ex vivo human DC, expanded from normal human peripheral blood mononuclear cells (PBMC), were pulsed with vaccine antigens and/or adjuvant, to determine the effect on their maturation and activation status. In these human studies, heat-killed Bacillus cereus was used in addition to HK B. anthracis as a positive control, since B. cereus is a related bacillus which causes gastrointestinal food-borne illness. Optimisation experiments to determine rPA (and B. cereus) doses demonstrated that similar to murine BMDC, pulsing of human DC with rPA significantly upregulated CD40 (p<0.01) and CD80 (p<0.001) (Figure 1b). Lymph-node homing marker CCR7 (upregulated on DC by maturation and activation) was also upregulated by rPA (p<0.001; Figure 1b). There was no change in expression of costimulatory markers CD83 or CD86 or in expression of receptors involved in DC recognition of bacteria or bacterial products (including Toll-like receptors 2 and 4; data not shown). Using an optimised rPA dose of 150 ng/ml for pulsing human DC, in combination with CpG, resulted in enhanced levels of CD40 and CD80 compared to rPA alone (Figure 1c), and the addition of HK B. anthracis to this combination enhanced expression even further (Figure 1d).

Cellular immune response to DC vaccination in combination with active immunisation

When splenocytes prepared from immunised mice were re-stimulated ex vivo with rPA, there was a significant increase in IFNγ SFC from mice actively immunised with rPA and alum compared to naive mice at days 7 (p<0.05; Figure 4a) and 14 (p<0.001; Figure 4b). The recall response to rPA at the earlier time point of 7 days post-immunisation, was even further enhanced in mice actively immunised with PA and alum together with transfection of DC vaccine (p<0.001; Figure 4a). By day 14, both the actively immunised (rPA and alum) and the combined immunisation groups (rPA and alum and DC vaccine) had a significantly elevated (p<0.01 and p<0.001, respectively) recall response compared with mice given unstimulated DC, with no significant difference between the two immunised groups.

Anti-PA response to DC vaccination in combination with active immunisation

Analysis of day 14 sera from mice that had been transduced with DCs pre-pulsed with rPA, CpG and HK B. anthracis showed that they had developed a specific IgG response to PA (Figure 5). Mice which had been immunised with rPA and alum, or with rPA and alum and also transduced with the DC vaccine, had significantly elevated (p<0.05) anti-rPA IgG titres, when compared with mice receiving stimulated DCs alone (DC vaccine). In terms of antibody induction however, the combination of active immunisation with passive transfection of puls ed DC, had no additive effect. In pilot studies we observed that the predominant IgG sub-class induced in mice either actively immunised with rPA & alum or adoptively transfused with rPA-pulsed DC, or both actively and passively immunised, was IgG1 (data not shown).

Post-challenge bacteriology and survival

Mice were challenged with 3×10^4 CFU B. anthracis STI i.p. (approximately 10 median lethal doses) 14 days post-immunisation. Mice treated with PBS only had a mean time to death of 3 days following challenge with 10MLD B. anthracis STI (data not
shown). Two days post-challenge, a cohort of mice from each treatment group was culled with spleens taken to ascertain bacterial loads. Mice receiving unstimulated DCs together with alum showed limited survival (Figure 6) with all animals dead within 5 days and a splenic bacterial load significantly greater than all other groups (Figure 7). Mice receiving only the DC vaccine had 60% survival but a significantly (p < 0.01) reduced bacterial load compared with the group receiving unstimulated DC with alum (negative controls), whilst mice immunised with rPA and alum showed 80% survival with a significantly (p < 0.05) reduced bacterial load compared with negative controls. However, mice immunised with both rPA and alum and transfused with the DC vaccine had 100% survival and a highly significant reduction in bacterial load within 2 days of challenge, compared to the negative controls (p < 0.001) and to the rPA and alum group (p < 0.05).

There was a statistically significant difference in the survival curves for the negative control (alum+BMDC&CpG) group compared with the group actively immunised with rPA & alum and also receiving rPA&HK B. anthracis-pulsed DC (p < 0.002). Comparison of survival curves between the negative control (alum+BMDC&CpG) group and the group receiving rPA&HK B. anthracis-pulsed DC only, was also statistically significant (p < 0.01).

Discussion

 Whilst there are currently licensed prophylactic vaccines for anthrax (e.g. Anthrax Vaccine Adsorbed, AVA and Anthrax Vaccine Precipitated, AVP) they have several limitations including a long primary schedule that requires multiple vaccinations (0, 3, 6 and 32 weeks currently for the UK AVP) to induce immunity, regular booster doses to maintain protective immunity and relatively poor development of CMI responses capable of clearing spores or vegetative bacteria. Furthermore, prophylactic antibiotic regimens require those suspected to have been exposed to take prolonged antibiotic courses (60 days) with the risk of residual
spores germinating after completion of the course and poor compliance to the regimen. Therefore a vaccine that can effectively induce CMI responses capable of reducing vegetative bacterial or spore burden is desirable, potentially by enhancing existing vaccines. In this study we examined the potential for DC transfusion to enhance conventional anthrax vaccination in terms of reducing the time required to develop protective immunity and enhancing the specific CMI response induced, with the rationale that this could significantly improve bacterial clearance post-exposure.

The in vitro co-stimulation of DCs with rPA, heat-killed B. anthracis and CpG oligonucleotides (DC vaccine) and administered i.d. to A/J mice. Spleens were taken at 7 days (a) and 14 days (b) post- vaccination and restimulated ex vivo with rPA. A one-way Anova with Tukey multiple comparison post-hoc test was performed on the results (* p<0.05, ** p<0.01, *** p<0.001). Error bars represent the standard error of the mean calculated from the means of three replicates from five animals. c) Proliferation of CD4+ naive human T cells following 5-day co-culture with syngeneic human DC pulsed with medium only, B. cereus or rPA (n = 5). After 2-way ANOVA analysis with Bonferroni corrections, there were no significant differences between B. cereus and medium only (basal)-pulsed DC regarding stimulatory capacity but rPA-pulsed DC were significantly more stimulatory than basal conditions at 4% and 6% (p<0.05 in both cases) and significantly more stimulatory than B. cereus pulsed DC at 6% (p<0.05). d) CD4+ naive human T-cell proliferation following 5-day co-culture with syngeneic DC pulsed with CpG only, rPA with CpG or combination of rPA, CpG and HK B. anthracis (n = 3). After 2-way ANOVA with Bonferroni corrections, there were no significant differences between CpG and rPA/CpG-pulsed human DC regarding stimulatory capacity but rPA/CpG/HK B. anthracis-pulsed human DC were significantly more stimulatory than both other conditions at 6% (p<0.001 in both cases).

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Figure 2. Stimulation of T cell responses. a and b) DCs were stimulated with rPA only (DC vaccine rPA only) or with PA, heat killed B. anthracis and CpG oligonucleotides (DC vaccine) and administered i.d. to A/J mice. Spleens were taken at 7 days (a) and 14 days (b) post- vaccination and restimulated ex vivo with rPA. A one-way Anova with Tukey multiple comparison post-hoc test was performed on the results (* p<0.05, ** p<0.01, *** p<0.001). Error bars represent the standard error of the mean calculated from the means of three replicates from five animals. c) Proliferation of CD4+ naive human T cells following 5-day co-culture with syngeneic human DC pulsed with medium only, B. cereus or rPA (n = 5). After 2-way ANOVA analysis with Bonferroni corrections, there were no significant differences between B. cereus and medium only (basal)-pulsed DC regarding stimulatory capacity but rPA-pulsed DC were significantly more stimulatory than basal conditions at 4% and 6% (p<0.05 in both cases) and significantly more stimulatory than B. cereus pulsed DC at 6% (p<0.05). d) CD4+ naive human T-cell proliferation following 5-day co-culture with syngeneic DC pulsed with CpG only, rPA with CpG or combination of rPA, CpG and HK B. anthracis (n = 3). After 2-way ANOVA with Bonferroni corrections, there were no significant differences between CpG and rPA/CpG-pulsed human DC regarding stimulatory capacity but rPA/CpG/HK B. anthracis-pulsed human DC were significantly more stimulatory than both other conditions at 6% (p<0.001 in both cases).

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Dendritic Cell Vaccination in Anthrax

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Dendritic Cell Vaccination in Anthrax

(a) Graphs showing the proportion of TGFβ+ T-cells and IL-6+ T-cells across different groups.

(b) Graphs showing the proportion of IL-17+ T-cells and IFNγ+ T-cells across different groups.

Statistical significance:
- p = 0.23
- p = 0.36
- p = 0.18
- p = 0.88
- p = 0.52
- p = 0.78
- p = 0.99
- p = 0.91
Furthermore human DC pulsed with rPA, caused a dose-related proliferative response in naïve human CD4\(^+\) T-cells, which was further enhanced when DC were pulsed with CpG and HK \textit{B. anthracis}, in addition to rPA and this was accompanied by a significantly enhanced secretion of TGF\(\beta\). The study of human DC-T cell interactions cannot exactly match the murine studies, since it is entirely based on \textit{ex vivo} T-cell stimulation, however these concurrent human studies supported the murine data demonstrating that rPA, HK \textit{B. anthracis} and CpG is an optimum activating combination for the DC. This allowed the optimum DC-activating combination of rPA+CpG+HKSTI to be deployed together with active immunisation in the mouse, to achieve accelerated T-cell mediated immune responses against \textit{B. anthracis}.

The efficacy of the combined immunisation regimen was stringently tested by challenging mice with multiple lethal doses of \textit{B. anthracis} only 14 days after immunisation. Whilst naïve mice rapidly succumbed to infection, 80\% of those immunised with rPA and alum survived. The DC vaccine alone protected 60\% of mice and this protection was attributed predominantly to the induction of a specific CMI response. By comparison, the DC vaccine, combined with rPA in alum immunisation, fully protected all the mice and these mice had a highly significant reduction in splenic bacteria as early as 2 days post-challenge. Mice receiving either the DC vaccine only, or rPA in alum only, had bacterial loads which although significantly reduced compared with naïve mice, were still elevated compared with the combined vaccine group.

This study provides proof-of-principle that a single transfusion of specifically activated DCs can augment the response to conventional active immunisation, with benefits in terms of accelerating time to immunity, specific CMI, survival and most significantly, clearance of \textit{B. anthracis} in the murine model. Whilst these are significant findings, the passive transfusion of pre-stimulated DCs is not a pragmatic approach to mass vaccination in the clinic. We are pursuing alternative approaches to enhancing CMI through the \textit{in situ} stimulation of DCs in the host, for example by targeting DCs with relevant antigens (such as PA and spore coat proteins) fused to an antibody directed at a DC surface receptor, such as DEC205, an approach that has been demonstrated successful in protecting against \textit{Y. pestis} in mice [5]. Here, we have used activated DCs prophylactically to enhance CMI, but appropriately activated DCs could also be used in a post-exposure context to augment the CMI response in the early phase of infection. In conclusion, the \textit{ex vivo} pulsing of DCs with pathogen-derived material and defined stimulatory antigens with CpG, has induced the maturation of DCs \textit{ex vivo} and is able to direct the development of specific and efficacious T cell responses in an \textit{in vivo} murine model of anthrax.

**Materials and Methods**

**Ethics Statement**

All animal procedures were performed in accordance with UK legislation as stated in the UK Animal (Scientific Procedures) Act...
1986. The Institutional Animal Care and Use Committee approved the Project licence (PPL 30/2488) which was granted on 02/11/2008.

All procedures with human blood samples were performed under Ethics Committee approval (reference 05/Q405/71 entitled ‘Tissue specific immune regulation by dendritic cells in the intestine and other sites’) from the Outer West London Research Ethics Committee (NHS) on 9 March 2010. An amendment to the protocol (Sub Study Version 1.0/dated 29 November 2010) was approved by the NHS on 14 February 2011. This protocol was reviewed by the U.S. Army Medical Research and Materiel Command (USAMRMC), Office of Research Protections (ORP), Human Research Protection Office (HRPO) and found to comply with applicable DOD, U.S. Army, and USAMRMC human subjects protection requirements. All human samples were obtained with written consent from the donor.

Mice

A/J mice were purchased from Charles River U.K. and held in specific pathogen-free facilities with free access to food and water and allowed to acclimatise for seven days prior to use. There is a positive correlation between the immunising dose of rPA and the protective antibody response when using A/J mice, making them a suitable model for anthrax infection studies [13]. Furthermore, A/J mice are deficient in the complement protein C5, making them susceptible to toxigenic B. anthracis [14]. Animals undergoing challenge were held in rigid isolators with both inflowing and out-flowing HEPA filtered air.

Murine DC culture method

DCs were prepared using a method modified from previous reports [15], [16]. Briefly, bone marrow was flushed from murine femurs and tibias and passed through a 40 μm cell sieve to create a single cell suspension. Bone marrow cells were washed in complete media (RPMI-1640 supplemented with 10% foetal calf serum, 1% penicillin-streptomycin-glutamine and 50 μM 2-mercaptoethanol) and red cells lysed before being washed again. Cells were seeded at $1 \times 10^6$ cells mL$^{-1}$ and cultured in a fully humidified atmosphere at 37°C with 5% CO$_2$; complete media was supplemented with

Figure 5. Antibody responses 14 days after either stimulated DCs, or rPA and alum, or both, were administered. Each point represents the mean of three replicates from five animals. A one-way Anova with Tukey multiple comparison post-hoc test was performed on the results (* p<0.05, ** p<0.01, *** p<0.001).

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Figure 6. Post Challenge survival. Groups of 5 A/J mice immunised with a DC vaccine (comprising of DCs stimulated overnight with rPA, heat-killed B. anthracis and CpG), rPA in alum, or rPA in alum plus DC vaccine, or left naïve, were challenged at 14 days post-immunisation with $3 \times 10^4$ CFU B. anthracis STI (i.p.) and monitored for survival over the subsequent 8 days. There was a statistically significant difference in the survival curves for the negative control (alum+BMDC&CpG) group compared with the group actively immunised with rPA&alum and also receiving rPA&HK B.anthracis-pulsed DC (p<0.002) by both the Mantel-Cox and Gehan-Breslow-Wilcoxon tests. Comparison of survival curves between the negative control (alum+BMDC&CpG) group and the group receiving rPA&HK B.anthracis-pulsed DC only, was also statistically significant (p<0.01) when both tests were applied.

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Figure 7. Enumeration of viable B. anthracis in the spleens of cohorts of mice two days post challenge. Each point represents the mean of three replicates from five animals, four from the naive group due to an early death. A one-way Anova with Tukey multiple comparison post-hoc test was performed on the results (* p<0.05, ** p<0.01, *** p<0.001).

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20 ng/ml of GM-CSF. Media was replaced at day 3 and 5 before loosely adherent cells were harvested at day 8. Cells were confirmed to be >90% CD11c+ by flow cytometry and used in stimulation assays.

**Human DC characterisation method**

Human blood was collected from healthy volunteers with no known autoimmune or inflammatory diseases, allergies or malignancies, following informed consent (EC number 05/ Q9405/71). Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation over Ficoll-Paque plus (Amersham Biosciences, Chalfont St. Giles, UK). Human low density cells (LDC) were obtained following Nycoprep centrifugation of 20 h.-cultured PBMC prior to antigenic pulsing or T-cell stimulation. Human DC were then identified as HLA-DR+ lineage cocktail (CD3/CD14/CD16/CD19/CD34) live cells by flow cytometry for analysis of co-stimulatory molecules and cytokine production. LDC used as a DC source are 98–100% HLA-DR+, with morphological characteristics of DC (both at optical and electron microscopy) and are potent stimulators of allogeneic naïve T cells [17], [18].

**Stimulation of dendritic cells**

Human and murine DCs were stimulated with rPA, CpG ODN (Invivogen) and heat killed *B. anthracis* prepared using a method previously reported [19]. Briefly, *B. anthracis* (STI strain) spores were harvested and washed three times by centrifugation before being resuspended in PBS. The spore suspension was inactivated by incubation in a water bath for 2 h. at 90°C with occasional shaking. After inactivation the culture was checked for viability by inoculating 10 mL broths with heat-killed suspension and incubating at 37°C for one week. 96-well plates were subsequently inoculated with the entire broth and incubated for a further 7 days. An absence of growth indicated the spore suspension to be inactivated.

**Flow cytometry to assess cellular activation**

Murine antibodies with the following specificities and conjugations were purchased from Biolegend, CD11c-PeCy7, CD80-PE, CD86-APC, CD40-FTTC, MHC-II PerPCy5.5, together with appropriate isotype matched controls. After staining, cells were fixed with 1% paraformaldehyde in 0.85% saline and stored in the refrigerator prior to acquisition on the flow cytometer, within 48 hours.

Human monoclonal antibodies with the following specificities and conjugations were used: TLR2-FTTC (TLR2.3), TLR4-FTTC (HTA125), CD40-PE (LOB7/6), DC exclusion cocktail-PE-Cy5 (CD3 (S4.1), CD14 (TUK1), CD16 (3G8), CD19 (SJ25-C1), CD34 (5B1)) were purchased from AbD Serotec (Oxford, UK). CD83-PE (HB15e), CD80-FTTC (L307.4), HLA-DR-FTTC/APC (G46-6), CD86-FTTC (24F), IL-10-APC (JES3-19F1), IL-12-PE (C11.5), IL-6-FTTC (MQ2-13A5), IL-17-PE (SCPL1362), IFNγ-APC (25723.11), FoxP3-PE (259D/C7), CD4-PE (RPA-T4), CD3-PE/PeCy5/APC (UCHT1), CD8-APC (SK1), CD45RO-PE (UCHL1), CD45RA-PECy5 (HI100) were purchased from BD Biosciences (Oxford, UK). CCR7-PE (150503) and TGFβ-PE (IC38B2) were purchased from R&D Systems (Abingdon, UK).

Appropriate isotype-matched control antibodies were purchased from the same manufacturers. After staining, cells were fixed with 1% paraformaldehyde in 0.85% saline and stored in the refrigerator prior to acquisition on the flow cytometer, within 48 hours.

**Cytokine production by human DC**

Intracellular cytokine production by DC was measured following antigenic pulsing of DC via comparison of monensin-treated DC (4 h.) and non-monensin-treated DC (incubated with medium only for 4 h.). Cells were then labelled for surface marker expression using monoclonal antibodies to identify DC as described above, fixed and permeabilised before labelling for cytokines prior to acquisition.

**Enrichment of CD4+ naïve T cells for human DC stimulation assays**

Blood from the same donor as the DC source was used to isolate human CD4+ naïve T cells. PBMC were depleted of CD14+, CD19+, HLA-DR+, CD45RO+ and CD8+ cells using immuno-magnetic beads following the manufacturer’s instructions. Flow cytometric analysis confirmed that>96% of these cells were CD45CD4+ T cells (data not shown).

**Human DC stimulation of T-cell assays**

Human DC4+ naïve T cells were CFSE-labelled and incubated for 5 days with enriched syngeneic DC at 2, 4 and 6% (of total T-cell number), providing dose-dependent proliferation of T cells in all cases. Cells were recovered following 5d. culture and CFSEproliferating cells were identified, analysed and quantified by flow cytometry.

**Immunisations**

DCs were prepared as above. For mouse immunisations, 2×106 DCs were stimulated with 10 μg/mL rPA, 6 μg/mL CpG and 10^4 CFU/mL heat-killed *B. anthracis* STI for 18 h. at 37°C. Cells were harvested and washed to remove excess antigen and resuspended in PBS. Mice, 5 per group, were immunised intradermally (i.d.) with 1×10^6 cells resuspended in 100 μl PBS on day 0 (the DC vaccine). Alternatively, mice were immunised with rPA and alum and received 10 μg rPA formulated in 100 μl of 0.26% v/v alum, intramuscularly (i.m.), on d.0. Mice receiving both DCs and rPA and alum, received these formulations at the same time on d.0.

**ELISPOT**

The number of rPA - specific IFNγ+ splenocytes from naïve and immunised mice was assessed using an ELISPOT assay. Briefly, 7 or 14 d. post-immunisation mice were culled, spleens removed and macerated through a cell sieve. Cells were pelleted and red cells removed using lysis buffer before being washed, counted and stored in the refrigerator prior to addition on the flow cytometer, within 48 hours.

**ELISA**

The anti-PA antibody titre was determined as previously reported [20]. Briefly, microtitre plates were coated overnight at 4°C with 5 mg ml^-1 rPA in PBS. Serum samples were double-diluted in PBS containing 1% w/v skimmed milk powder and incubated for 2 h. at 37°C. Binding of serum antibody was detected using horse-radish peroxidase conjugated goat anti-mouse IgG, diluted 1:2,000 in 500 μl of PBS, and the substrate 2,2′-Azino-bis (3-ethylbenzthiazoline-sulfonic acid) (1.09 mM ABTS). Titres were presented as the end-point dilution, which gave an absorbance of
challenge dose was calculated by culturing the inoculum on nutrient agar plates for 48 h. Groups of ten mice were challenged intra-peritoneally (i.p.) and monitored, with those showing signs of severe illness humanely culled. Survivors were culled after 8d. Actual challenge dose was calculated by culturing the inoculum on nutrient agar plates for 48 h. Groups of ten mice were challenged intra-peritoneally (i.p.) and monitored, with those showing signs of severe illness humanely culled. Survivors were culled after 8d.

Bacteriology

Five mice per group were culled for bacteriology 2d. after challenge. Spleens were macerated through a wire mesh into sterile PBS. Splenocyte suspensions were serially diluted and 100 μL of each suspension added to nutrient agar in triplicate. Plates were incubated at 37°C for 48 h. before colony forming units (CFU) were enumerated. Plates were incubated at 37°C for 48 h. before colony forming units (CFU) were enumerated.

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Statistical analysis

Statistical analysis using paired and unpaired t-test, and one- and two-way Anova was performed using GraphPad Prism as stated in figure legends, with calculation of mean and standard error of the mean (SEM). Samples were analysed using a one-way-Anova with a Tukey post-hoc test (* p<0.05, ** p<0.01, *** p<0.001). For comparison of survival curves, the log-rank Mantel-Cox and the Gehan-Breslow-Wilcoxon tests were applied.

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Author Contributions

Conceived and designed the experiments: IJTT ERM NRE SCK DW. Performed the experiments: IJTT ERM MGS DW. Analyzed the data: IJTT ERM SCK DW. Contributed reagents/materials/analysis tools: NRE ERM IJTT. Wrote the paper: IJTT REM SCK DW.

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