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Analysis of Anthrax and Plague Biowarfare Vaccine Interactions with Human Monocyte-Derived Dendritic Cells[1]

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The anti-biowarfare anthrax and plague vaccines require repeated dosing to achieve adequate protection. To test the hypothesis that this limited immunogenicity results from the nature of vaccine interactions with the host innate immune system, we investigated molecular and cellular interactions between vaccines, dendritic cells (DCs), and T cells and explored the potential for adjuvants (pertussis) to boost induction of host immunity. Human monocyte-derived DCs were matured in the presence of vaccines and analyzed for their ability to induce Th1/Th2 development from naive T cells, expression of cell surface maturation/costimulation molecules, and cytokine production. The vaccines showed different behavior patterns. Although the plague vaccine is equivalent to control maturation factors in maturation and stimulation of DCs and induces strong MLR and Th outgrowth, the anthrax vaccine is a poor inducer of DC maturation, as indicated by low levels of HLA-DR, CD86, and CD83 induction and minimal proinflammatory cytokine production. Interestingly, however, anthrax vaccine-treated DCs stimulate Th1 and Th2 outgrowth and a limited MLR response. There was no sustained negative modulatory effects of the anthrax vaccine on DCs, and its limited stimulatory effects could be overridden by coculture with pertussis. These results were supported by analysis of anthrax vaccine recall responses in subjects vaccinated using pertussis as an adjuvant, who demonstrate anthrax-specific effector T cell responses. These data show that the anthrax vaccine is a suboptimal DC stimulus that may in part explain the observation that it requires repeated administration in vivo and offer a rational basis for the use of complementary DC-maturing adjuvants in combined immunotherapy. ***The Journal of Immunology,*** **2005, 175: 7235–7243.**

The advent of vaccine schedules for common childhood epidemic infectious diseases over recent decades has led to their eradication in most industrialized societies. More recently, therefore, the attention of vaccinologists has turned to more complex challenges, including that of biological warfare and possible bioterrorism. Among those agents considered to be category A threats are anthrax (*Bacillus anthracis*) and plague (*Yersinia pestis*) (1–4). For both agents, vaccines have been in existence for years, but both have limitations (3). The anthrax vaccine, for example, requires doses at 2 and 4 wk, with boosters at 6, 12, and 18 mo and then annually (5). Protection against plague can be achieved after two doses at an interval of 1–4 wk, with boosting every 6 mo (6, 7). Even under these circumstances, the protection afforded by vaccines from inhalational anthrax and pneumonic plague may not be ideal (3). The complexity of achieving adequate protection is further increased by the fact that targets for bioterrorism are typically unspecified, resulting in the potential need for rapid induction of protection, possibly against multiple agents.

The goal of vaccine administration is the safe induction of adaptive immunity to the given pathogen. Ideal vaccines should afford similar levels and quality of immunity to those achieved through the wild-type infection. Typically, this includes a balance of Th1, Th2, and regulatory T cells, as well as the generation of high-affinity Abs of the appropriate class and long-lived immunological memory (8). The central immune cell responsible for directing the magnitude and quality of an immune response is the dendritic cell (DC).[3] DCs are professional APCs, responsible for de novo priming of immune responses and efficiently stimulating memory responses (8–10). The interaction between DCs and naive T cells determines the strength, quality, and breadth of the T and B cell response through a variety of molecular mechanisms including levels of peptide-MHC complexes, costimulatory molecules, and secretion of cytokines and chemokines (11).

In peripheral tissues, DCs exist in an immature form that is efficient at taking up and processing Ags (12). After activation, DCs become competent APCs bearing high levels of costimulatory molecules, presenting MHC-bound antigenic peptides to naive, Ag-specific T lymphocytes that they encounter after migration to the local lymph node. In their sentinel role, DCs are constitutively distributed throughout most tissues of the body, particularly localized to those sites that comprise the external barriers, such as skin and mucosal surfaces (8, 13, 14). In this study, the DC is likely to be one of the first immune cells to encounter the wild-type organism, or its vaccine equivalent, and therefore the outcome of this encounter is critical to the host immune response.

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[3] Abbreviations used in this paper: DC, dendritic cell; WCP, whole cell pertussis vaccine; PA, protective Ag; MF, maturation-inducing factor; biowarfare vaccines, anti-biowarfare vaccinations; iDC, immature DC; LF, lethal factor; EF, edema factor; CBA, cytokine bead array; 7-AAD, 7-amino-actinomycin D.

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We reasoned that the explanation for the limited immune response to vaccines such as anthrax and plague could lie in this initial interaction between the vaccine and immature DC (iDC). In the present study, therefore, we used an in vitro strategy for the examination of vaccine-DC encounters and subsequent DC maturity, secretory capacity, and induction of T cell polarization. Through this approach, we demonstrate that inadequate stimulation of DCs by the anthrax vaccine may be a factor underlying its limited immunogenicity in vivo. In contrast, at high concentrations, the plague vaccine is capable of full DC maturation and induction of effector responses. Adapting our in vitro strategy to examine the potential for beneficial or negative effects of vaccine combinations and adjuvants, we were able to show that combinations that include powerful adjuvant agents such as whole-cell pertussis (WCP) can enhance the limited stimulatory effects of the anthrax vaccine, a concept supported by analysis of recall responses in anthrax/WCP vaccinees.

Materials and Methods

We generated iDCs from five healthy human donors and exposed them to vaccines to examine the major checkpoints in the events leading to T cell priming in the lymph node. Our overall strategy is shown in Fig. 1. This study was approved by the local research ethics committee.

Generation of immature monocyte-derived DCs

PBMCs from healthy laboratory donors were isolated by density gradient centrifugation on Lymphoprep (Nycomed). PBMCs were then layered onto a Percoll (Amersham Biosciences) gradient, consisting of three density layers (1.076, 1.059, and 1.045 g/ml), and centrifuged at 1750 × *g* for 45 min. The light density fraction, containing predominantly monocytes, was seeded into 24-well culture plates at a density of 0.5×10^6 cells/ml in IMDM (Invitrogen Life Technologies) containing 86 μg/L gentamicin (Sigma-Aldrich), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies) supplemented with 1% FCS (PAA Laboratories). Cytofluorimetric analysis showed that the purification procedure yielded >90% pure $CD14^+$ cells. After 1 h at 37°C, nonadherent cells were removed, and adherent cells were cultured in IMDM/FCS supplemented with IL-4 (250 IU/ml) and GM-CSF (500 IU/ml; both cytokines from Strathmann Biotec) to obtain immature monocyte-derived DCs. On day 3, the supplemented media was refreshed, and after 6 days, the iDCs were ready for stimulation. iDCs were washed and analyzed by cytofluorimetric analysis for CD1a, CD14, and CD3 expression. The mean percentage (±SD) of $CD1a^+$, $CD14^+$, and $CD3^+$ cells was 92% (2.6), 7.5% (2.4), and 2.8% (1.6), respectively, from five experiments.

Vaccine preparations

The United Kingdom human anthrax vaccine consists of a protein precipitate from the supernatant fluid of cultures of the Sterne strain of *B. anthracis*. The major immunogen is protective Ag (PA), the nontoxic, cell-binding component of the anthrax toxin complex. The vaccine also contains lethal factors (LFs) and edema factors (EFs). The concentration of PA in the vaccine preparation is 1.3–2.2 μg/ml (whole molecule and fragments), and the concentration of LF is 0.4–0.7 μg/ml (G. Griffiths and M. Hudson, personal communication; provisional data to be updated and validated using GLP functional assays/immunoassays). An EF is present in very low levels below the detection limit of the assay method. This alum-precipitated human anthrax vaccine (product license no. PL1511/0037) was produced by The Centre for Applied Microbiology and Research (Porton Down, Salisbury, Wiltshire, U.K.) for the United Kingdom Department of Health. In our study, the anthrax vaccine was used over a range of quantities, which achieved a PA concentration of 0.13–43 pg/ml (i.e., vaccine diluted between 1/10,000 and 1/30).

The plague vaccine (CSL Limited) consists of a suspension of agar-grown, heat-killed organisms of *Y. pestis* in saline at 3×10^9 organisms/ml and was used in our studies in the range $0.3–100 \times 10^6$/ml (i.e., vaccine diluted between 1/10,000 and 1/30). The strains used in this preparation were obtained from the Haffkine Institute (Mumbai/Bombay, India), and their virulence was confirmed by demonstration of lethal effect in rats.

The WCP vaccine consists of killed whole-cell preparation of *Bordetella pertussis* W28, prepared at The Centre for Applied Microbiology and Research to the original Burroughs-Wellcome procedure for the preparation of a single WCP vaccine and provided at a strength of 4×10^{10} organisms/ml (product license no. 208/10/99). In our studies, the pertussis vaccine was used in the concentration range $0.4–4 \times 10^7$ organisms/ml (i.e., vaccine diluted between 1/10,000 and 1/30).

In preliminary studies to examine DC maturation and effector function by analysis of accessory molecule expression, cytokine production, and cellular toxicity, vaccines were used across the range of concentrations stated above. Single concentrations were then selected as those at which optimal DC activation was observed at 48 h. These selected concentrations were used in time-course studies (6, 16, 24, 48, and 72 h) and in the examination of DC interaction with CD40L, MLR, and polarization of effector T cell responses.

Examining cytokine potential of iDCs and degree of maturation

Immature DCs were stimulated at a density of 8×10^4 cells/200 μl in 96-well plates in IMDM and 1% FCS with either vaccines or various control preparations representing maturation-inducing factors (MFs) IL-1β (10 ng/ml), TNF-α (50 ng/ml) (both cytokines from Strathmann Biotec AG), and LPS (100 ng/ml) (Sigma-Aldrich); the Th1-type stimulant IFN-γ (1000 U/ml; R&D Systems), which drives the development of DCs that promote Th1 responses; and PGE2 (10^{-6} M; Sigma-Aldrich), which promotes Th2 responses. Supernatants were harvested and stored at −80°C for subsequent analysis of cytokine secretion using the cytokine bead array (CBA) inflammatory kit assay (BD Biosciences) and a FACSCalibur flow cytometer (BD Biosciences) according to the manufacturer's instructions. In brief, we used four bead populations with distinct fluorescence intensities, coated with capture Ab specific for IL-12p70, IL-6, IL-10, and TNF-α proteins. Supernatant samples were incubated with human cytokine capture beads and stained with PE detection reagent. After incubation for 3 h at room temperature, samples were washed and acquired using the FACSCalibur. Data were analyzed using CellQuest and CBA Analysis Software 1.1 (both from BD Biosciences). The lower detection limit for IL-12p70 was 1.9 pg/ml, for IL-6 was 2.5 pg/ml, for IL-10 was 3.3 pg/ml, and for TNF-α was 3.7 pg/ml.

iDCs were also cultured under identical conditions to examine the effects of vaccines on maturation. DCs were then harvested, washed extensively to remove all supplements, and stained for expression of cell-surface molecules representing maturation and activation by flow cytometry. Mouse anti-human mAbs against the following molecules were used: CD1a-FITC, CD83-FITC, and CD86-PE (all from Serotec); and HLA-DR-PerCP, CD3-FITC, CD14-FITC, CD19-FITC, and CD56-FITC (all from BD Biosciences). In addition, cellular toxicity was evaluated using 7-amino-actinomycin D (7-AAD; final concentration, 1 μg/ml; Calbiochem). At least 5000 events gated on forward and side scatter were analyzed using the CellQuest program (BD Biosciences), with dead ($7\text{-}AAD^+$) cells excluded. Corresponding isotype Abs were used to establish the quadrants and markers for analysis.

Effects of vaccines on the T cell stimulatory potential of DCs

Vaccine effects on the T cell stimulatory potential of DCs were examined using the MLR. Immature DCs were exposed to vaccines for 48 h at the following concentrations, which had elicited optimal effects on DCs in the preliminary studies: anthrax at 0.43 pg/ml of PA; plague at 10×10^6 organisms/ml; pertussis at 1×10^6 organisms/ml. DCs were harvested,

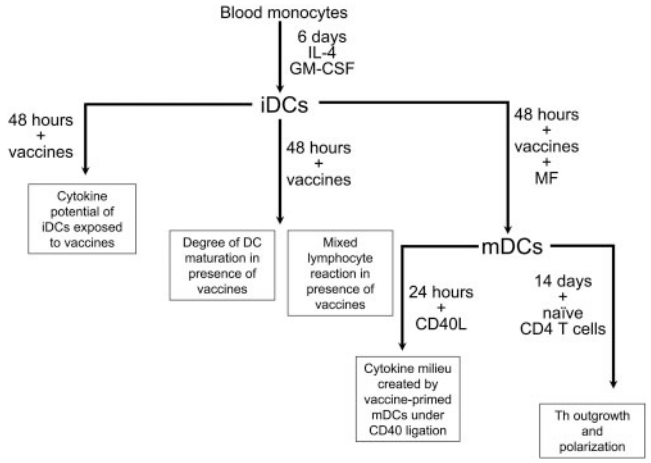

FIGURE 1. Scheme used to examine checkpoints in DC maturation and generation of Th effector cells. mDCs, Mature DCs.

washed extensively, and cultured at a range of cells/well (100–10,000) in 96-well flat-bottom plates in the presence of 50,000/well allo-$CD4^+$ T cells, purified from PBMCs using the Isolation kit II (Miltenyi Biotec). After 5 days, [^{3}H]thymidine (0.5 μCi/well) was added, cells were harvested, and proliferation was measured by liquid scintillation spectroscopy.

Effects of vaccines on cytokine production by mature DCs

To examine vaccine effects on cytokine production by mature DCs, iDCs were first matured for 48 h in the presence of vaccines as described above, with the addition of the conventional MFs (IL-1β at 10 ng/ml, TNF-α at 50 ng/ml, and LPS at 100 ng/ml final concentrations). In these experiments, vaccines were used at the following concentrations, which had elicited optimal effects on DCs in the preliminary studies: anthrax at 0.43 pg/ml of PA; plague at 10×10^6 organisms/ml; pertussis at 1×10^6 organisms/ml. Mature DCs were then harvested, washed extensively, and cultured (4×10^4 cells/well) in 96-well flat-bottom plates in the presence of an equivalent number of human CD40L-expressing mouse plasmacytoid cells (J558 cells; a gift from Dr. P. Lane, Medical Research Council Center for Immune Regulation, University of Birmingham, Birmingham, U.K.) in IMDM containing 10% FCS in a final volume of 200 μl. Supernatants were harvested after 24 h and stored at -80°C for analysis of cytokine secretion measured using the CBA inflammatory kit as described above.

Effects of vaccines on naive Th cell polarization by mature DCs

To examine vaccine effects on naive Th cell polarization by mature DCs, iDCs were first matured for 48 h in the presence of vaccines (at the same concentrations as above) supplemented with conventional MFs (IL-1β (10 ng/ml), TNF-α (50 ng/ml), and LPS (100 ng/ml)).

Naive ($CD4^+CD45RA^+CD45RO^-$) cells were purified from PBMCs by negative selection using a $CD4^+$/$CD45RO^-$ kit (R&D Systems) and were >98% pure as assessed by flow cytometry. Naive $CD4^+$ T cells (2×10^4 cells/200 μl of IMDM with 10% FCS) were cocultured with 5×10^3 DCs matured in the presence of vaccines plus MF and staphylococcal enterotoxin B (final concentration, 100 pg/ml; Sigma-Aldrich), in 96-well flat-bottom plates. On day 5 of these cultures, recombinant human IL-2 (final concentration, 10 U/ml; Cetus Corporation) was added, and the cultures were expanded for the next 9 days. On day 14, the quiescent Th cells were restimulated with PMA (10 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) for 6 h, and during the last 5 h, brefeldin A (10 μg/ml; Sigma-Aldrich) was present, to detect the intracellular production of IL-4 and IFN-γ using specific mAbs (both from BD Pharmingen) by flow cytometry. Briefly, the cells were fixed in paraformaldehyde (2%; Sigma-Aldrich), permeabilized with saponin (0.1%; Sigma-Aldrich), and labeled with FITC-conjugated anti-IFN-γ mAb (BD Biosciences) and PE-conjugated anti-IL-4 (BD Biosciences). The cells were evaluated by FACSCalibur (BD Biosciences). At least 10,000 events gated on forward and side scatter were analyzed using the CellQuest program (BD Biosciences). Corresponding isotype Abs were used to establish the quadrants for analysis.

Modulation of vaccine effects on DCs by adjuvant

To investigate the potential for effects of anthrax vaccine to be countered by adjuvant, all of the studies were also conducted in the presence of WCP extract.

ELISPOT analysis of recall T cell responses to the anthrax vaccine

Blood samples were obtained from volunteers attending the Gulf War Illness Research Unit at King's College London as part of a cross-sectional stage II immunological analysis of veterans of the 1990–1991 Persian Gulf War, the details of which have been reported previously (15). The study had ethical committee approval, and informed consent was obtained from each subject. Acquisition for the current study was between 2002 and 2005. In all cases, exposure to the anthrax vaccine with WCP as an adjuvant was recorded in personal medical records, and vaccination was conducted as described previously (16) (⟨www.mod.uk/issues/gulfwar/info/medical/mcm.htm⟩). Fresh heparinized blood was obtained from 25 veterans and 8 healthy control subjects without history of anthrax/anthrax vaccine exposure. Cytokine ELISPOT analysis was performed as described previously (17, 18) using U-Cytech kits for IFN-γ, IL-2, IL-4, and IL-13 and the following stimuli: medium alone, anthrax vaccine (diluted 1/3000), and tetanus toxoid vaccine (1/1000). Plates were dried, and spots of >80 μm were counted in a BioReader 3000 (Biosys). Results are reported as spots per 300,000 cells.

Statistical analysis

Comparisons of cytokine secretion by DCs under different experimental conditions were made using one-way ANOVA and the Dunn multiple comparison test. Comparison of ELISPOT responses were made using the Mann-Whitney *U* test. Calculations were made using Prism 4 software, and p values <0.05 were considered significant.

Results

Phenotypic analysis of DC maturation and cytokine production in the presence of anthrax and plague vaccines

We first studied the direct effects of the anthrax and plague vaccines on the maturation of monocyte-derived iDCs and their cytokine production. DCs cultured in the presence of control preparations comprising TNF-α, IL-1β, and LPS (MF) showed maturation as expected after 48 h. This was evident from the induction of CD83 expression and the marked up-regulation of HLA-DR and CD86, the key molecules for T cell stimulation (Fig. 2, *B–D*, filled bars), as well as the production of the Th1-stimulatory cytokine IL-12p70 and proinflammatory cytokines TNF-α, IL-6, and IL-10 (Fig. 3, *A–D*, filled bars).

Our initial experiments aimed to establish optimal vaccine concentrations for additional in vitro studies. After 48-h cultures with vaccines, iDCs were assessed for viability, surface molecule expression, and cytokine production. As shown in Fig. 2*A*, cell viability of iDCs was preserved in the presence of a range of concentrations of the plague vaccine. On the basis that it offered optimal expression of accessory molecules (Fig. 2, *B–D*) and secretion of cytokines (Fig. 3, *A–D*), a dilution of the plague vaccine of 1/300 (equivalent to 10^7 organisms/ml) was selected for use in subsequent experiments. At this dilution, the plague vaccine was able to activate iDCs to a similar level, in terms of cytokine production and expression of CD83, HLA-DR, and CD86, to that achieved with MF. In contrast, for the anthrax vaccine, cell death increased markedly at dilutions of <1/300 (Fig. 2*A*). At dilutions between 1/300 and 1/10,000, cell death was equivalent to nontreated iDCs, and across this range, surface molecule expression and cytokine production did not vary greatly (Figs. 2, *B–D*, and 3, *A–D*). In subsequent experiments, a dilution of 1/3000 was selected.

These selected dilutions were used to examine the optimal time course for iDC stimulation. iDCs showed optimal cell viability and stimulation, as judged by the balance between surface marker expression, cytokine production, and cell death, at 48 h in the presence of either MF or the biowarfare vaccines (Figs. 2, *E–G*, and 3, *E* and *F*).

Under the optimal conditions that we established, it was evident that the two biowarfare vaccines behaved differently in terms of iDC maturation. Whereas the plague vaccine was able to up-regulate surface molecules and induce cytokine secretion to levels equivalent to those observed with MF, the anthrax vaccine, in direct contrast, induced very low levels of maturation, such that the surface phenotype and cytokine secretion of DCs cultured in their presence resembled the immature state (Figs. 2 and 3). Although stimulants such as MF can up-regulate HLA-DR expression by three to five times, levels of HLA-DR expression on DCs after a 48-h stimulation with the anthrax vaccine were unchanged. Similarly, although MF up-regulated costimulatory molecule (e.g., CD86) expression by three to eight times, anthrax vaccine-exposed DCs had levels of CD86 expression similar to those observed in the immature state. Likewise, CD83, a surface molecule characteristically appearing on DCs along with the process of maturation, as shown here, increased 4- to 5-fold after induction with MF but was expressed at very low levels after coculture of iDCs with the anthrax vaccine (Fig. 2*D*). The anthrax vaccine triggered very little

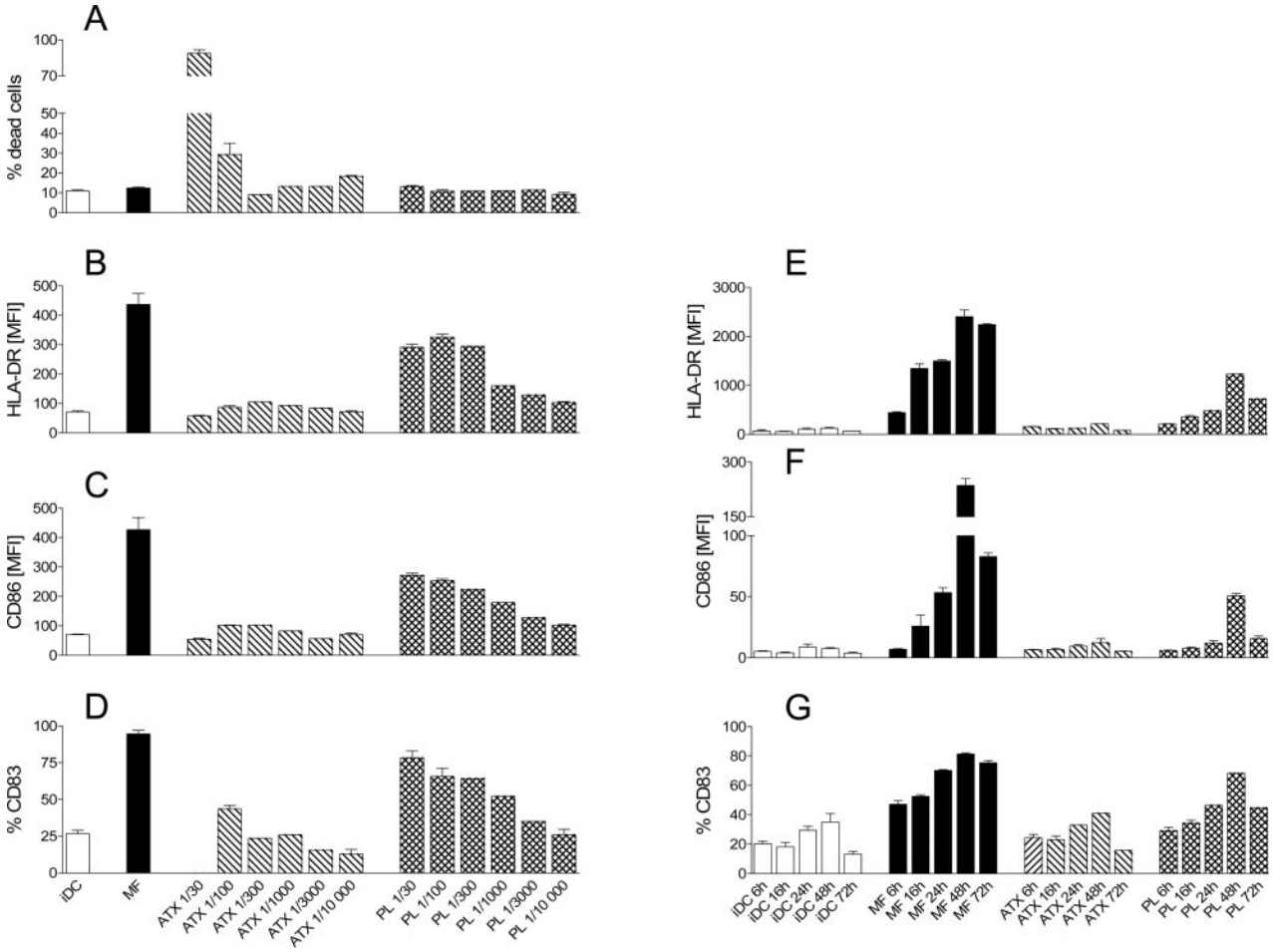

FIGURE 2. Optimization of vaccine concentration and time course for detection of DC maturation. Bar charts show flow cytometric analysis of immature monocyte-derived DCs cultured in the presence of medium alone and remaining iDCs, or in the presence of MFs (IL-1β, TNF-α, and LPS), or in the presence of a range of concentrations of anti-biowarfare vaccines for anthrax (ATX) and plague (PL). DCs have been labeled with 7-AAD for live/dead analysis (*A*), anti-HLA-DR (*B*), anti-CD86 (*C*), and anti-CD83 (*D*). Combining data for cytokine production under the same conditions (see Fig. 3, *A–D*) indicates that the optimal dilutions were 1/3000 for ATX and 1/300 for PL. These concentrations were used in time course studies (*E–G*) that indicate an optimal culture period of 48 h for DC maturation. Bars represent mean levels (error bars are SDs) for triplicate measurements on the same cultures and are representative of seven independent experiments on five different donors. MFI, Mean fluorescence intensity.

IL-12p70 secretion (Fig. 3*A*), achieving levels similar to or less than unstimulated iDCs and significantly lower than the level achieved with MF alone ($p < 0.01$ compared with mean levels in seven independent experiments on five different donors at an anthrax vaccine dilution of 1/3000). Likewise, TNF-α, IL-6, and IL-10 were undetectable or were produced in amounts similar to or below the level observed for iDCs and significantly lower than in the presence of MF ($p < 0.01$ compared with means from seven independent experiments on five different donors).

Overall, these data on the acquisition of T cell-stimulatory molecules and cytokine secretion after coculture with biowarfare vaccines indicate that the anthrax vaccine does not induce DC maturation or cytokine production when assessed using the conventional markers shown here.

Effects of vaccines on the T cell-stimulatory potential of DCs

In light of these findings, we next sought to examine whether DCs exposed to biowarfare vaccines acquired the capacity to stimulate MLR. This was assessed using a fixed number of allogeneic responder $CD4^+$ T cells and varying numbers of DCs that had been exposed to vaccines for 48 h. We found that plague and pertussis vaccines have a high capacity to stimulate allogeneic $CD4^+$ T cells that is directly comparable to DCs exposed to MF. In contrast, DCs exposed to the anthrax vaccine stimulated $CD4^+$ T cells suboptimally, but at a level higher than iDCs (Fig. 4*A*).

CD40L-induced cytokine production by DCs matured in the presence of vaccines

We next sought to establish the cytokine milieu generated by DCs matured in the presence of the anthrax and plague vaccines on encountering CD40L-expressing cells as a surrogate for interaction with lymph node T cells.

Because the anthrax vaccine alone did not induce a mature DC phenotype, and the state of maturity influences the capacity of DCs to drive Th1 or Th2 responses, to perform these experiments, DCs were matured in the presence of vaccines as well as conventional MFs (IL-1β, TNF-α, and LPS). Under these conditions, maturation in the presence of the anthrax vaccine was equivalent to that achieved with MF alone (Table I and Fig. 5). IL-12p70, TNF-α, IL-6, and IL-10 production reached levels similar to or beyond those achieved in the presence of MF alone. However, it is noteworthy that in each of five experiments the presence of anthrax induced IL-12p70 production at levels higher than those seen with MF alone, although this difference did not reach statistical significance. Overall, these

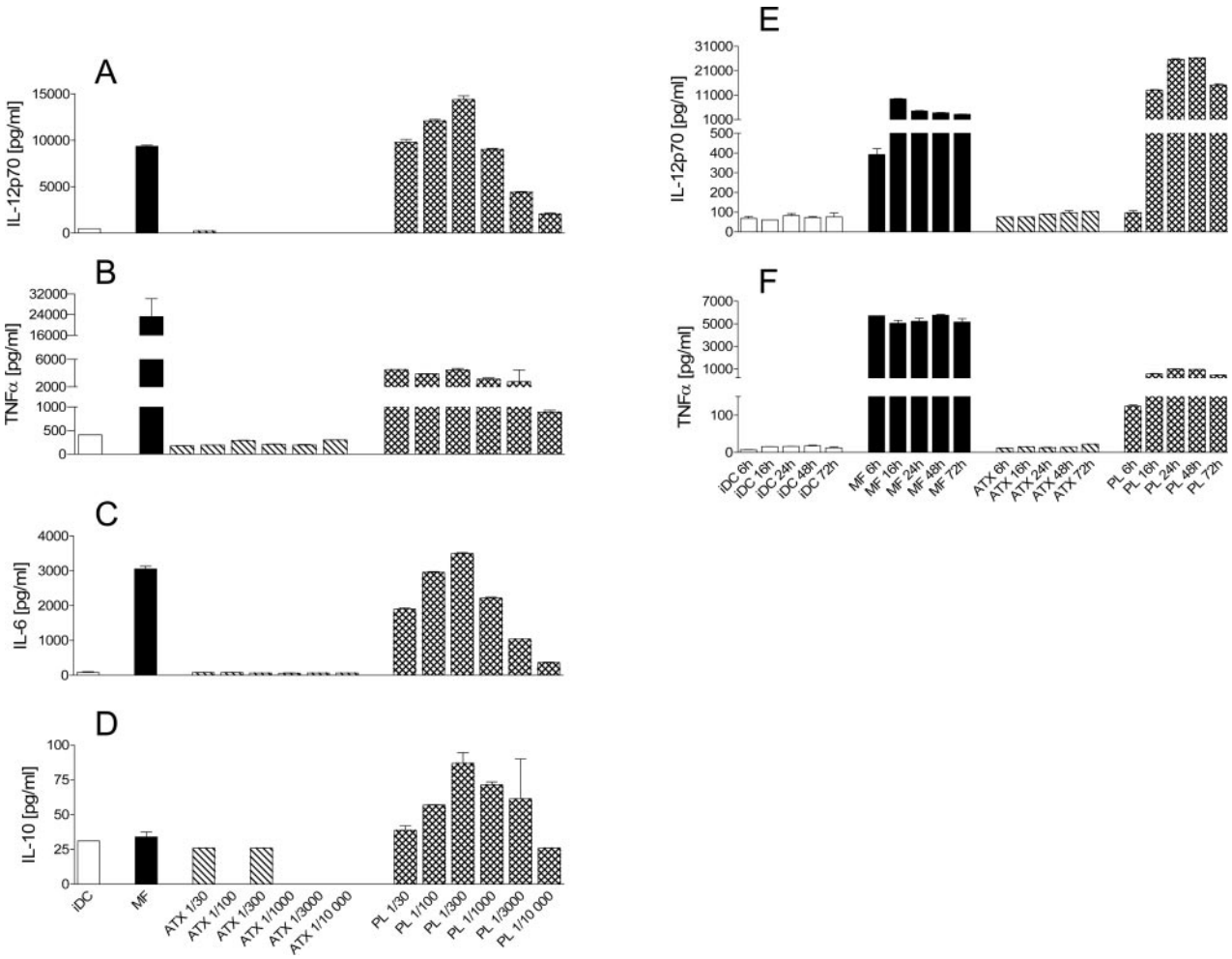

FIGURE 3. Bar charts showing multiplex cytokine analysis of immature monocyte-derived DCs cultured in the presence of medium alone and remaining iDCs, or in the presence of MFs (IL-1β, TNF-α, and LPS), or in the presence of a range of concentrations of anti-biowarfare vaccines for anthrax (ATX) and plague (PL). DC supernatants have been analyzed for secretion of IL-12p70 (*A*), TNF-α (*B*), IL-6 (*C*), and IL-10 (*D*). Combining data for surface marker expression under the same conditions (see Fig. 2, *A–D*) indicates that the optimal dilutions were 1/3000 for ATX and 1/300 for PL. These concentrations were used in time course studies (*E* and *F*) for IL-12p70 and TNF-α that indicate an optimal culture period of 48 h for DC maturation. Bars represent mean levels (error bars are SDs) for triplicate measurements on the same cultures and are representative of seven independent experiments on five different donors.

observations indicate that the anthrax vaccine had no inhibitory effect on mature DC interaction with T cells.

Nature of Th effector cell polarization by anthrax and plague vaccines

Mature effector DCs obtained after a 48-h coculture in the presence of vaccines supplemented with MF were used to stimulate naive CD4 T cells in the presence of staphylococcal enterotoxin B to generate polarized effector Th cells. In keeping with previous reports, DCs matured with conventional MFs (IL-1β, TNF-α, and LPS) induce the development of a mixture of IFN-γ-producing, Th1- and IL-4-producing Th2 effector cells; the addition of IFN-γ leads to a strongly polarized Th1 effector cell response, and the addition of PGE2 biases responses toward IL-4-producing Th2 cells (Fig. 6).

DCs matured in the presence of MF supplemented with the anthrax or plague vaccine induced a mixed effector Th response, in that both Th1 and Th2 cells were generated (Fig. 6). For both vaccines, the percentage of Th1 cell outgrowth was less than that seen in the presence of IFN-γ but more than or equivalent to that seen in the presence of PGE2 or MF alone. For both vaccines, generation of IL-4-producing Th2 cells was similar to that achieved with the PGE2 control and greater than that seen for MF alone. These data suggest that both vaccines are capable of stimulating naive Th outgrowth. In the case of plague, this result might be expected on the basis of cytokine production and influence on DC maturation (Figs. 2 and 3). However, the result for anthrax is surprising, given the failure of the anthrax vaccine to stimulate cytokine production or DC maturation as measured by conventional markers (Figs. 2 and 3).

Effect of biowarfare vaccines on DCs matured with LPS

Because the anthrax vaccine did not induce DC maturation as assessed by conventional markers, we examined whether there was evidence for an inhibitory effect of this agent on DCs. iDCs were exposed to biowarfare vaccines for 24 h, washed extensively, and stimulated with LPS (1, 10, or 100 ng/ml) for 16 h. There was no evidence for inhibition of LPS-induced expression of maturation markers or cytokine production by the anthrax vaccine (Fig. 7) at any LPS concentration. In fact, in six experiments with two separate donors, LPS-induced IL-12p70 and TNF-α production was always enhanced by the addition of the anthrax vaccine (Fig. 7). These data indicate that the anthrax vaccine, although showing limited stimulatory activity alone, is able to synergize with other agents to influence DC function.

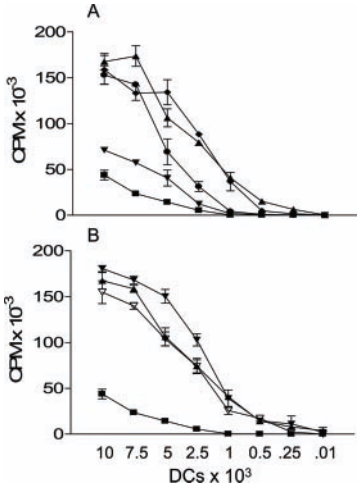

FIGURE 4. MLR of 50,000 allogeneic CD4$^+$ T cells to a range of DC numbers after DC exposure to different vaccine conditions. *A*, Proliferation of CD4$^+$ T cells after coculture with iDC (■), anthrax vaccine (▼), plague vaccine (●), pertussis vaccine (♦), and MFs (▲). The plague and pertussis vaccines give rise to DCs that evoke robust MLR response equivalent to MF alone. MLR response to anthrax vaccine-treated DCs is higher than for iDCs but falls short of that achieved with MF. *B*, Proliferation of CD4$^+$ T cells after coculture with iDCs (■), MF alone (▲), and anthrax vaccine plus pertussis (▽), or plus MF (▼). These data indicate that the anthrax vaccine has no sustained inhibitory effect on the ability of DCs to induce MLR. Data represent means of triplicates from a single experiment, and the error bars are SEMs. Data from a single donor are shown and are representative of data from three independent experiments from three different donors.

Modulation of DC maturation status and function by adjuvant

Our results indicate that the anthrax vaccine fails to induce DC maturation or cytokine production as assessed using conventional markers. As a consequence, expansion of effector T cells in vivo could, in theory, be limited or less sustained. These limitations could theoretically be counteracted through the use of an appropriate adjuvant. To examine this, we analyzed the adjuvant effect of whole-cell extract of *B. pertussis* on the different checkpoints of DC function. Pertussis was selected because it has previously been coadministered with the anthrax vaccine to enhance immunogenicity and protection from potential biological warfare attack.

Using our in vitro approach, we assessed the effects of adding WCP extract to DCs cocultured with the anthrax biowarfare vaccine. The WCP extract alone proved a powerful stimulator of DC maturation. At dilutions <1/3000, WCP was toxic to DCs as assessed by staining with 7-AAD. However, at a dilution of 1/3000, WCP induced DC maturation (91% CD83$^+$ DCs; mean fluorescence intensity for HLA-DR and CD86 staining was 456 and 189 units, respectively). Likewise, WCP diluted 1/3000 induced a mean 1955 pg/ml (SD 54) TNF-α and 1260 pg/ml (SD 192) IL-12p70. These markers of DC maturation for WCP were similar to those obtained with MF alone as shown in Figs. 2 and 3 (filled bars). In addition, WCP appeared to have a strong overall Th1 priming effect (Fig. 6).

Using WCP in cocultures with the anthrax vaccine, there was no apparent modulatory effect on DC maturation or cytokine production by the anthrax vaccine. Thus, iDCs showed appropriate maturation in the presence of WCP and the anthrax vaccine combined (Fig. 8). Cytokine production by mature DCs in the combined presence of WCP and the anthrax vaccine was similar to that for WCP alone (Fig. 5), whereas the effect of adding WCP to biowarfare vaccines was a polarization of naive CD4 T cells toward Th1 similar to that observed for WCP alone (Fig. 6). These data indicate that the anthrax vaccine, despite inducing only limited DC maturation when judged by conventional markers, does not lead to any sustained inhibitory effects on DC maturation or effector function.

Table I. *Phenotypic characteristics of DCs exposed to vaccines in the presence of MFs*

Cell Type	Maturation Stimulus	Maturation Markers		
		HLA-DR	CD86	CD83
iDC		90	36	6
Mature DC	MF	243	98	66
Mature DC	Anthrax + MF	257	150	60
Mature DC	Plague + MF	318	222	62
Mature DC	Pertussis + MF	424	306	78

Detection of recall responses to the anthrax vaccine in a cohort of vaccinees

Our in vitro data suggested that the limited DC stimulation achieved by the anthrax vaccine could be overcome in the presence of an adjuvant such as WCP. Indeed, this is a strategy used in vivo to enhance the immunogenicity of the anthrax vaccine. To examine the efficacy of this approach, we next investigated whether recall T cell responses against the anthrax vaccine could be detected by cytokine ELISPOT in control subjects (anthrax vaccine naive) and vaccinated subjects (military personnel who had received the anthrax vaccine coadministered with WCP as an adjuvant). Anthrax-specific recall responses were clearly detectable for both Th1 and Th2 cytokines in the vaccinated group but not in the naive controls (Fig. 9). There were significant differences in the number of anthrax-specific spots between vaccinated and naive individuals for IFN-γ ($p < 0.05$), IL-2 ($p < 0.01$), and IL-13 ($p < 0.0001$).

Discussion

In the present study, we have used existing technologies for examining DC responses to pathogens to develop an in vitro strategy for examining the interaction between vaccines and DCs. We have applied this approach to increase our understanding of the immune response engendered by two vaccine preparations, anthrax and plague, that may be of critical importance in protection from exposure to the respective biological warfare agents. Our data show that the plague vaccine preparation triggers maturation and effector function of DCs. In contrast, using the same measures of DC activation, the anthrax vaccine invokes minimal maturation and very limited effector function. In the case of the anthrax vaccine, this may explain the requirement for repeated dosing to obtain protection in vivo.

The anthrax and plague vaccines are known to be inefficient immunogens, requiring repeated and frequent administration. As a consequence, the ability of public health systems to plan for possible bioterrorism is severely impaired. Not only is the degree of protection unpredictable, but so is the length of time taken to achieve it, and any possible confounding effects of multiple vaccinations given in the same short space of time. An in vitro model, in which it is possible to play out interaction between vaccine and iDCs, may therefore prove useful in evaluating vaccine effects. Vaccines could fail, or engender only limited protection, for at least two possible reasons: either as a result of not stimulating DC maturation or through the presence of toxins and proteins with inhibitory effects on DC function. The in vitro model can be used to explore the relative contribution of these scenarios and also to examine measures that could be used to redress the balance, such as the use of adjuvants.

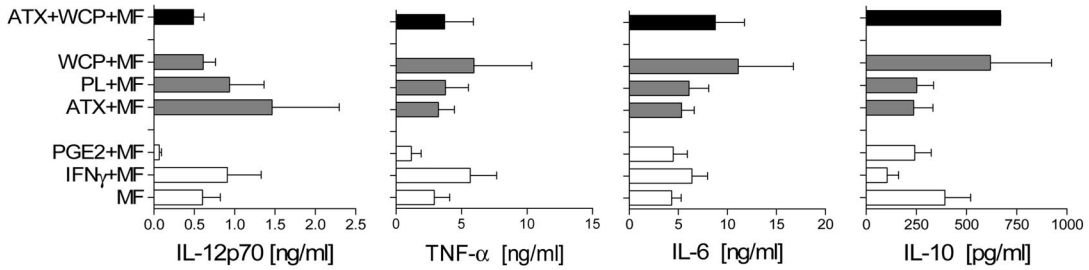

FIGURE 5. Bar charts showing cytokine production by DCs exposed at the mature stage for 24 h in the presence of CD40 ligation to various control stimuli, vaccines, or combinations of vaccines, always in the presence of MFs. IFN-γ and PGE2 (plus MF) are used to represent Th1 and Th2 polarizing stimuli, respectively. Bars represent mean (SD) cytokine levels measured in triplicates from cultures obtained in a single representative experiment of five replicated studies. ATX, Anthrax; PL, plague.

Our studies indicate that during its encounter with iDCs, the anthrax vaccine induces limited maturation or production of key proinflammatory cytokines. This finding resonates with the recent report that the anthrax lethal toxin, present in our vaccine preparation, severely impairs DC function through disruption of immune cell MAPK signaling networks (19). In that report, DCs exposed to lethal toxin failed to up-regulate costimulatory molecules or make proinflammatory cytokines and did not effectively stimulate Ag-specific T cells in vivo. There are some similarities between these findings and those made in the present study when DCs were exposed to the vaccine, although we were unable to demonstrate inhibition of LPS-induced responses. This may reflect differences in the preparations used, or the fact that the final concentrations of PA and LF achieved in our cultures are below those showing clear inhibitory effects in the study by Agrawal et al. (19). An additional possibility is that factors present in the anthrax vaccine preparation, but not LF or PA, are responsible for effects on DCs. The combination of PA, a nontoxic, cell-binding component of the anthrax toxin complex, with EFs produces an edema toxin that induces increased intracellular cAMP levels in susceptible cells (20). This inhibits neutrophil phagocytosis (21) and differentially down-regulates LPS-induced production of TNF-α and IL-6 by increasing the intracellular cAMP levels in monocytes (22). Additionally, factors that up-regulate cAMP, such as PGE2 (23), cholera toxin (24), and histamine (25), are all known to induce Th2-type responses, and in the present study, anthrax-primed, mature DCs generated a predominant Th2-type response.

Clearly, there are important implications both for the generation of effective immunity during encounter with wild-type *B. anthracis* and also for anthrax vaccine design. However, our strategy of in vitro studies has the capacity to indicate manipulations that may overcome any undesirable effects. First, we were able to show that, for the most part, the limited DC maturation and cytokine production induced by the anthrax vaccine was correctable. For example, DCs matured in the presence of conventional MFs plus vaccine produced appropriate amounts of some of the major proinflammatory cytokines

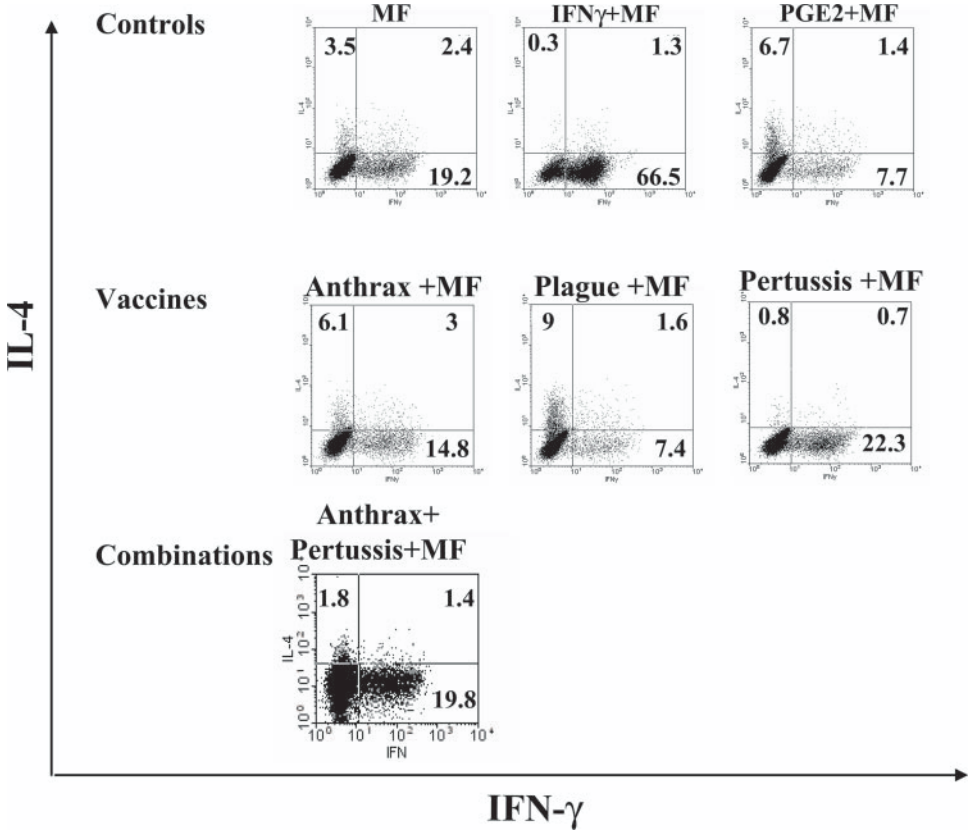

FIGURE 6. Dot plot flow cytometric analyses of IFN-γ (*x*-axis) vs IL-4 (*y*-axis) staining of T cells expanded and polarized by DCs matured for 48 h under the conditions shown. The figures in quadrants represent the percentage of stained cells. Representative data from one of five different donors are shown.

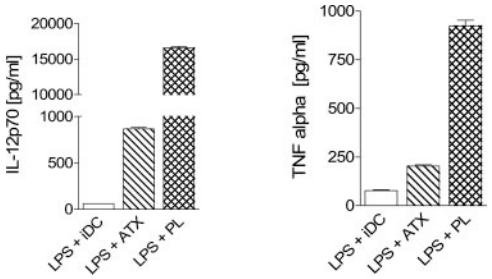

FIGURE 7. Effect of anthrax biowarfare vaccine on LPS-stimulated DCs. The graph shows cytokine production by iDCs exposed initially to anthrax and plague vaccines and subsequently stimulated with LPS for 24 h. Data are representative of six experiments on two donors. Bars represent the means of triplicates, and error bars represent the SD. ATX, Anthrax; PL, plague vaccine.

(TNF-α, IL-6) and were less liable to Th2 polarization when compared with vaccine alone. This has important implications for the possible use of adjuvants to overcome poor immunogenicity. It is also consistent with the fact that repeated, short-term vaccination achieves effective host protection, presumably as a result of inducing escalating levels of local inflammation that in turn provides DC maturation signals.

It is of interest that under conditions designed to examine Th cell outgrowth, both the anthrax and plague vaccines promote the generation of Th2 effector cells. It is possible that this response occurs naturally as a result of exposure to wild-type *B. anthracis* or *Y. pestis* organisms, but to date no study on the T cell response under these conditions has been conducted. The current literature is limited to observations on the type of Ab response generated as the result of natural infection compared with vaccine administration. IgG1 and IgG3 class Abs are generally detected after natural infection compared with all of the IgG subclasses after vaccine administration, and it is noteworthy that IgG4 isotype Abs are indicative of a Th2 immune response (26). A final possibility, in the case of the anthrax vaccine at least, is the effect of alum, a known Th2-polarizing adjuvant. However, it is known that alum does not have direct effects on DC activation (27).

Despite the fact that the anthrax vaccine invoked limited DC maturation, it was able to induce a predominant Th2 response. This is not typical of iDCs and is more surprising given the high production of IL-12p70 after CD40 ligation. It is possible that Th2-type vaccine effector responses are due to OX40 ligand interaction, which is known to promote Th2 cells (28, 29), or other factors, which are as yet unknown. Equally possible is that the anthrax vaccine, by not maturing DCs and not triggering the expression of costimulatory molecules on DCs, results in an iDC-like phenotype that is known to produce more IL-12p70 than the mature cells (30).

To counter the limited immunogenicity of the anthrax and plague vaccines, WCP extract was added to the vaccination regimen as an adjuvant for United Kingdom troops deployed to the first Persian Gulf War. Pertussis has a powerful effect on DC maturation and activation, with marked proinflammatory cytokine production and Th1 polarization (29). In our study, examination of the effect of pertussis on DC-anthrax vaccine interactions showed that the limited effects of this biowarfare vaccine could be overcome. A mature DC phenotype was achieved along with good Th1 outgrowth. Indeed, our ex vivo cytokine ELISPOT data indicate that Th1 and Th2 anthrax-specific responses are induced and long-lived in individuals receiving combined anthrax and pertussis vaccines, although we were unable to document the relative benefit of adjuvant because subjects that received the anthrax vaccine alone are not available for study. In contrast with the limited effects of the

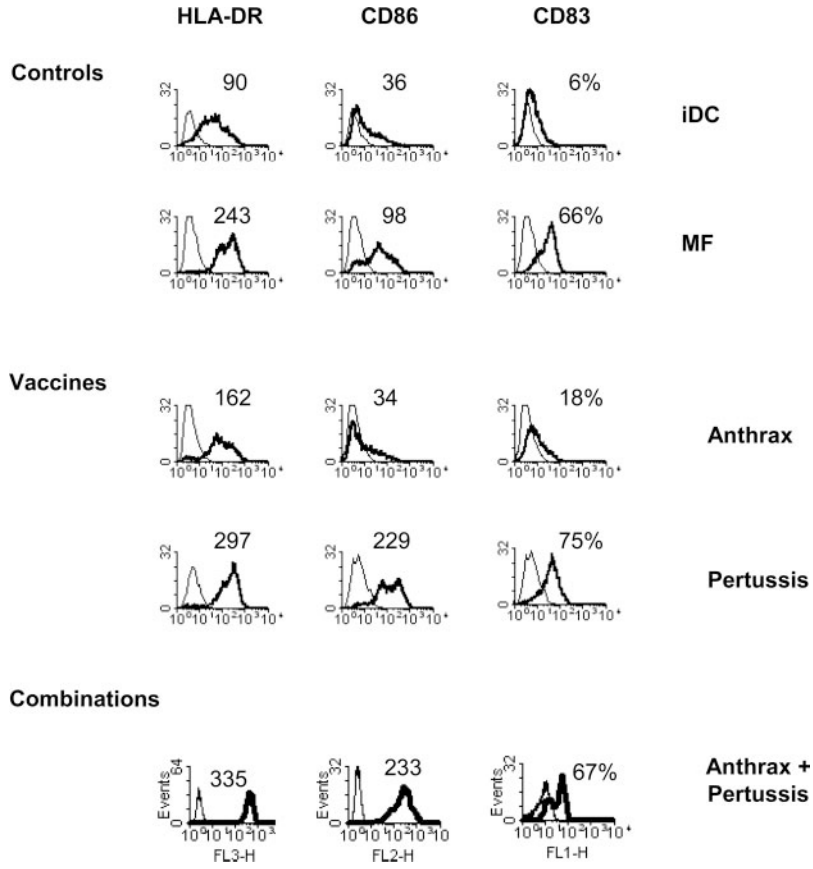

FIGURE 8. Effects of WCP in combination with the anthrax vaccine on DC maturation. Histograms of flow cytometric analysis of acquisition of maturation markers on DCs exposed at the immature stage to control stimuli, single vaccine preparations, and combinations of vaccines for 48 h are shown. The thin line represents isotype control Ab staining; the bold line represents the designated mAb. The histograms represent mean fluorescent intensity for HLA-DR and CD86 and the percentage of positive cells set at the 99th percentile of the isotype control for CD83. Representative data from one of five different donors are shown.

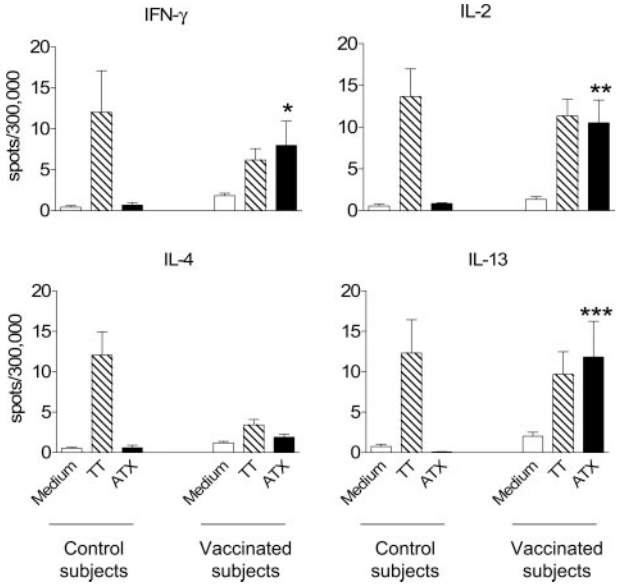

FIGURE 9. Recall T cell responses detected by cytokine ELISPOT against tetanus toxoid (TT) and anthrax (ATX) vaccines in control subjects (anthrax vaccine naive) and military personnel who had received anthrax vaccine coadministered with WCP as an adjuvant. Bars represent the mean number of spots counted for each cytokine in triplicate, and error bars are SEM. Anthrax-specific recall responses are clearly detectable for both Th1 and Th2 cytokines in the vaccinated group but not in the naive controls. There are significant differences in the number of anthrax-specific spots between vaccinated and naive individuals for IFN-γ (*, $p < 0.05$), IL-2 (**, $p < 0.01$), and IL-13 (***, $p < 0.0001$). Mean background (medium alone) spots ranged between 0 and 4 and were not different between the groups.

anthrax vaccine, the plague vaccine was efficient in induction of DC maturation and effector function. It seems unlikely, therefore, that the requirement for repeated multiple administrations of the plague vaccine is explained by an inability to activate the immune system via DCs.

In summary, anthrax and plague vaccines are poor immunogens in vivo; in the case of anthrax, this may be a result, at least in part, of its limited ability to trigger the maturation process of iDCs and generate an appropriate proinflammatory cytokine milieu, which are crucial for initiation of effector immune responses. Our study also supports, through in vitro and in vivo data, the potential of DC-activating adjuvants to overcome such effects.

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Disclosures

The authors have no financial conflict of interest.

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