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Effect of *Bacillus anthracis* virulence factors on human dendritic cell activation

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Summary

Bacillus anthracis possesses three primary virulence factors: capsule, lethal toxin (LT), and edema toxin (ET). Dendritic cells (DCs) are critical to innate and acquired immunity and represent potential targets for these factors. We examined the ability of *B. anthracis* spores and bacilli to stimulate human monocyte-derived DC (MDDC), primary myeloid DC (mDC), and plasmacytoid DC (pDC) cytokine secretion. Exposure of MDDCs and mDCs to spores or vegetative bacilli of the genetically complete strain UT500 induced significantly increased cytokine secretion. Spores lacking genes required for capsule biosynthesis stimulated significantly higher cytokine secretion than UT500 spores from mDCs, but not MDDCs. In contrast, bacilli lacking capsule stimulated significantly higher cytokine secretion than UT500 bacilli in both MDDCs and mDCs. Spores or bacilli lacking both LT and ET stimulated significantly higher cytokine secretion than UT500 spores or bacilli, respectively, in both mDCs and MDDCs. pDCs exposed to spores or bacilli did not produce significant amounts of cytokines even when virulence factors were absent. In conclusion, *B. anthracis* employs toxins as well as capsule to inhibit human MDDC and mDC cytokine secretion, whereas human pDCs respond poorly even when capsule or both toxins are absent.

Keywords

Human myeloid DC; Human plasmacytoid DC; *B. anthracis*; *B. anthracis* toxins; *B. anthracis* capsule

Introduction

Bacillus anthracis, the Gram-positive, nonmotile, spore-forming bacterium that causes anthrax, possesses three primary virulence factors; an extracellular poly-D-glutamate (D-PGA) capsule and the secreted toxins, lethal toxin (LT) and edema toxin (ET), that are induced by physiologic temperature, CO₂, and bicarbonate concentrations found in mammalian hosts [1–3]. Capsule is produced by the enzymes CapA, CapB, and CapC encoded by the *capBCAD* operon located on virulence plasmid pXO2. LT and ET are binary toxins composed of enzymatic A-components lethal factor (LF) or edema factor (EF), respectively, sharing the B-component protective antigen (PA), which translocates LF and EF into the cytoplasm [4]. LF,

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EF, and PA are encoded by *lef*, *cya*, and *paga* respectively, contained on virulence plasmid pXO1 [5–10].

The D-PGA capsule is critical to virulence in that deletion of the *capBCAD* operon from the pXO1 + pXO2+ strain UT500 results in a 10,000-fold increase in LD50 [11]. The capsule, which forms the outermost layer of bacilli, is a weak immunogen [12] and inhibits phagocytosis by mouse macrophages [13]. LF, a Zn²⁺-dependent protease, cleaves and inactivates several human mitogen-activated protein kinase kinases (MAPKKs) critical to innate and adaptive immune responses [14–17]. Purified LT has deleterious effects on macrophages and DCs, including induction of apoptosis and inhibition of cytokine secretion [18–23], EF, a calmodulin-activated adenylate cyclase, also inhibits innate immune functions, including phagocytosis by neutrophils and macrophages, and cytokine secretion by macrophages and DCs [22–25].

Based on current understanding, anthrax disease progresses through several stages including spore deposition, spore phagocytosis, spore germination during migration to lymph nodes, proliferation of bacilli in lymph nodes, and systemic dissemination of bacilli [26–28]. The relatively short time period between initial infection and death [29] suggests that immune system failure lies primarily in the innate response during early disease stages to detect bacteria and/or generate responses to inflammatory signals. Pathogens commonly disable innate immune responses by inactivating DCs [30], which detect pathogens at environmental interfaces resulting in cytokine secretion and migration to draining lymph nodes. DC responses may be particularly important to anthrax as DCs extensively take up and transport spores to lymph nodes in mouse studies [23,31].

Understanding interactions between *B. anthracis* and human DCs is critical to extrapolating information from animal models and facilitating development of new therapeutics. Previous work on *B. anthracis* virulence factor effects on DCs used purified LT [21] or toxin-deficient mutants derived from strain Sterne 7702, which lacks plasmid pXO2 containing *capBCAD* and possibly other genes contributing to virulence [22,23,27,32]. Of these studies, some examined murine DCs [22,23], and human DC studies used monocyte-derived DCs exclusively [21,27,32].

Here we assessed the impact of capsule, LT and ET on secretion by human DC subsets of cytokines important to innate and adaptive immune responses following *in vitro* exposure to *B. anthracis*. We used a genetically complete pXO1 + pXO2+ strain (UT500) and UT500-derived isogenic deletion mutant strains lacking the genes required for production of capsule, LF, EF, or both LF and EF. We examined cytokine secretion by three types of human peripheral blood-derived DCs: primary mDCs and pDCs isolated from peripheral blood, and monocyte-derived DCs (MDDCs). Because the different stages of *B. anthracis* infection are characterized by distinct anatomical compartments and bacterial forms, we examined different subsets of DCs likely associated with these compartments, as well as both spore and vegetative forms.

Subjects and methods

B. anthracis strains

The fully toxigenic capsule-forming strain UT500 (pXO1+ pXO2+) was constructed previously by transducing plasmid pXO2 from strain 6602 (Pasteur) into strain 7702 (Sterne) [33]. UT538 (Δ *capBCAD* [Sp^r]), UT539 (Δ *lef* [Kan^r]), UT540 (Δ *cya* [Kan^r]), UT541 (Δ *lef* Δ *cyo* [Kan^r, Sp^r]) were derived previously from UT500 by targeted deletion of genes encoding capsule biosynthetic enzymes, LF, EF, or both LF and EF respectively [11,34].

Preparation of spores and vegetative bacilli

Spores and vegetative bacilli were prepared as previously described [34], Spore stocks were confirmed by microscopy to contain more than 95% spore forms.

Human MDDC preparation, MDDCs were prepared as described previously [35,36], with the following modifications. Peripheral blood mononuclear cells isolated from anonymous human donor buffy coats (United Blood Services; Albuquerque, NM) by Ficoll-Paque Plus centrifugation (GE Healthcare, Uppsala, Sweden) were incubated at 4°C to induce cell aggregation, layered over FBS, re-aggregated at 4°C, washed twice in PBS containing 0.6 mmol/l ethylenediaminetetraacetate (EDTA), and resuspended in PBS with 2% FBS, 2 mmol/l EDTA (Gibco; Grand Island, NY). T cells, NK cells, B cells, and neutrophils were removed with antibody-linked magnetic beads recognizing CD3, CD16, CD19, or CD56 (Miltenyi Biotec, Auburn, CA). Cell preparations were seeded onto 10-cm culture dishes at $2\text{--}3 \times 10^6$ cells/ml in Dendritic Cell Maturation Medium (DCMM) (Sigma-Aldrich, St. Louis, MO) containing 2 mmol/l L-glu-tamine, 100U/ml penicillin, 100 µg/ml streptomycin, 55 µmol/l 2-mercaptoethanol (2-ME), and incubated with interleukin (IL)-4 (1000 IU/ml) and GM-CSF (50 ng/ml) (Invitrogen; Carlsbad, CA), with cytokine replenishment at day 3, to yield monocyte-derived DCs at day 6 (MDDCs).

Human primary DC isolation. Peripheral blood mononuclear cells were isolated by Ficoll-Paque centrifugation of buffy coats (United Blood Services; Albuquerque, NM) followed by antibody-linked magnetic bead depletion of cells expressing CD3, CD14, CD16, CD19, CD56, or Glycophorin A (Miltenyi Biotec; Auburn, CA), Cells labeled with anti-CD123-PE, anti-HLA-DR-PerCP, anti-CD11c-APC, and FITC-conjugated Lineage Markers (CD3, CD19, CD16, CD56, CD14) (BD Pharmingen; San Jose, CA) were processed by fluorescence-activated cell sorting (FACS) with MoFlo™ High-Performance Cell Sorter (Dako Cytomation; Ft, Collins, CO). Gating strategies were determined using unstained and single-stained (compensation) controls. DCs, which were identified as Lin, HLA-DR⁺, were categorized as mDCs or pDCs by expression of CD11c or CD123, respectively. By post-sort analysis, mDC preparations were 84–98% pure (average purity 89%), with 0.2–0.6% contaminating pDCs, pDC preparations were 90–99% pure (average purity 94%), with 0.3–0.6% contaminating mDCs. Cells were resuspended in DCMM with 2 mmol/l glutamine and 55 µmol/l 2-ME, and in the case of pDCs, 10 ng/ml IL-3 (R&D Systems; Minneapolis, MN) was added.

B. anthracis cultures with DCs

Spores or bacilli were added to DCs at a multiplicity of infection (MOI) of 1 in 96-well round-bottom plates in DCMM containing 2 mmol/l glutamine, 55 µmol/l 2-ME. Spore experiments were performed with 50,000 MDDCs, 25,000 mDCs or 25,000 pDCs. Bacilli experiments were performed with 25,000 cells for each DC type. MOIs were determined in each experiment by CFU measurement of inocula. MOIs between 0.6 and 1.8 were considered usable, as cytokine secretion was proportional to MOI in this range for both spores and bacilli (data not shown). MOIs of virulence factor mutants did not differ from UT500 by more than 2-fold in any given experiment. Following inoculation, plates were centrifuged 5 minutes at 500 rpm and incubated at 37°C with 5% CO₂. Ciprofloxacin (final concentration 1.0 µg/ml) was added 1 hour after spore inoculation or 3 hours after bacilli inoculation. Spore germination was determined by susceptibility to killing after incubation at 65°C for 30 minutes, as measured by CFU count. At 24 hours post-inoculation, plates were centrifuged 10 minutes at 1000 rpm, and supernatants stored at –80°C. Positive controls for MDDCs and mDCs consisted of lipopolysaccharide (LPS) from *Escherichia coli* strain 0111:B4 (Sigma-Aldrich, St. Louis, MO) at 1 µg/ml, and influenza strain A/PR/8/34 (H1N1) virus (Advanced Biotechnologies Incorporated; Columbia, MD) at MOI of 10 for pDCs. Experiments were conducted in an ABSL-3 facility.

Cytokine quantitation

Cytokine concentrations were measured using antibody-linked beads recognizing 21 different cytokines (BioSource-Invitrogen; Carlsbad, CA) and a Luminex 100 reader (Luminex Corporation, Austin, TX), and raw data were analyzed with the StatLIA statistical analysis program (Brendan Scientific, Carlsbad, CA). Statistical analysis of cytokine concentrations was performed with GraphPad Prism (La Jolla, CA).

Results

Determination of DC-*B. anthracis* culture conditions

Inhalational anthrax is carried out by airborne spores that deposit in the lung and germinate in response to phagocytosis as they are transported to draining lymph nodes [28]. Bacilli predominate for the remainder of the infection and account for proliferation, systemic dissemination, and toxin production. Because DCs may respond differently to spores and bacilli, we exposed DCs to each form separately. Spores and bacilli were generated from *B. anthracis* strain UT500, a genetically complete pXO1+ pXO2+ strain that produces capsule, LT and ET [33], as well as from UT500-derived isogenic virulence factor deletion mutants lacking the capsule biosynthesis operon, LF, EF, or both LF and EF. Preliminary studies determined that UT500 spores or bacilli inoculated at an MOI of 1 stimulated cytokine secretion in MDDCs, and that higher MOIs did not significantly affect cytokine levels (data not shown).

Spores encountered by DCs are rapidly taken up and germinate. In exposing DCs to spores we aimed to expose DCs to pathogen-associated molecular patterns (PAMPs) and other potential immunomodulatory factors contained in dormant spores or expressed during germination, while limiting exposure to bacilli and bacillary stage-specific processes including replication. MDDCs and mDCs were incubated with UT500 and mutant spores for 1 hour. Over 95% of spores of each strain initiated germination within 20 minutes after addition to DC cultures as determined by susceptibility to killing by incubation at 65°C for 30 minutes (data not shown). Previous studies showed that at one hour post-infection, human MDDCs and mouse bone marrow-derived DCs contain endocytosed spores [22,27]. Therefore, the antibiotic ciprofloxacin was added one hour after spore inoculation with each DC type, resulting in complete killing of bacteria as determined by plating for CFU (data not shown). For each strain, numbers of total bacteria recovered from MDDC or mDC cultures did not change markedly over the 1 hour incubation period, typically increasing between 1.0-fold and 1.7-fold (Figures 1A and 1C). Addition of ciprofloxacin at 1 hour postinfection resulted in MDDC, mDC and pDC viabilities at 24 hours postinfection similar to respective unexposed controls, regardless of which bacterial strain was present in the culture (Supplemental Figure 1).

In studying responses of DCs exposed to bacilli, our goals were to (1) use bacilli grown under conditions to induce virulence factor expression, including a fully developed capsule in appropriate strains, prior to initiation of contact with DCs; (2) allow sufficient time for appropriate strains to express and secrete toxins during *In vitro* cultures with DCs; and (3) avoid significant losses in DC viability that would complicate interpretation of data. Pilot studies determined that addition of ciprofloxacin 3 hours postinfection allowed these conditions to be met. In culture medium without DCs, all five strains proliferated at similar rates (data not shown). When co-cultured with MDDCs, although bacterial numbers of each strain increased over the 3-hour incubation period, acapsular *capBCAD*⁻ bacilli proliferated at a lower rate than UT500 or any of the toxin mutant strains (Figure 1B). In contrast, when co-cultured with mDCs, *capBCAD*⁻, UT500, and toxin mutant bacilli proliferated at similar rates (Figure 1D). These findings suggest that MDDCs, but not mDCs, possess *B. anthracis* growth-limiting functions that are inhibited by capsule but unaffected by either toxin. MDDCs and mDCs incubated with bacilli showed slight viability decreases at 24 hours postinoculation relative to unexposed

controls, but decreases were similar whether incubated with UT500 or any of the mutant strains (Supplemental Figure 1). pDCs showed larger viability decreases relative to unexposed controls than did MDDCs or mDCs, but viabilities were similar whether incubated with UT500 or mutant strains.

Human MDDC, mDC and pDC cytokine secretion postexposure to spores and bacilli of *B. anthracis* parent strain UT500

UT500 spores-DC subsets—We first determined for each DC type whether UT500 spores stimulate cytokine secretion. LPS was used as a positive control for MDDCs and mDCs, and influenza A virus for pDCs. In MDDCs and mDCs, UT500 spores stimulated large increases in secretion of proinflammatory cytokines including IL-12p40, tumor necrosis factor (TNF)- α , and IL-6 (Figures 2A and 2B), as well as MIP-1 α , MIP-1 β , IL-8, IL-10, GM-CSF, RANTES, and GRO- α (data not shown), ranging from one to two orders of magnitude higher than unexposed controls. No increases in IL-1 β , IL-1 α , G-CSF, IFN- α , or eotaxin secretion were observed (data not shown). In pDCs, UT500 spores failed to stimulate elevated cytokine secretion, in contrast to influenza A virus which stimulated large amounts of IFN- α and TNF- α (Figure 2C).

UT500 bacilli-DC subsets—In MDDCs and mDCs, UT500 bacilli, like UT500 spores, stimulated large increases in secretion of IL-12p40, TNF- α , and IL-6, ranging from one to two orders of magnitude higher than unexposed controls (Figures 3A and 3B). UT500 bacilli failed to stimulate pDC cytokine secretion (Figure 3C).

Role of capsule in MDDC, mDC, and pDC cytokine secretion after exposure to spores and bacilli

Spores-DC subsets—We next asked whether capsule formation impacts cytokine secretion by DCs exposed to spores, *capBCAD*[−] spores, which are genetically incapable of forming capsule, stimulated several-fold higher IL-12p40, IL-6 and TNF- α secretion than UT500 spores in mDCs, but not in MDDCs (Figures 4A and 4C; data not shown). Nevertheless, in both MDDCs and mDCs, *capBCAD*[−] spores stimulated significantly higher cytokine secretion than unexposed controls (data not shown). Like UT500 spores, *capBCAD*[−] spores failed to stimulate IFN- α or IL-6 secretion by pDCs (Figure 4E).

Bacilli-DC subsets—*capBCAD*[−] bacilli stimulated several-fold higher IL-12p40, IL-6, and TNF- α secretion than UT500 bacilli not only from mDCs but also from MDDCs (Figure 4B and 4D; data not shown). *capBCAD*[−] bacilli failed to stimulate pDC secretion of any cytokines, including TNF- α (Figure 4F; data not shown).

Role of toxins in MDDC, mDC, and pDC cytokine secretion after exposure to spores and bacilli

Spores-DC subsets—In MDDCs and mDCs, LF-/EF-deficient spores stimulated small, but statistically significant, increases in IL-12p40 and IL-6 secretion relative to UT500 spores (Figures 5A and 5C). In contrast, single-toxin mutant spores deficient in only LF or only EF stimulated similar levels of cytokine secretion as UT500 spores in both MDDCs and mDCs, suggesting that the two toxins function redundantly to inhibit cytokine secretion. Again, pDCs showed no cytokine response to spores deficient in either or both toxins (Figure 5E).

Bacilli-DC subsets—In MDDCs and mDCs, LF-/EF-deficient bacilli stimulated several-fold increases in IL-12p40, IL-6 and TNF- α secretion relative to UT500 bacilli, although in MDDCs the increase in IL-6 secretion was not statistically significant (Figures 5B and 5D; data not shown). EF-deficient bacilli stimulated similar levels of IL-12p40 and IL-6 as UT500

bacilli in MDDCs and mDCs. LF-deficient bacilli stimulated similar levels of IL-12p40 and IL-6 as UT500 bacilli in MDDCs, but significantly higher levels in mDCs. Interestingly, in mDCs, LF-deficient bacilli stimulated a similar amount of IL-6 secretion as LF-/EF-deficient bacilli, but less than half the amount of IL-12p40. This result suggests that in mDCs, LF acts independently of EF to inhibit IL-6 secretion, but combines with EF to inhibit IL-12p40 secretion. pDCs showed statistically significant increases in IFN- α and IL-6 secretion in response to LF-/EF- bacilli as compared with UT500 bacilli (Figure 5F), but IL-6 quantities were negligible compared with MDDCs and mDCs exposed to LF-/EF- bacilli, and IFN- α quantities were only slightly above the limit of detection.

Discussion

In human beings, *B. anthracis* causes three forms of anthrax disease according to the portal of entry—namely, subcutaneous, gastrointestinal, and inhalational. Inhalational anthrax, in particular, is associated with a high mortality rate, and is the form most relevant to biologic warfare. In this form, *B. anthracis* proliferates to high titers within several days of spore deposition in the lungs, indicating the failure of innate immunity. Studies point toward inhibition of multiple innate immune functions of several cell types by the three primary *B. anthracis* virulence factors—namely, capsule, LT, and ET. Among innate immune cells are DCs, which secrete cytokines in response to pathogen-associated molecular patterns (PAMPs) [30]. In the mouse model, lung DCs have been implicated as the primary cell to take up and carry deposited spores to draining lymph nodes [31]. Optimal DC responses require a sequence of events frequently targeted by viral and bacterial virulence factors. This sequence includes initiation of contact with pathogens, phagocytosis, phagolysosome formation and degradation of the pathogen, and activation of signaling pathways leading to cytokine secretion and cell maturation [30,37,38]. We sought to extend previous work into the effect of *B. anthracis* virulence factors on DCs [19,21,22,27,32] by addressing several issues relevant to human anthrax infection: (1) does a fully virulent, pX01 + pX02+ *B. anthracis* strain stimulate human DC cytokine secretion; (2) do human DCs respond differently to spores than to bacilli; (3) do the primary *B. anthracis* virulence factors, in particular capsule, inhibit DC responses; and (4) do cytokine responses vary between DC subtypes?

Two prominent DC categories include resident DCs recruited from the bloodstream to undiseased tissue during the course of normal homeostasis to act as sentinels, and inflammatory DCs recruited to diseased tissue as monocytes that subsequently undergo cytokine-induced differentiation [39,40]. Therefore, primary mDCs and pDCs, which are DCs directly purified from peripheral blood under steady-state conditions, may be better surrogates for resident human DCs than are MDDCs, which by virtue of cytokine-induced differentiation may closely resemble inflammatory DCs [39]. Because both resident and inflammatory DCs are likely to be relevant to *B. anthracis* pathogenesis, we examined responses of surrogates for both types.

Each form of *B. anthracis* performs specific functions in pathogenesis. A relatively small number of spores cause infection and germinate shortly thereafter, while bacilli proliferate, produce capsule and toxins, and eventually disseminate systemically during the remainder of the disease. Therefore, the innate immune response to bacilli, especially during early stages of bacillary proliferation in the lymph node, may be as or more important than the response to spores. Previous studies examined DCs exposed to spores [21–23,27,32], but bacilli and spores bear several obvious structural and compositional differences [41] and hence may elicit distinct DC responses. In addition, virulence factors could affect responses to spores or bacilli differently depending on how each form stimulates DC signaling pathways. Therefore, we assessed DCs responses to spores and bacilli in separate experiments.

We found that spores from the fully virulent pX01 + pX02+ strain UT500 stimulated cytokine secretion in MDDCs and mDCs, consistent with previous findings using the pX02-deficient Sterne 7702 strain [22,27,32]. We conclude that exposure to *B. anthracis* spores rapidly activates signaling pathways leading to cytokine secretion in human mDCs and MDDCs. Previous work showing that killed spores activate murine splenocyte cytokine secretion through multiple MyD88-dependent pathways [42] raises the possibility that live spores activate analogous pathways in human mDCs and MDDCs, but relevant pathogen related receptors (PRRs) and PAMPs await identification.

We found that UT500 bacilli also stimulated cytokine secretion by both mDCs and MDDCs. Although we cannot rule out the possibility that DCs were stimulated by bacterial degradation products resulting from ciprofloxacin killing, this result suggests that presence of capsule alone does not prevent mDC or MDDC responses. Previous work implicates TLR2 [43] and multiple MyD88-dependent pathways [42,43] in the response to killed bacilli, suggesting standard Gram-positive PAMPs such as PGN, Upoteichoic acid (LTA), or putative *B. anthracis* lipoproteins [44] as candidate ligands.

Role of capsule in regulating DC cytokine secretion

Although capsule is associated primarily with bacilli, the quantities of capsule present in dormant spores or produced during germination have not been well-characterized and remain a potential factor in DC responses. We found that *capBCAD*⁻ spores stimulated increased cytokine secretion relative to UT500 spores in mDCs, but not in MDDCs. This difference between MDDC and mDC responses suggests that either MDDCs are more resistant to capsular effects, or that MDDCs allow less capsule production during co-incubation than do mDCs, possibly because of greater or more rapid MDDC phagocytic and/or killing ability as a result of *in vitro* maturation. One implication of these findings is that capsule production may begin at the outset of infection to inhibit the response of lung-resident DCs to inhaled spores.

Our findings showed that *capBCAD*⁻ bacilli stimulated increased cytokine secretion in both MDDCs and mDCs relative to UT500 bacilli. In fact, capsule impacted cytokine secretion in MDDCs and mDCs to a similar degree as the combined effect of LF and EF, in keeping with the importance of capsule to overall virulence in animal model studies [11]. How capsule inhibits MDDC and mDC cytokine secretion is unclear but could occur through a variety of mechanisms. As the outermost layer of bacilli, capsule is positioned to physically block host PRRs from internally located bacterial PAMPs. Furthermore, capsule has been shown to inhibit phagocytosis by neutrophils and macrophages [13,45] and could act similarly on MDDCs and mDCs. Reduction in DC phagocytosis could result in diminished cytokine secretion by decreasing the surface area of DC-pathogen contact, preventing bacterial digestion and concomitant PAMP release, or allowing increased LT and ET production by promoting bacterial survival and proliferation. Indeed, we observed that encapsulated bacilli strains, regardless of toxin gene deletions, showed higher proliferation rates than *capBCAD*⁻ bacilli during incubation with MDDCs. However, the inhibitory effect of capsule on cytokine secretion is unlikely to be mediated solely by increased toxin production as a result of greater bacterial proliferation, because in mDC cultures, *capBCAD*⁻ bacilli stimulated markedly higher cytokine secretion than UT500 bacilli but proliferated to a similar degree as UT500 bacilli. Clearly, our results warrant further studies into mechanisms of the capsule's effect on human DCs.

Role of toxins in regulating DC cytokine secretion

In MDDCs and mDCs, LF-/EF-deficient spores stimulated small but statistically significant increases in IL-12p40 and IL-6 secretion relative to UT500 spores, indicating that both toxins were secreted in sufficient quantities prior to ciprofloxacin killing to have an appreciable

impact on DCs. Indeed, promoters of genes encoding PA, LF, and EF are active soon after spore germination in murine alveolar macrophages [26]. In contrast, single-toxin mutant spores deficient in either LF or EF stimulated similar levels of IL-12p40, IL-6, and TNF- α secretion by MDDCs and mDCs as did UT500 spores, indicating that at least in this experimental setting the effects of LF and EF on cytokine secretion are redundant and of similar magnitude. This result is consistent with previous reports showing purified LT and ET are each capable of inhibiting cytokine secretion [21,22,25].

LF-/EF-deficient bacilli, like LF-/EF-deficient spores, stimulated greater cytokine secretion than UT500 bacilli in both MDDCs and mDCs. Notably, differences in elicited cytokine secretion between the LF-/EF-deficient strain and UT500 were greater in bacilli experiments than in spore experiments. We attribute this difference to the longer co-incubation period of DCs with bacilli than with spores prior to addition of antibiotic, and the fact that vegetative bacilli are already in a toxin-producing state while spores must germinate before beginning toxin production. Hence we would predict that longer incubation times of spores with DCs prior to addition of ciprofloxacin would lead to larger differences in elicited cytokine secretion between LF-/EF-spores and UT500 spores.

In MDDCs, single-toxin mutant bacilli lacking either LF or EF stimulated similar levels of IL-12p40, IL-6, and TNF- α secretion as UT500 bacilli, indicating that the two toxins have redundant effects of similar magnitude. Like MDDCs, mDCs showed no increase in TNF- α secretion in response to single mutant LF-deficient or EF-deficient bacilli as compared with UT500 bacilli. However, mDCs secreted significantly higher quantities of IL-12p40 and IL-6 in response to LF-deficient bacilli than to UT500 bacilli, but similar quantities in response to EF-deficient bacilli as to UT500 bacilli, indicating a larger role for LF than EF in inhibiting secretion of these cytokines by mDCs.

Our finding that LF and EF each inhibit MDDC and mDC cytokine secretion agrees with a previous study using mouse bone marrow-derived DCs exposed to spores from toxin mutants derived from the pX01 + pX02- Sterne 7702 strain [22]. The previous study differed slightly from ours in finding that single toxin-deficient mutants stimulated significant elevations in cytokine secretion as compared with the reference strain, whereas our spore exposure experiments did not. Additionally, the previous study found that EF, but not LF, inhibited IL-12 secretion, whereas our MDDC and mDC bacilli exposure experiments found that both LF and EF inhibit IL-12p40 secretion. These differences could result from differences between mouse and human cells, tissue of DC origin, effects of other genes on pX02 (which is absent from the Sterne strain), or antibiotic properties. Gentamicin, used in the previous study, penetrates cells less readily than ciprofloxacin [46], which we used to simulate effects in humans following recommended anthrax treatment guidelines. Gentamicin's reduced cellular penetration may allow a longer time period for bacterial survival and toxin production, thus magnifying the effects of each toxin.

We found that pDC cytokine secretion was minimal in response to spores and bacilli even when capsule or both toxins were absent. This lack of responsiveness is consistent with pDC expression of TLR7 and TLR9, which detect single-stranded RNA and CpG DNA motifs respectively, to the exclusion of other TLRs [47,48]. In addition, pDCs respond to TLR7 and TLR9 agonists with large amounts of IFN- α secretion, a response more appropriate for viral than for bacterial infection [49]. Therefore, we predict that direct interaction between human pDCs and *B. anthracis* has minimal impact on the innate immune response during early stages of infection.

***In vivo* significance**

In these studies, antibiotic was added to prevent bacterial overgrowth and allow assessment of early DC responses. In the absence of antibiotic, *in vivo* toxin concentrations will likely exceed those present in our experiments as vegetative bacilli continue toxin production and the effective MOI rises as a result of bacterial replication. Given the concentration-dependent effects of each toxin [21,22], we expect that increases in *in vivo* incubation times and bacterial numbers would reduce DC cytokine secretion to levels below those observed in our experiments because of direct inhibition of cytokine secretion and/or DC apoptosis [19] or lysis [50]. Other *in vivo* factors potentially relevant to pathogenesis include the contribution of connective tissue together with blood and lymph flow to distribution of bacilli and toxins.

Taking these considerations into account, our experiments suggest that resident lung DCs respond to spore uptake with a burst of cytokine secretion as they migrate and enter lymph nodes. If the spore burden is small, the resulting host innate immune response may be adequate to clear infection. However a sufficiently large spore burden may generate enough LT, ET, and capsule to inhibit cytokine secretion and establish an environment favorable to bacterial survival and replication. As bacterial numbers and incubation times increase, elevated concentrations of LT and ET would inhibit functions of inflammatory DCs and other immune cells recruited from the bloodstream. Systemic toxin concentrations would eventually increase and allow bacilli to disseminate and seed target organs.

Our studies raise several questions. LT and ET have been shown to disrupt MARK and cAMP pathways, respectively, but it is not known whether they act upon a common downstream signaling factor controlling cytokine secretion or on distinct factors, nor is it known whether they affect secretory pathways. Although we used cytokine secretion as a readout for DC activation, future studies should also assess effects of LT, ET, and capsule on other processes integral to DC activation such as antigen presentation.

In summary, we found that both spores and bacilli from a genetically complete pX01+ pX02 + *B. anthracis* strain stimulated secretion of proinflammatory cytokines in human MDDCs and mDCs, LF and EF exerted inhibitory effects on spore- and bacilli-induced cytokine secretion that were redundant in MDDCs, but LF had a markedly stronger effect than EF in mDCs. Our findings are consistent with a model in which infecting *B. anthracis* spores produce capsule and toxins soon after germination to compromise the ability of resident DCs to initiate innate immune functions that would otherwise help eliminate spores. Bacilli formed subsequently in lymph nodes continue to produce capsule and toxins in order to inhibit immune cell recruitment and inactivate any cells that manage to be recruited.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

B. anthracis

Bacillus anthracis

DC

	dendritic cell
EF	edema factor
ET	edema toxin
IFN	Interferon
IL	interleukin
LF	lethal factor
LT	lethal toxin
mDC	primary myeloid DC
MDDC	human monocyte- derived DC
PA	protective antigen
pDC	plasmacytoid DC
TNF	tumor necrosis factor

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Figure 1.

Bacterial colony-forming units (CFU) in spore and bacilli experiments with human monocyte-derived dendritic cells (MDDCs) and primary myeloid dendritic cells (mDCs). Bacteria were quantified by plating for CFU at timepoints between addition of spores (A, C) or bacilli (B, D), and addition of ciprofloxacin, for MDDC (A, B) and mDC (C, D) cultures (1 hour postinfection for spore experiments, 3 hours for bacilli experiments). Results shown are averages of two (spore) or three (bacilli) independent experiments. Error bars indicate standard error of mean. Data points are slightly offset along x-axis to aid viewing.

Figure 2.

Human DC cytokine secretion after exposure to spores of *B. anthracis* strain UT500 (*capBCAD* +, LT+, ET+). Cytokines were measured in cell culture supernatants 24 hours after spore exposure at an MOI of 1 for (A) MDDCs, (B) primary mDCs, and (C) primary pDCs. As positive controls, *Escherichia coli* LPS was added at 1 μ g/ml to MDDC and mDC cultures, and influenza virus at a MOI of 10 to pDC cultures. Graphs depict mean concentrations from five (MDDC and pDC) or six (mDC) independent experiments, each from a different individual human donor. Error bars indicate standard error of the mean. Statistical significance of cytokine concentration differences between DCs exposed to UT500 spores, LPS or influenza, versus the respective unexposed DC type, were calculated by the one-tailed paired *t*-test; **p* < 0.05, ***p* < 0.01.

Figure 3.

Cytokine secretion by human DCs after exposure to bacilli from *B. anthraxis* strain UT500 (*capBCAD*⁺, LT⁺, ET⁺). Cytokines were measured in cell culture supernatants 24 hours after exposure of (A) MDDCs, (B) mDCs, and (C) pDCs to bacilli at MOI of 1. As positive controls, LPS was added at 1 μ /ml to MDDC and mDC cultures, and influenza virus at a MOI of 10 to pDC cultures. Graphs for each DC type depict mean concentrations from at least six (MDDC), five (mDC) or three (pDC) independent experiments, each from a different individual human donor. Error bars indicate standard error of the mean. Statistical significance of cytokine concentration differences between UT500 bacilli-exposed DCs, versus the respective unexposed DC type, were calculated by the one-tailed paired *t*-test; **p* < 0.05, ***p* < 0.01.

Figure 4.

Cytokine secretion by human DCs after exposure to spores or bacilli from a UT500-derived mutant strain lacking the *capBCAD* operon required for capsule formation. Cytokines were measured in cell culture supernatants 24 hours after exposure to spores or bacilli at MOI of 1 for (A, B) MDDCs, (C, D) mDCs, and (E, F) pDCs. Graphs for each DC type depict mean concentrations from at least three independent experiments, each from a different individual human donor. Error bars indicate standard error of the mean. Statistical significance of cytokine concentration differences between DCs exposed to *capBCAD*⁻ mutant spores or bacilli, versus UT500 spores or bacilli, respectively, were calculated by the one-tailed, paired *t*-test; **p* < 0.05, ***p* < 0.01.

Figure 5.

Cytokine secretion by human DCs after exposure to spores or bacilli of UT500-derived mutant strains containing deletions of genes required for production of Lethal Toxin (LT-), Edema Toxin (ET-), or both toxins (LT-/ET-). Cytokines were measured in cell culture supernatants 24 hours after exposure to spores or bacilli at MOI of 1 for (A, B) MDDCs, (C, D) mDCs, and (E, F) pDCs. Graphs for each DC type depict mean concentrations from at least three independent experiments, each from a different individual human donor. Error bars indicate standard error of the mean. Statistical significance of cytokine concentration differences between DCs exposed to toxin-mutant spores or bacilli, versus UT500 spores or bacilli, respectively, were calculated by the one-tailed paired *t*-test; **p* < 0.05, ***p* < 0.01.