Suppression of Dendritic Cell Activation by Anthrax Lethal Toxin and Edema Toxin Depends on Multiple Factors Including Cell Source, Stimulus Used, and Function Tested

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Bacillus anthracis produces lethal toxin (LT) and edema toxin (ET), and they suppress the function of LPS-stimulated dendritic cells (DCs). Because DCs respond differently to various microbial stimuli, we compared toxin effects in bone marrow DCs stimulated with either LPS or *Legionella pneumophila* (Lp). LT, not ET, was more toxic for cells from BALB/c than from C57BL/6 (B6) as measured by 7-AAD uptake; however, ET suppressed CD11c expression. LT suppressed IL-12, IL-6, and TNF- α in cells from BALB/c and B6 mice but increased IL-1 β in LPS-stimulated cultures. ET also suppressed IL-12 and TNF- α , but increased IL-6 and IL-1 β in Lp-stimulated cells from B6. Regarding maturation marker expression, LT increased MHCII and CD86 while suppressing CD40 and CD80; ET generally decreased marker expression across all groups. We conclude that the suppression of cytokine production by anthrax toxins is dependent on variables, including the source of the DCs, the type of stimulus and cytokine measured, and the individual toxin tested. However, LT and ET enhancement or suppression of maturation marker expression is more related to the marker studied than the stimuli or cell source. Anthrax toxins are not uniformly suppressive of DC function but instead can increase function under defined conditions.

Introduction

CONCERN FOR ANTHRAX DISEASE escalated in the medical and scientific community around the world following the use of the anthrax agent as a biological weapon on U.S. soil in 2001 (Atlas, 2002). *Bacillus anthracis*, the etiological agent of anthrax, is a gram-positive, spore-forming bacterial pathogen of both humans and animals (Dixon *et al.*, 1999; Mock and Fouet, 2001). The bacilli are covered by an antiphagocytic, polyglutamic capsule that helps to evade host immunity and mediate the early invasive stage of infection (Mock and Fouet, 2001). Toxic virulence factors are also produced by *B. anthracis* such as lethal toxin (LT) and edema toxin (ET), together termed anthrax toxins (Duesbery and Vande Woude, 1999; Collier and Young, 2003; Mourez, 2004). These exotoxins are comprised of three secreted polypeptides—protective antigen (PA), lethal factor (LF), and edema factor (EF), which form the anthrax toxins in binary complexes, that is, PA + LF = LT or PA + EF = ET (Collier and Young, 2003; Mourez, 2004). PA facilitates the translocation of either factor across cell membrane through receptor-mediated endocytosis (Collier and Young, 2003; Mourez, 2004). Once inside the cell, LF is a zinc-dependent metalloprotease that cleaves most members of the MAPK kinases family and disrupts intracellular signaling, resulting

in proinflammatory response suppression (Collier and Young, 2003; Mourez, 2004). EF is a calcium- and calmodulin-dependent adenylate cyclase that increases the intracellular concentration of cAMP, resulting in immune cell modulation (Collier and Young, 2003; Mourez, 2004). Although LT has been shown in various studies to be more lethal than its counter part (Pezard *et al.*, 1991), it is speculated that these toxins work together to promote disease (Tournier *et al.*, 2005; Baldari *et al.*, 2006).

Dendritic cells (DCs) are efficient antigen-presenting cells and are crucial in both adaptive and innate immunity (Liu, 2001; Shortman and Liu, 2002; Agrawal *et al.*, 2003). Immature DCs are immunological sensors and are positioned in the periphery to detect pathogen-associated molecular pattern (PAMP). DCs become mature en route to secondary lymphoid organs where they present processed antigen to naïve T cells to launch an adaptive immune response (Liu, 2001; Shortman and Liu, 2002; Agrawal *et al.*, 2003). LT has been reported to induce necrosis in BALB/c-derived DCs, but apoptosis in cells from C57BL/6 (B6) mice *in vitro*; DCs from humans mirrored the response seen in the latter (Alileche *et al.*, 2005; Baldari *et al.*, 2006). Agrawal and his colleagues were the first to demonstrate that anthrax toxins suppress the function of DCs. Using purified LT, they showed that the function of mouse splenic DCs was

compromised *in vitro* and that the toxin prevented DCs from maturing by suppressing surface activation marker expression induced by LPS (Agrawal *et al.*, 2003). Additional work by Tournier's lab using bone marrow-derived DCs (BM-DCs) instead of splenic derived showed that the both toxins could suppress or enhance cytokine production depending on the cytokine tested, and concluded that the toxins cooperate to suppress the innate immune response (Tournier *et al.*, 2005). In both studies, the authors proposed that this disruption might impair the immune system from controlling a *B. anthracis* infection; it is also proposed that disrupting the DC-T cell interaction might result in making the host susceptible to infection with other microbes following toxin exposure (Starnbach and Collier, 2003). To study further the effects of anthrax toxins on DC function including the modulation of response to infection with another intracellular microbe, we designed studies to test DC function following not only activation by endotoxin (LPS) treatment but also activation by infection with *Legionella pneumophila* (Lp).

Lp is an intracellular, gram-negative bacterial pathogen causing Legionnaires' disease (Friedman *et al.*, 2002; Neild and Roy, 2003). This disease is prevalent among immune-compromised individuals, including young children, aged persons, transplant patients, patients receiving corticosteroids, and patients suffering from AIDS (Kikuchi *et al.*, 2005). Our lab has previously shown that infection of DCs with *Legionella* induces a spectrum of cellular changes consistent with Th1 immune polarization (Lu *et al.*, 2006a, 2006b; Newton *et al.*, 2007). Lp infection stimulated the production of polarizing cytokines such as IL-12, IL-6, and TNF- α along with increased MHC class II expression (Lu *et al.*, 2006a, 2006b; Newton *et al.*, 2007). In addition, unlike LPS stimulation, Lp stimulates DCs through TLR 2 and TLR 9 rather than TLR 4 (Newton *et al.*, 2007). DCs stimulated with Lp elicit distinct immune responses (Kikuchi *et al.*, 2004), and the molecular and cellular mechanisms mediating these responses differ in many ways to those following LPS or *B. anthracis* stimulation. Accordingly, to more fully understand the effects of anthrax toxins on DC function, we designed studies to test their effects on cells stimulated with another well-defined intracellular pathogen, *Legionella*, and compare the responses to cells stimulated with LPS. Our data support the conclusion that LT and ET are not uniformly suppressive of DC function but rather modulate function up or down depending on variables such as the function tested, the microbial stimulus used, and the genetic variation in innate immune response mechanisms in the host cell.

Materials and Methods

Mice and bacteria

BALB/c and C57BL/6 (B6) were purchased from NCI-Harlan (Fredrick, MD). They were used at 9–13 weeks of age. They were housed and cared for at the University of South Florida Health Sciences Center Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. A virulent strain of Lp (M124), serogroup 1 isolate from a case of Legionnaires' disease at Tampa General Hospital (Tampa, FL), was cultured on buffered charcoal yeast extract plates for 48 h from a passage 3 stock stored at -80°C . The concentration of bac-

teria was determined by spectrophotometer. For infection of DC cultures, see below.

Isolation and purification of DCs

The femurs and tibias were removed from euthanized mice. The bone marrow cells were flushed out of the leg bones with buffer RPMI 1640 and antibiotic/antimycotic solution (Sigma, St. Louis, MO). The red blood cells in the suspension were lysed using ammonium-chloride potassium, and the cells were resuspended in RPMI 1640 supplemented with 50 μM 2-ME, 10% BGS, antibiotics, 100 mM L-glutamine, and 5 ng/mL GM-CSF (BD Pharmingen, San Diego, CA). Briefly, on day 0, 1×10^6 /mL cells were seeded in 3 mL medium in six-well plates (Costar, Cambridge, MA). On day 1, nonadherent cells were removed by gentle washing, and the wells were replaced with fresh medium. On day 3, the wells were replenished with nutrients by replacing 1 mL of fresh medium. After day 9 in culture, DCs were loosely attached to culture plates and were easily harvested by gently rinsing for subsequent use. For further purification by magnetic cell sorting, an AutoMACSTM Separator Pro (Miltenyi Biotec, Auburn, CA) was used according to the manufacturer's protocol. Up to 10^8 total GM-CSF-enriched cells were resuspended in running buffer (PBS plus 0.5%, culture grade bovine serum albumin, and 0.75 mg/mL EDTA) at room temperature and then incubated with CD11c Microbeads N418 (Miltenyi Biotec) for 15 min at $4-8^{\circ}\text{C}$. Cells were resuspended in buffer (10^7 cells/mL) and sorted with AutoMACSTM Separator Pro using program "possel" for positive selection.

Anthrax toxin treatment

The recombinant toxin components PA, LF, and EF, purchased from List Biological Laboratories (Campbell, CA), were reconstituted in sterile, 1% BSA in PBS buffer and stored in aliquots at -80°C according to the manufacturer's instruction. DCs were preincubated with PA 200 ng/mL alone or with LF 0.01–50 ng/mL, EF 0.1–50 ng/mL, or in combination, 25 ng/mL each, for 5 h, at 37°C , in complete RPMI (RPMI 1640 supplemented with 50 μM 2-ME, 5% FCS, 100 mM L-glutamine). The cells were washed with HBSS, resuspended in culture medium, and stimulated with either LPS (1 μg /mL) or Lp for 24 h. For *Legionella* infection, DCs in culture medium were infected for 50 min with viable bacteria at a 10–20:1 ratio (bacteria-to-cells) and then washed to remove excess extracellular bacteria. Culture cells and supernatants were harvested after 24 h and analyzed for cytokines, viability, and surface marker expression.

Flow cytometry analysis and cell viability

Surface markers were analyzed by flow cytometry. Following treatment and incubation for 24 h, DCs were resuspended to 1×10^6 cells/mL in PBS containing 2% fetal calf serum, Fc receptors blocked with anti-Fc receptor Fc γ RII/RIII antibodies for 15 min on ice, stained with fluorescein isothiocyanate (FITC)-, R-phycoerythrin (PE)-, and allophycocyanin (APC)-conjugated monoclonal antibodies to CD11c, CD40, CD80 (B7.1), CD86 (B7.2), and MHC II (BD PharmingenTM, San Jose, CA) for an additional 30 min. To assess cell viability, labeled cells were centrifuged, resuspended,

and incubated for 5 min on ice with 7-amino-actinomycin D (7-AAD) (BD Pharmingen), and then suspended in 1 mL 2% FCS-PBS buffer. Stained cells were analyzed by flow cytometry using FACSCanto II (Becton-Dickinson, Mountain View, CA) and the program BD FACSDiva Software v5.0.1 (Becton-Dickinson). Some data were further analyzed using FlowJo 7 (TreeStar, San Carlos, CA) to exclude dead (7-AAD-positive) cells.

Enzyme-linked immunosorbent assay

The proinflammatory cytokines IL-12p40/p70, IL-6, IL-1 β , and TNF- α were measured in 24-h DC culture supernatants by ELISA. Medium-bind, 96-well Costar enzyme immunoassay plates were coated with anti-murine IL-12p40/p70 (Pharmingen, San Diego, CA) in NaHCO₃, pH 8.2. After 2 h at 37°C, the plates were blocked for 1 h at 37°C. Culture supernatants and serial dilution of murine IL-12p40/p70 standard (Pharmingen) were added for 1 h, followed by biotinylated anti-murine IL-12p40/p70 for 1 h, and then horse radish peroxidase for 30 min. After the substrate TMB (Sigma) was added, plates were allowed to develop for 15–45 min, and stopped with H₂SO₄. Units were calculated from the standard curve, which was performed for each plate. The plates were washed between each step with two to five changes of nanopure water. IL-6 ELISA was performed by the same protocol with anti-IL-6 in PBS, biotinylated anti-IL-6 antibody, and recombinant IL-6 for standards. IL-1 β and TNF- α ELISA were coated with anti-IL-1 β and anti-TNF- α antibodies, respectively, in carbonate, pH 9.5. Biotinylated and recombinant antibodies were used accordingly.

Statistical analysis

The data approximately follow a normal distribution, and the observations were independent to each other; therefore, the real values in ng/mL or pg/mL from any two groups were compared by one-tailed *t*-test (unequal variance). Based on the $\alpha = 0.05$ level, statistical significance is noted by ♦ and * between compared sample groups where $p < 0.05$. The values in Figure 1 are expressed as percent of control wherein the control is the *Legionella* only (Lp only) treated group.

Results

Modulation by LT and ET of DC cytokine production in response to Lp infection

It has been reported that LT suppresses cytokine production by DCs stimulated with LPS and that the effective toxin concentration was in the $\mu\text{g/mL}$ range (Agrawal *et al.*, 2003). However, other studies involving BM-DCs rather than splenic and stimulated with other stimuli such as *B. anthracis* spores suggested that suppression of cytokine production was not uniformly observed (Tournier *et al.*, 2005). In fact, LT treatment increased certain cytokines as did treatment with ET (Tournier *et al.*, 2005), suggesting that the toxin effects may differ depending upon the source of the DC and the cell stimulus used. To extend these studies, therefore, we examined the effect of various toxin concentrations in bone marrow DC cultures infected with the intracellular pathogen, Lp (Fig. 1). DCs were cultured and treated with 200 ng/mL PA for 6 h and increasing concentrations of either LF (0.01–

50 ng/mL) or EF (0.1–50 ng/mL) followed by infection with Lp for 50 min. The cells were washed and resuspended in medium and cultured for an additional 18 h. Culture supernatants were collected and analyzed by ELISA for IL-12, IL-6, IL-1 β , and TNF- α . PA treatment alone did not significantly affect cytokine production; however, LF combined with PA dose dependently decreased the production of all four cytokines (Fig. 1A). ET plus PA treatment also suppressed IL-12 and TNF- α at 50 ng/mL, but significantly increased the production of IL-6 and IL-1 β even at relatively low concentrations (Fig. 1B). These toxin effects were attenuated using toxins heated to 56°C for 35 min (data not shown), confirming previous reports (Tournier *et al.*, 2005) that only active toxins modulate cytokines in DCs. These results suggested that LT treatment was uniformly more suppressive when added to Lp-infected DCs, while ET was less suppressive and even capable of enhancing cytokine production depending upon the concentration of the toxin.

LT treatment is more toxic than ET in vitro for DCs from BALB/c mice

The above studies were done using partially purified CD11c⁺ cell preparations from BALB/c mice. However, DCs from this mouse strain have been reported to be sensitive to the toxic effects of LT, and because the above studies showed LT suppressed cytokine production, we measured DC viability in cells from BALB/c mice and B6 mice, which are less susceptible to killing by LT (Friedlander *et al.*, 1993; Moayeri *et al.*, 2003; Alileche *et al.*, 2005; Tournier *et al.*, 2005). We also felt it important to study toxin effects in DC preparations purified by positive selection with anti-CD11c magnetic microbeads. DCs from both mouse strains were isolated, purified, and treated or not with either LT or ET, and stimulated with either LPS or Lp infection for 24 h; the cells were then incubated with the impermeable, DNA intercalating dye, 7-AAD, followed by flow cytometry analysis to assess viability. The results showed that DCs from both strains lose the CD11c marker following 24 h incubation without GM-CSF (Fig. 2) going from >95% CD11c positive to between 60% and 70% positive after 24 h. Treatment with LPS alone had no effect on viability in either strain, but LT pretreated cells from BALB/c mice with LPS decreased cell viability after 24 h (Fig. 2B); viability of cells from B6 mice was less affected by the LT. Treatment with ET had no effect on viability. Interestingly, ET treatment did significantly suppress the expression of CD11c in cells from both mouse strains (Fig. 2B, C). Results with Lp-stimulated cells showed that infection of the cultures significantly increased CD11c expression but decreased viability due to the apoptotic effect of Lp (Husmann and Johnson, 1994; Byrne and Swanson, 1998; Kirby *et al.*, 1998; Alli *et al.*, 2000; Neumeister *et al.*, 2002; Zink *et al.*, 2002) with cells from B6 mice somewhat more sensitive (Fig. 2C). Treatment with LT decreased cell viability even more in cells from BALB/c mice and decreased the expression of CD11c in cells from B6 mice. ET treatment was not as toxic as LT; however, overall it decreased the expression of CD11c in both strains as with LPS-stimulated cells. From these results, it is clear that LT is more toxic than ET for cultured DCs from BALB/c mice when the cells are stimulated with either LPS or Lp; however, ET has greater potency in suppressing CD11c expression in both groups. Because

FIG. 1. LT (PA + LF) and ET (PA + EF) modulate BALB/c DC cytokine production in response to *Legionella pneumophila* infection. DC cultures were pretreated for 5–6 h with protective antigen (PA) 200 ng/mL and either (A) LF at 0.01–50 ng/mL or (B) EF at 0.1–50 ng/mL. The cultures were then infected with Lp for 50 min, and supernatants were analyzed 18 h later by ELISA for IL-12p40/p70, IL-6, IL-1 β , and TNF- α . The results are expressed as percentage of the Lp only control and are the means of four to six experiments. * $p < 0.05$, compared to Lp control.

cells from B6 mice were more resistant to toxicity, they were included in subsequent cytokine studies. In addition, we decided to gate on the CD11c⁺ cells that were also 7AAD negative in subsequent activation surface marker studies to determine the toxin effects on viable, CD11c⁺ DCs.

LT and ET can either enhance or suppress cytokine production in purified DC cultures

Having established that LT was more toxic than ET for DCs from BALB/c mice, we reexamined the effects on cytokine production of both toxins in affinity-purified DC cultures from both BALB/c (sensitive) and B6 (resistant) mice stimulated with either LPS or Lp. It was hypothesized that cytokine suppression by LT would be greater in cells from BALB/c mice because of its toxic effect on these cells. DCs were purified and stimulated for 24 h, and supernatants harvested and analyzed for IL-12, IL-6, IL-1 β , and TNF- α . Figure 3A shows that for IL-12 production LT was suppressive only in cells from BALB/c, while ET was suppressive in cells from both sources and following both stimuli. For IL-6 (Fig. 3B), LT was suppressive only in BALB/c cells stimulated with LPS, while in cells stimulated with Lp, the toxin was suppressive in both strains. Since in Figure 2 we

showed that LT is more toxic for BALB/c cells, it is possible that suppression of IL-12 and IL-6 in BALB/c cells is partly due to the toxic effect of the toxin. ET treatment moderately suppressed IL-6 following LPS stimulation but enhanced the cytokine response in cells from B6 mice stimulated with Lp. IL-1 β levels were surprisingly enhanced in cells from both strains following LT treatment and LPS stimulation, but were suppressed in both cell groups following Lp stimulation. ET treatment, on the other hand, had the reverse effect causing a suppression following LPS treatment and enhancement following Lp stimulation (Fig. 3C). TNF- α levels were suppressed in all groups by both toxins (Fig. 3D). It appears from the results that LT and ET could suppress and enhance cytokine production in DC cultures and that this was more dependent upon the cytokine measured and the cell stimulus than on the mouse strain source of the cells. LT suppressed cytokines such as IL-12 and IL-6 in cells from sensitive BALB/c, the toxin could also suppress IL-1 β and TNF- α equally in cells from both mouse strains. Yet it increased IL-1 β production from both strains following LPS stimulation. ET was also surprisingly quite suppressive in cells from both strains, but it too could enhance select cytokines induced by the two different stimuli independent of the mouse strain used. Clearly the modulation of cytokine

FIG. 2. LT (PA + LF) treatment is more toxic than ET (PA + EF) for BALB/c purified DCs. DCs from BALB/c and C57BL/6 (B6) mice were purified by magnetic cell sorting, incubated, and analyzed by flow cytometry for CD11c expression and viability by 7-AAD uptake. (A) DCs from both mouse strains were ~95% CD11c⁺ and viable at the start of incubation. Purified DCs were pretreated for 5–6 h with protective antigen (PA) 200 ng/mL and either LF at 50 ng/mL or EF at 50 ng/mL, and then stimulated for 24 h with either (B) LPS (1 µg/mL) or (C) *Legionella* (10–20:1 bacteria-to-cell ratio). The data are representative of three to four experiments.

production by anthrax toxins is dependent on many variables, including the source of the cells, the type of stimulus and cytokine measured, and the individual toxin tested.

LT and ET differentially stimulate DC maturation marker expression

The above studies showed that LT and ET can modulate the production of cytokines associated with immune maturation and polarization of the DCs. To further examine toxin effects on DC maturation, we examined their effects on DC

maturation marker development following stimulation with LPS and Lp in purified, CD11c⁺ cells from both BALB/c and B6 mice. The markers studied were those important in adaptive immunity, including MHC II, CD40, CD80 (B7-1), and CD86 (B7-2). Stimulated and toxin-treated cells were stained with fluorescent antibodies and analyzed by flow cytometry. Maturation marker expression was analyzed on CD11c⁺ cells that were also viable as judged by 7-AAD exclusion. Surprisingly, LT greatly increased the expression of MHCII across both strains and stimuli (Fig. 4A), while ET had a moderate suppressive effect. Regarding CD40

FIG. 3. LT (PA + LF) and ET (PA + EF) can either enhance or suppress cytokine production in purified DC cultures. Affinity-purified DCs from BALB/c and C57BL/6 (B6) mice were pretreated for 5–6 h with protective antigen (PA) 200 ng/mL and either LF (50 ng/mL) or EF (50 ng/mL), and then stimulated for 24 h with either LPS (1 μ g/mL) or Lp (10–20:1 bacteria-to-cell ratio). Culture supernatants were harvested and analyzed by ELISA for (A) IL-12p40/p70, (B) IL-6, (C) IL-1 β , and (D) TNF- α . Data are the mean $\pm$ SEM cytokine concentrations in culture supernatants from three to four independent experiments. $\blacklozenge$ $p < 0.05$, compared to DC only; $*$ $p < 0.05$, compared to LPS or Lp only.

expression, both toxins suppressed the response to LPS, while, on the other hand, LT increased CD40 expression in Lp-stimulated cells (Fig. 4B). Suppression by both toxins was generally observed across all groups for CD80 marker expression (Fig. 4C), while enhancement of the CD86 marker was observed except in the case of LPS-stimulated B6 cells, where ET significantly suppressed marker development (Fig. 4D). From these results it appears that LT and ET can either enhance or suppress maturation marker expression and that this is generally more related to the marker studied than the cell stimulus or cells source. LT tended to increase MHCII and CD86 while suppressing CD40 and CD80, and ET, on the other hand, tended to decrease all of the markers.

Combined toxin treatment mimics the LT effect

To study the effect of toxins combined, DCs were pretreated with PA 200 ng/mL together with LF and EF,

25 ng/mL each, for 5 h, followed by stimulation for 24 h. The culture supernatants were assayed for secretion of IL-12, IL-6, IL-1 β , and TNF- α by ELISA, and the results were combined with those from Figure 3. Interestingly, we observed two distinct patterns of response as seen in Figure 5A and B. In the first (Fig. 5A), the combination treatment and LT had the same effect on cytokine production, and this was different from ET. For example, combination treatment and LT treatment increased the LPS-induced IL-1 β response in BALB/c mice, while ET suppressed the response (Fig. 5A). In the second pattern (Fig. 5B), the combination treatment and the individual toxin treatments all had the same effect on cytokine production, and this was suppressive. Since each toxin is of different intracellular enzyme, that is, metalloprotease and adenylate cyclase, it appears from these results that these enzymes may interfere with each other or have varying levels of control over cytokine production depending upon the biochemical program activated within the cell.

FIG. 4. LT (PA + LF) and ET (PA + EF) differentially stimulate DC maturation marker expression. Affinity-purified DCs from BALB/c and C57BL/6 (B6) mice were pretreated for 5–6 h with protective antigen (PA) 200 ng/mL and either LF (50 ng/mL) or EF (50 ng/mL), and then stimulated for 24 h with either LPS (1 μ g/mL) or Lp (10–20:1 bacteria-to-cell ratio). Cells were harvested and analyzed by flow cytometry for the expression of (A) MHC class II, (B) CD40, (C) CD80, and (D) CD86. Data are the mean $\pm$ SEM percent positive of the gated population from three to four independent experiments. ♦ $p < 0.05$, compared to DC only; * $p < 0.05$, compared to LPS or Lp only.

Discussion

Previous reports have shown a role of LT and ET in immune suppression, thus facilitating *B. anthracis* infection and disease (Baldari *et al.*, 2006). A number of molecular mechanisms for disrupting signaling pathways have been described for the toxins that can impair the functions of many immune cells, including T-cells (Comer *et al.*, 2005; Fang *et al.*, 2005; Paccani *et al.*, 2005), B-cells (Fang *et al.*, 2006), neutrophils (O'Brien *et al.*, 1985; Wright and Mandell, 1986; During *et al.*, 2005), monocytes (Kassam *et al.*, 2005), DCs (Agrawal *et al.*, 2003; Alileche *et al.*, 2005; Brittingham *et al.*, 2005; Tournier *et al.*, 2005; Cleret *et al.*, 2006; Reig *et al.*, 2008), and macrophages (Pellizzari *et al.*, 1999; Erwin *et al.*, 2001; Berg-

man *et al.*, 2005; Cui *et al.*, 2006). DCs are potent antigen presenting cells and play an important role in innate and adaptive immunity (Banchereau *et al.*, 2000; Liu, 2001; Pulendran *et al.*, 2001; Shortman and Liu, 2002). These cells are now known to have highly diverse characteristics when isolated from various areas in the host and the characteristics diversify even further when the cells are stimulated by various microbial antigens. To date, the effect of anthrax toxins on DC maturation to only two microbial antigens, LPS and anthrax spores, has been reported. To more fully understand toxin effects, we wanted to study cells treated with a third type of stimulus, *Legionella*, a bacterial agent known to infect and alter DC maturation (Lu *et al.*, 2006a, 2006b; Rogers *et al.*, 2007). Our findings show that LT and ET can either enhance

FIG. 5. Combined toxin treatment mimics the LT effect. **(A)** Combination mimics the response of LT. **(B)** Combination is not different from either toxin alone. Affinity-purified DCs from BALB/c and C57BL/6 (B6) mice were pretreated for 5–6 h with protective antigen (PA) 200 ng/mL and either LF (50 ng/mL) or EF (50 ng/mL) or both (25 + 25 ng/mL), and then stimulated for 24 h with either LPS (1 μ g/mL) or Lp (10–20:1 bacteria-to-cell ratio). Culture supernatants were harvested and analyzed by ELISA. Data are the mean $\pm$ SEM cytokine concentrations in culture supernatants from three to four independent experiments. $\blacklozenge$ $p < 0.05$, compared to DC only; $*$ $p < 0.05$, compared to LPS or Lp only.

or suppress DC functions, and that the outcome is dependent upon several factors, including the agent used to stimulate the cells, the DC function tested, and the genetic background of the DC donor mice.

Several reports showed that LT is more toxic for BALB/c-derived BM-DCs than for cells isolated from B6 mice (Alileche *et al.*, 2005; Tournier *et al.*, 2005; Reig *et al.*, 2008), and our current results support these findings. LT toxicity occurred within 24 h in LPS-stimulated cultures (Fig. 2B); however, in Lp-stimulated cells, LT increased toxicity in BALB/c cells, but had an attenuating effect in B6 cells on the enhanced toxicity induced by this intracellular pathogen (Fig. 2C). On the other hand, in contrast to LT, ET treatment was not toxic in cultures from either mouse strain. In addition to BM-DCs, LT toxicity has also been reported in mouse macrophages (Moayeri *et al.*, 2003, 2004) and in DC cultures derived from spleen (Agrawal *et al.*, 2003) and lung (Cleret *et al.*, 2006). The mechanisms of toxicity appear to involve both caspase-dependent and -independent mechanisms and also to depend on the extent of activation/maturation of the DCs (Alileche *et al.*, 2005; Reig *et al.*, 2008). In fact, in DCs from B6 mice, LT toxicity was attenuated in matured cells pretreated with microbial stimuli such as LPS (Reig *et al.*, 2008). In our studies, the cells were not pretreated with LPS but rather with LT followed by LPS for 24 h; cell damage, as measured by 7-AAD uptake, increased eightfold in BALB/c mice while only fourfold in B6 mice. Thus, the mouse strain selective toxicity occurred prior to cell maturation by LPS. In contrast to LPS treatment, our results with Lp-stimulated cells were quite different, in that as expected, treatment with the intracellular pathogen was more harmful than LPS for DCs from both mouse strains. Although LT increased toxicity in BALB/c cells, it surprisingly attenuated the toxicity in B6 cells. *Legionella* infection was more toxic in these cells, and it is likely that LT treatment suppressed the intracellular life cycle of Lp and thus its apoptotic effect (Husmann and Johnson, 1994; Byrne and Swanson, 1998; Kirby *et al.*, 1998; Alli *et al.*, 2000; Neumeister *et al.*, 2002; Zink *et al.*, 2002). Because the LT toxic effect in BALB/c mice occurred within 24 h, it is possible that this early toxicity is due to necrosis rather than apoptosis as suggested by others (Alileche *et al.*, 2005). This cytotoxic effect has also been correlated with the presence of *Kif1c* gene (Watters *et al.*, 2001; McAllister *et al.*, 2003) and early necrosis via Nalp1b activation (Alileche *et al.*, 2005; Reig *et al.*, 2008).

Studies by Pezard's group first reported that LT, not ET, accounts for the toxin mortality in mice (Pezard *et al.*, 1991); however, two recent studies showed a significant effect of ET in whole-animal mortality studies (Firoved *et al.*, 2005; Voth *et al.*, 2005). Whether ET is more toxic than LT *in vivo* remains unclear; however, we show ET is less toxic on cultured DCs, and this is consistent with other *in vitro* studies (Tournier *et al.*, 2005). In addition to toxicity, we show for the first time that ET suppressed the LPS- and Lp-induced expression of the CD11c lymph node homing receptor (Fig. 2B, C). This finding is in contrast to an earlier report showing that CD11c expression was increased 24 h following phagocytosis of *B. anthracis* spores (Brittingham *et al.*, 2005). This study used human monocyte-derived DCs stimulated with attenuated, toxin negative spores, and these were as potent as fully pathogenic spores in increasing CD11c expression. Thus, the effect on CD11c in this study probably had little to do with

toxin production. Since CD11c expression may facilitate DC migration to the lymph node and promote the development of adaptive immunity, it is possible that the CD11c suppression by ET may prevent effective DC migration and lead to progression of the infection following spore germination.

The DC cytokine profile (Fig. 3) indicated that production was differentially regulated by both toxins. In addition, the effect of either LT or ET on any one cytokine varied depending on the type of stimuli and/or mouse strains. Our results with LPS- and Lp-stimulated BM-DCs treated with either LT or ET support the data of Tournier *et al.* and Cleret *et al.* as they observed a similar type of differential regulation by toxins studies using BM-DC and lung DC, respectively, stimulated with nontoxigenic (LF⁻/EF⁻) mutant anthrax spores RP42 (Tournier *et al.*, 2005; Cleret *et al.*, 2006). Further, even though there were three related studies examining the effect of LT on LPS-activated DC *in vitro* (Agrawal *et al.*, 2003; Alileche *et al.*, 2005; Reig *et al.*, 2008), ours is the only study also investigating the effect of ET treatment on LPS-activated DCs. LT, in addition to its lethality, was generally suppressive on IL-12, IL-6, and TNF- α in BALB/c-derived DCs stimulated with either LPS or Lp. This was also seen in LPS-activated splenic DCs (Agrawal *et al.*, 2003) and lung DCs (Cleret *et al.*, 2006) from BALB/c mice. This is consistent with the action of LT, which is a metalloprotease that cleaves the N-terminus of many MAP kinases responsible for the production of many proinflammatory cytokines (Duesbery *et al.*, 1998; Pellizzari *et al.*, 1999; Vitale *et al.*, 2000; Park *et al.*, 2002; Tournier *et al.*, 2005). However, in our hands, LT was not uniformly suppressive. For example, it minimally stimulated IL-12 production in B6 cultures and robustly increased IL1 β in LPS-stimulated cultures from both mouse strains. The mechanism of this increase is not known at this time.

ET, though nontoxic in our study, is nonetheless an important factor contributing to the pathogenesis of anthrax infection and is speculated to work synergistically with LT (Pezard *et al.*, 1995; Tournier *et al.*, 2005; Cui *et al.*, 2007). We showed that ET suppressed IL-12 and TNF- α in both BALB/c- and B6-derived DC cultures, which is consistent with the results from DC stimulated with mutant anthrax spores (Tournier *et al.*, 2005; Cleret *et al.*, 2006). However, ET also increased cytokine production such as IL-6 in Lp-stimulated B6 DCs and IL-1 β in Lp-stimulated DCs from both strains (Fig. 3). This enhancement is similar to that seen in lung DCs stimulated with anthrax spores (Cleret *et al.*, 2006) and human monocytes stimulated with LPS (Hoover *et al.*, 1994). ET is an adenylate cyclase and increases intracellular cAMP in target cells, thus modulating many physiological processes (Leppla, 1982; Drum *et al.*, 2002). It has been shown that elevated cAMP or its analogs contributed to the increase of IL-6 in human monocytes (Hoover *et al.*, 1994) and the increase of IL-10 in DCs (Kambayashi *et al.*, 2001) and splenocytes (He *et al.*, 2000). Our results suggest that this ET effect may be extended to the production of IL-1 β in Lp-infected BM-DCs.

LT and ET are both encoded on the same plasmid and coexpressed in nature upon exposure to *B. anthracis* infection. Therefore, in addition to individual toxins, we also investigated the effects of combined toxin treatment (Fig. 5). We observed two distinct patterns of response with combination treatment either mimicking only the LT effect on cytokine production, or the combination treatment mimicking the

effect of LT or ET treatment only. Our findings were consistent with the previous work that showed that LT plus ET induced cytokine suppression in BALB/c lung and bone marrow DCs stimulated with mutant spores (Tournier *et al.*, 2005; Cleret *et al.*, 2006); moreover, the attenuated IL-12 response from B6 was also reported by Tournier *et al.* Studies in which stimulated human and mouse DCs treated with spores expressing both toxins also confirmed the suppressive effect of the combination (Brittingham *et al.*, 2005; Tournier *et al.*, 2005; Cleret *et al.*, 2006). Interestingly, however, the speculation that the toxins may work synergistically has not been shown in any of these studies, including our own. The mechanism by which the LT effect dominates over ET is not clear; however, we speculate that under certain conditions of DC activation, LT-based metalloprotease activity is enzymatically more efficient than ET-based adenylyl cyclase activity or the metalloprotease inactivates either the adenylyl cyclase itself or its downstream signaling cascade.

In addition to cytokines, we also studied the effect of LT and ET on DC maturation marker development following stimulation with LPS or Lp. Unlike with toxin effects on viability and cytokines wherein the source of the cells and the type of stimulus contributed to the outcome, with marker expression, these variables had a lesser influence. For example, LT treatment enhanced MHC class II and CD86 expression across all groups, while ET was generally suppressive across all groups for all of the markers. Our results with LPS are at variance with those obtained with splenic DCs (Agrawal *et al.*, 2003), wherein it was reported that all markers were suppressed by LT, while we saw an increase in MHC class II and CD86. Other studies with lung DCs infected with anthrax spores or treated with toxin showed little change in MHC class II and CD86 expression (Cleret *et al.*, 2006), while those with BM-DCs stimulated with anthrax spores in the presence or absence of toxins showed an increase in CD86 similar to what we saw.

In conclusion, the modulating effect of anthrax toxins on DC maturation and function has been reported using culture systems of splenic, lung, and bone marrow cells; microbial stimulation by anthrax and LPS; and cells from toxin sensitive and resistant strains. Our results with BM-DCs from both strains and stimulated with LPS and Lp confirm the relative toxic nature of LT on cells from BALB/c mice; however, we also show that LT and ET can attenuate DC toxicity due to intracellular infection by an agent such as Lp, suggesting the modulation of necrosis and apoptosis by anthrax toxins is dependent upon the relative activity of these processes within the cell. Our results also confirm previous reports that LT suppresses IL-12, IL-6, and TNF- α in LPS-stimulated DCs from BALB/c mice, however, we also show for the first time that LT can increase IL-12 in B6 cells and IL-1 β in cells from both strains. LT also increased the expression of MHC II and CD86, which was not observed in studies using splenic and lung DCs. Regarding the effect of ET, we showed for the first time that it suppressed the homing receptor, CD11c, in response to LPS and Lp stimulation, but increased the production of IL-6 in Lp-stimulated B6 cells as well as IL-1 β in cells from both strains. Together, the data support the conclusion that anthrax toxins are not uniformly suppressive of DC function but rather modulate function up or down depending on variables such as the function tested,

the stimulus used to activate the DCs, and genetic variation in innate immune response mechanisms in the host cell.

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Disclosure Statement

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