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Anthrax Lethal Toxin Has Direct and Potent Inhibitory Effects on B Cell Proliferation and Immunoglobulin Production¹

Hui Fang, Lixin Xu, Trina Y. Chen, Julianne M. Cyr, and David M. Frucht²

Protective host immune responses to anthrax infection in humans and animal models are characterized by the development of neutralizing Abs against the receptor-binding anthrax protective Ag (PA), which, together with the lethal factor (LF) protease, composes anthrax lethal toxin (LT). We now report that B cells, in turn, are targets for LT. Anthrax PA directly binds primary B cells, resulting in the LF-dependent cleavage of the MAPK kinases (MAPKKs) and disrupted signaling to downstream MAPK targets. Although not directly lethal to B cells, anthrax LT treatment causes severe B cell dysfunction, greatly reducing proliferative responses to IL-4-, anti-IgM-, and/or anti-CD40 stimulation. Moreover, B cells treated with anthrax LT in vitro or isolated from mice treated with anthrax LT in vivo have a markedly diminished capacity to proliferate and produce IgM in response to TLR-2 and TLR-4 ligands. The suppressive effects of anthrax LT on B cell function occur at picomolar concentrations in vitro and at sublethal doses in vivo. These results indicate that anthrax LT directly inhibits the function of B cells in vitro and in vivo, revealing a potential mechanism through which the pathogen could bypass protective immune responses. *The Journal of Immunology*, 2006, 176: 6155–6161.

B *acillus anthracis* has several critical virulence factors that are encoded on the plasmids pXO1 and pXO2. The latter plasmid contains the genes required for capsule synthesis, whereas pXO1 encodes anthrax protective Ag (PA),³ lethal factor (LF), and edema factor (EF) (1). Anthrax PA binds to one of two cellular receptors, anthrax toxin receptor-1 or capillary morphogenesis gene-2/anthrax toxin receptor-2 (2–4). Following binding, anthrax PA is cleaved by cellular furin and heptamerizes, forming binding sites for EF and/or LF. The receptor complex is then internalized within endocytotic vessels that are subsequently acidified, transforming the PA heptamer into a pore and allowing intracellular translocation of the active enzymes EF (an adenylate cyclase) and LF (a protease targeting MAPKKs) (5–7).

We have focused our studies on identifying the critical immune cell targets for anthrax LT. The effects of anthrax LT in modulating host macrophage function have been of longstanding interest in the field, as these professional phagocytic cells are critical initial targets of infection (8–10). More recent studies have addressed the pleiotropic effects of anthrax LT. For example, anthrax LT also directly disrupts the function of dendritic cells (11, 12), neutrophils (13–16), and endothelial cells (17, 18). Most recently, we and others demonstrated that T lymphocytes bind and internalize anthrax

LT, which then cleaves and inactivates MAPKKs, thereby disrupting intracellular signaling leading to IL-2, IL-4, and IFN- γ production (19–21). Although survivors of the anthrax bioterrorism attacks of 2001 developed robust humoral responses to infection (22), highlighting the role of B cells in host resistance, a direct role of anthrax LT on B cell function had not been previously addressed.

We herein report that B cells are direct targets for anthrax LT, both in vitro and in vivo, leading to proteolysis of MAPKKs and disrupted downstream signal transduction. In addition, anthrax LT blocks MAPKK-dependent cellular B cell proliferation and IgM production at low/sublethal concentrations. These potent and direct effects on B cell function represent a previously unrecognized mechanism through which anthrax LT could attenuate protective humoral immune responses that are associated with host resistance to infection.

Materials and Methods

Primary B cell isolation and culture conditions

Murine B cells were isolated from the spleens of 8- to 12-wk-old BALB/c mice, in accordance with an animal protocol approved by the Food and Drug Administration Institutional Animal Care and Use Committee. The B cell population was purified using negative selection with the MACS mouse B cell isolation kit, following the manufacturer's suggested protocol (Miltenyi Biotec). The purity of the B cells was evaluated by flow cytometry (>99.6% CD45R/B220⁺). No differences in viability (always >95% viable) or yield trends (consistently around 30 million cells/spleen) were observed in B cells isolated from untreated vs anthrax LT-treated mice.

Primary human B cells were isolated from apheresed PBMC obtained from anonymous donors at the Department of Transfusion Medicine in the Clinical Center, National Institutes of Health as part of a protocol approved by the FDA institutional review board. The lymphocyte fraction was separated by elutriation using standard procedures and kindly provided by the laboratory of Dr. Kathleen Clouse. Human B cells were isolated by positive selection using anti-CD19 immunomagnets, following the manufacturer's suggested protocol (Miltenyi Biotec). Human B cells obtained in this manner were determined to be >95% CD19⁺ by flow cytometry analysis. Human B cells were cultured in complete RPMI 1640 medium (complete RPMI) containing 10% FBS (HyClone), 2 mM L-glutamine (Invitrogen Life Technologies), 10 mM HEPES buffer, 1 mM sodium pyruvate (Quality Biological), and 1% antibiotic/antimycotic (Sigma-Aldrich). Murine B

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³ Abbreviations used in this paper: PA, protective antigen; LT, lethal toxin; LF, lethal factor; LF-M, protease-deficient lethal factor mutant; EF, edema factor; MAPKK, MAPK kinase.

cell culture conditions also included 50 μM 2-ME (Sigma-Aldrich). Cell viability was assessed by trypan blue exclusion.

Reagents and antibodies

Recombinant anthrax PA and LF were purchased commercially and were stored in 1:1 glycerol-water at -20°C (List Biological Laboratories) for *in vitro* studies. Unless otherwise indicated, anthrax LT was administered in excess at concentrations of 2.5 $\mu\text{g}/\text{ml}$ PA and 1 $\mu\text{g}/\text{ml}$ LF. In selected experiments a proteolytically inactive mutant of LF was used as a negative control (E687C substitution in zinc binding site that eliminates enzymatic activity; List Biological Laboratories). LPS (Sigma-Aldrich) was used at a concentration of 25 $\mu\text{g}/\text{ml}$. Pam₃Cys-Ser-(Lys)₄, a synthetic TLR2 agonist, was purchased from Calbiochem and was used at a concentration of 1 $\mu\text{g}/\text{ml}$. Recombinant mouse IL-4 (R&D Systems), anti-mouse CD40 (BD Biosciences), and anti-mouse-IgM (Jackson ImmunoResearch Laboratories) were used for proliferation assays at concentrations of 5 ng/ml, 5 $\mu\text{g}/\text{ml}$, and 5 $\mu\text{g}/\text{ml}$, respectively. FITC-labeled anti-CD45R/B220 (BD Biosciences) and FITC-labeled anti-CD19 (BD Biosciences) were used for flow cytometry analysis of isolated B cells to assess purity.

Anthrax PA binding assays

Purified murine or human B cells were cultured at 4°C for 30 min with FITC-labeled anthrax PA (50 $\mu\text{g}/\text{ml}$; List Biological Laboratories) in the presence or absence of unlabeled anthrax PA (150 $\mu\text{g}/\text{ml}$) to confirm specific binding. Stained cells were then washed with PBS and analyzed by flow cytometry (see below). Unstained cells were analyzed in parallel to establish background levels of autofluorescence.

B cell viability assays

The viability of B cells was assessed using propidium iodide (PI) staining (BD Pharmingen). Briefly, 10^5 cells were washed in PBS at 4°C and then resuspended in 100 μl of binding buffer (0.1% BSA in PBS). PI solution was then added to the cells with gentle vortexing (final concentration 2 $\mu\text{g}/\text{ml}$), and the cells were subsequently maintained for 15 min at room temperature in the dark. Binding buffer (0.4 ml) was then added to each sample. Samples were then analyzed by flow cytometry for PI staining.

ELISA

Primary B cells were cultured in complete RPMI for 4 to 5 h, washed with RPMI 1640, and then stimulated as indicated in the presence or the absence of anthrax LT for 7 days. Cell-free supernatants were assayed for IgM concentration by ELISA using standard methods. Briefly, samples were incubated in 96-well Immulon 4 HBX plates (Fisher), which had been precoated overnight at 4°C with goat anti-mouse κ (2 $\mu\text{g}/\text{ml}$; Southern Biotechnology Associates) in a commercially prepared coating solution (KPL). Following incubation at room temperature for 1 h, the plates were washed and incubated with HRP-conjugated anti-mouse IgM (Southern Biotechnology Associates) for secondary staining. ELISA plates were then washed and incubated with TMB peroxidase substrate. Absorbance at 405 nm was measured using a Dynatech ELISA plate reader. Murine IgM (BD Biosciences) was used to generate standard curves for calculating sample levels of IgM.

Proliferation assays

Murine B cells ($1-2 \times 10^5$) at a concentration of $1-2 \times 10^6$ cells/ml were added in triplicate to 96-well culture plates (Corning Glassworks). Cells were stimulated in the indicated conditions at 37°C in a humidified incubator for 24 h. Subsequently, the cultures were treated for 18 h with 1 μCi of tritiated thymidine (Amersham Biosciences). Cells were lysed, and incorporated tritiated thymidine was measured using a 1205 Betaplate liquid scintillation counter (PerkinElmer Wallac).

Western blotting

Western blotting was performed using standard techniques as previously described (23). Briefly, cells were lysed on ice for 30 min in a buffer containing 20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 1% Triton X-100, and a protease inhibitor mixture. Protein extracts were obtained from centrifuged lysates and were loaded on 4–12% NuPAGE gradient gels (Invitrogen Life Sciences). Following electrophoretic separation and transfer to 0.2 μm nitrocellulose membranes (Bio-Rad), the membranes were analyzed using the following primary Abs: anti-MEK-1 (1/1000; BD Biosciences); anti-MEK-2 and β -Actin (1/250 and 1/1000, respectively; Santa Cruz Biotechnology); anti-p44/42 MAPK, anti-phospho-p44/42 MAPK, anti-phospho-JNK, anti-JNK, anti-phospho-p38 MAPK, anti-p38 MAPK (1/1000; Cell Signaling

Technology). HRP-conjugated anti-mouse (1/2000; Amersham Biosciences), anti-rabbit (1/2000; Amersham Biosciences), or anti-goat (1/5000; Abcam) were used as secondary Abs.

Flow cytometry

Flow cytometry was performed using standard techniques on a FACSCalibur flow cytometer (BD Biosciences). Analysis was performed using Cell Quest Pro software (BD Immunocytometry Systems).

Murine *in vivo* studies

Mice were treated with varying doses of anthrax LT as indicated, using a fixed ratio of LF/PA of 1:2.5. LF and PA were resuspended in PBS and injected *i.p.* into mice in a total volume of 1.0 ml of PBS. As a negative control, selected mice were treated with PBS alone. Mice were sacrificed 3 h after treatment, and spleens were harvested for primary B cell isolation as previously described. Primary B cells were then evaluated for proliferation and IgM production.

Results

Primary murine and human B cells are targets for anthrax LT

In previous studies, we and others showed that anthrax LT has a direct inhibitory effect on CD4⁺ T cells, which are critical modulators of cell-mediated immune responses (19–21). However, as effective humoral responses have been shown to correlate with survival during anthrax infections (24–27), it was also important to determine whether *Bacillus anthracis* modulates Ab production through direct actions on B cells as well. To this end, we studied murine splenic B cells that were purified using negative selection with immunomagnetic beads (routinely >99% CD45R/B220⁺). As shown in Fig. 1A (*left panel*), FITC-labeled anthrax PA bound to murine B cells. Specificity of this interaction was confirmed by coincubation with unlabeled anthrax PA, which competed with FITC-PA binding. In parallel experiments, human B cells were purified from the lymphocyte fraction of elutriated PBMC using positive selection with immunomagnetic beads (routinely >95% CD19⁺). As was observed with murine B cells, human B cells bound FITC-labeled anthrax PA, and coincubation with unlabeled anthrax PA specifically competed with this binding (Fig. 1A, *right panel*).

We next investigated whether murine and human B cells were capable of internalizing the PA/LF toxin complex, ultimately leading to the intracellular release of LF and proteolytic inactivation of MAPKKs. As is shown Fig. 1B, anthrax LT treatment led to the rapid cleavage and degradation of MEK-1 in murine B cells (<30 min), as demonstrated by the disappearance of full-length MEK-1 and corresponding appearance of the cleaved form. Degradation of full-length MEK-2 was apparent within 1–2 h, although the Ab used to detect MEK-2 was directed at the distal N terminus and was not capable of detecting cleaved MEK-2. Anthrax LT also induced the cleavage of MEK-3, MEK-4, and MEK-6, but not MEK-5 (not shown). These results were consistent with a previous study showing that MEK-5 is resistant to the proteolytic activity of anthrax LT (28). In addition, we confirmed that the anthrax LT-dependent cleavage of MAPKKs in B cells requires the proteolytic activity of anthrax LF. In contrast to anthrax LT generated using wild-type LF, anthrax LT generated using proteolytically inactive mutant LF (LF-M) failed to cleave MEK-1 or MEK-2 in murine B cells (Fig. 1C). As has been observed in CD4⁺ T cells, anthrax LT was not directly toxic to murine B cells. Murine B cells treated with anthrax LT at the highest dose used in our studies (1 $\mu\text{g}/\text{ml}$ LF/2.5 $\mu\text{g}/\text{ml}$ PA) showed no decrease in viability compared with untreated control cells for at least 48 h following treatment (Fig. 1D).

The effect of anthrax LT on B cells was not species specific, as anthrax LT treatment of primary human B cells led to degradation of MEK-1 and MEK-2 as well (Fig. 1E). As was observed in murine B cells, the reduction in the levels of full-length human

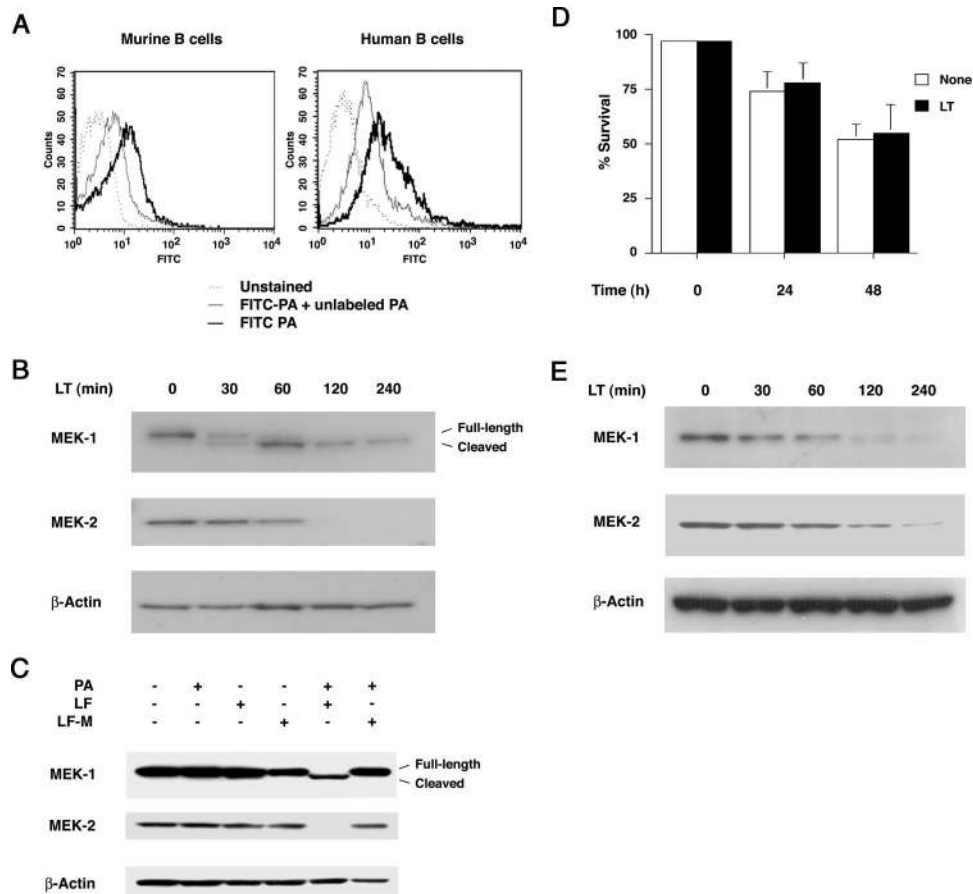


FIGURE 1. Anthrax LT cleaves MAPKKs in primary murine and human B cells. *A*, Purified murine (*left panel*) or human (*right panel*) B cells were stained with FITC-labeled anthrax PA in the presence or absence of unlabeled anthrax PA as indicated in the *Materials and Methods* section. Unstained cells were analyzed to establish background autofluorescence as shown. *B*, Murine B cells were stimulated with anthrax LT (1 $\mu\text{g/ml}$ LF, 2.5 $\mu\text{g/ml}$ PA) for varying time periods as indicated (0–240 min). Protein levels of MEK-1, MEK-2, and β -actin in protein lysates obtained from these cultures were assessed by Western blotting. *C*, Murine B cells were stimulated for 3 h in the presence or absence of anthrax PA, LF, and/or a proteolytically inactive LF mutant protein (LF-M) as indicated (1 $\mu\text{g/ml}$ LF or LF-M, 2.5 $\mu\text{g/ml}$ PA). Protein levels of MEK-1, MEK-2, and β -actin in cell lysates obtained from these cultures were assessed by Western blotting. *D*, Murine B cells were stimulated with or without anthrax LT (1 $\mu\text{g/ml}$ LF, 2.5 $\mu\text{g/ml}$ PA) for varying lengths of time as indicated. B cell viability was assessed using PI staining as described. *E*, Human B cells were stimulated with anthrax LT (1 $\mu\text{g/ml}$ LF, 2.5 $\mu\text{g/ml}$ PA) for varying time periods as indicated (0–240 min). Protein levels of MEK-1, MEK-2, and β -actin in protein lysates obtained from these cultures were assessed by Western blotting. Representative experiments that showed the identical qualitative trend are shown for *A*, *B*, *C*, and *E*. *D*, Data generated from three independent experiments, with error bars indicating interassay SD.

MEK-1 was evident within 1 h of treatment, whereas effects on full-length MEK-2 were delayed (1–2 h). However, in contrast to experiments with murine B cells, the presence of cleaved MEK-1 was difficult to detect in anthrax LT-treated human B cells (only a faint band at 1–2 h), suggesting that the cleaved form might be rapidly degraded in human B cells.

Anthrax LT disrupts MAPKK-dependent signaling in primary murine B cells

We next examined the effect of cotreatment of anthrax LT-treated murine B cells with agonists of the TLRs predicted to be stimulated during anthrax infection. TLR-4 is stimulated by the anthrax anthrolysin O protein, whereas TLR-2 is stimulated by the Gram-positive cell wall components, peptidoglycan and lipoteichoic acid (29, 30). Neither LPS, a TLR-4 agonist (31–33), nor Pam₃-Cys-Ser-(Lys)₄, a synthetic lipopeptide TLR-2 agonist (34), blocked the effect of anthrax LT to induce the degradation of the MAPKKs, MEK-1, or MEK-2 (Fig. 2A). These data suggested that anthrax LT might disrupt MAPKK-dependent TLR signaling and thereby down-modulate host B cell responses to bacterial products from *B. anthracis* that signal through these pathways.

To investigate whether anthrax LT disrupts TLR signaling, we cultured murine B cells for 3 h with anthrax LT and then treated them in the presence or absence of the Pam₃-Cys-Ser-(Lys)₄. Cell signaling to downstream MAPK targets was then assessed by Western blotting signaling. As shown in Fig. 2B, treatment of primary B cells with anthrax LT had no effect on total levels of ERK, JNK, or p38 MAPK but had a substantial blockade effect on Pam₃-Cys-Ser-(Lys)₄ signaling involving these molecules. For example, although robust phosphorylation of p44/p42 ERK was observed in Pam₃-Cys-Ser-(Lys)₄-stimulated B cells in the absence of anthrax LT, phosphorylation of p44/42 was not detected in cells pretreated with anthrax LT (Fig. 2B, *top panels*). Moreover, MAPKK-dependent signal transduction to another family of downstream MAPK substrates, the JNKs, was disrupted by anthrax LT as well. At baseline or after treatment with anthrax LT alone, little to no phospho-JNK was detectable by Western blotting (Fig. 2B, *middle panels*). Stimulation of B cells with Pam₃-Cys-Ser-(Lys)₄ led to the phosphorylation of both p46 JNK and p54 JNK in anthrax LT-free conditions. However, anthrax LT treatment had marked effects on Pam₃-Cys-Ser-(Lys)₄-dependent signaling, blocking the phosphorylation of p46/p54 JNK in response to Pam₃-Cys-Ser-(Lys)₄.

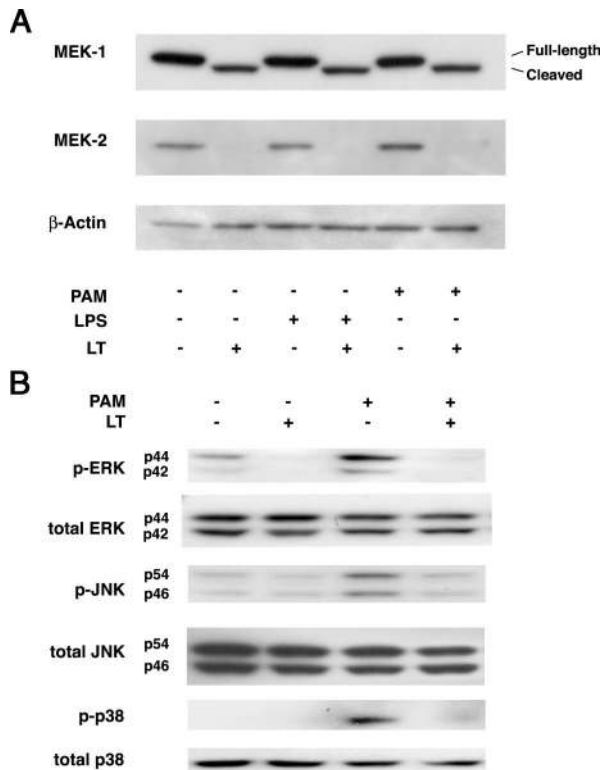


FIGURE 2. Anthrax LT disrupts MAPKK-dependent signaling in murine B cells. *A*, Purified murine B cells were cultured in the presence or absence of anthrax LT (1 μ g/ml LF, 2.5 μ g/ml PA), LPS (25 μ g/ml), and/or Pam₃-Cys-Ser-(Lys)₄ (PAM, 1 μ g/ml) for 24 h as indicated. Protein levels of MEK-1, MEK-2, and β -Actin in protein lysates obtained from these cultures were assessed by Western blotting. *B*, Murine B cells were precultured in the presence or absence of anthrax LT (1 μ g/ml LF, 2.5 μ g/ml PA) for 3 h. Selected cultures were then re-stimulated for 15 min with Pam₃-Cys-Ser-(Lys)₄ (PAM) as indicated. Protein levels of phospho-JNK (p-JNK), total JNK, phospho-ERK (p-ERK), total ERK, phospho-p38 (p-p38), and total p38 in lysates generated from these cultures were assessed by Western blotting using cell lysates. Representative experiments are shown from two (*A*) or four (*B*) independent experiments that showed the identical qualitative trend.

stimulation. Finally, anthrax LT also blocked Pam₃-Cys-Ser-(Lys)₄-dependent signaling to p38 MAPK (Fig. 2*B*, bottom panels). Taken together these data demonstrated that anthrax LT-induced degradation of MAPKKs disrupts signaling to their downstream MAPK substrates following stimulation of B cells with the TLR-2 agonist, Pam₃-Cys-Ser-(Lys)₄.

Anthrax LT blocks proliferation of primary murine B cells

We could not exclude the possibility that residual MAPKK signaling, in combination with other parallel signaling pathways, could overcome the effect of anthrax LT, thereby allowing normal B cell function. It was, therefore, important to evaluate directly the role of anthrax LT in modulating primary functions of B cells, two of which are proliferation and Ab production. We first investigated whether disruption of MAPKK-dependent signaling pathways by anthrax LT would affect the ability of primary B cells to proliferate. Primary murine B cells were stimulated with IL-4, LPS, anti-IgM, anti-CD40, combined IL-4 and anti-CD40, or combined anti-IgM and anti-CD40 in the presence or absence of anthrax LT. As shown in Fig. 3*A*, anthrax LT markedly reduced proliferative responses in all of these conditions.

The most potent single agent inducer of B cell proliferation in our stimulation panel was the TLR-4 agonist, LPS (31–33). In

separate experiments (Fig. 3*B*), we compared the effect of anthrax LT on TLR-4-dependent proliferation with its effect on TLR-2-dependent proliferation, using the synthetic TLR-2 agonist, Pam₃-Cys-Ser-(Lys)₄. In the absence of anthrax LT, treatment with the synthetic TLR-2 agonist, Pam₃-Cys-Ser-(Lys)₄, resulted in robust B cell proliferative responses (>150-fold increase over baseline; Fig. 3*B*). However, similar to its effect on LPS proliferative responses (Fig. 3, *A* and *B*), cotreatment with anthrax LT blocked proliferative responses to Pam₃-Cys-Ser-(Lys)₄. Neither anthrax PA nor LF alone blocked the proliferative responses of B cells stimulated by TLR agonists; only the combination of anthrax PA and LF (anthrax LT) had this anti-proliferative effect. In addition, anthrax LT generated using a protease-deficient LF mutant (LF-M) did not have an inhibitory effect on B cell proliferation, indicating that this effect was dependent upon MAPKK cleavage (Fig. 3*B*). Decreased proliferative responses in anthrax LT-treated B cells were not likely due to lethal toxicity, as no difference in cell viability was detected in cultures treated with or without anthrax LT (Fig. 1*D*).

In separate dose-response experiments, we determined the EC₅₀ of each of the two anthrax LT components in blocking B cell proliferation. As shown in Fig. 3*C*, half-maximal blockade of proliferation was observed at a LF concentration of 1 ng/ml in the presence of nonlimiting concentrations of PA. In the presence of nonlimiting concentrations of LF, half-maximal blockade of proliferation was measured at a PA concentration of 0.25–2.5 ng/ml. These concentrations of LF and PA were \sim 1 Log lower than those required to block IL-2 production in CD4⁺ T cells (20).

Anthrax LT inhibits MAPKK-dependent IgM production by murine B cells

A critical function of B cells is to produce pathogen-specific immunoglobulins during infections. Our studies with anti-IgM indicated that anthrax LT blocks proliferative responses to BCR stimulation. We next sought to determine whether anthrax LT affects the production of IgM in response to the TLR ligands, Pam₃-Cys-Ser-(Lys)₄ or LPS. As shown in Fig. 3*D*, treatment of B cell cultures with either of these TLR ligands markedly enhanced the production of IgM in the absence of anthrax LT. However, treatment of B cell cultures with anthrax LT entirely blocked Pam₃-Cys-Ser-(Lys)₄-induced and LPS-induced IgM production. We next confirmed that the blockade of IgM production by anthrax LT required the cleavage of MAPKK, using a proteolytically inactive LF mutant (LF-M). Anthrax LT generated with LF-M had no effect on TLR-dependent IgM induction (Fig. 3*D*). There was no decrease in the viability of 7-day cultures treated with anthrax LT (1 μ g LF/2.5 μ g PA), indicating that the effect of anthrax LT on IgM production was not likely due to the confounding factor of lethal toxicity (52 \pm 6% viability in untreated cultures vs 55 \pm 4% viability in LT-treated cultures, three independent experiments, not shown graphically). Taken together, these data demonstrated that anthrax LT specifically blocks IgM production in vitro through its proteolytic activity on MAPKK target molecules.

Anthrax LT targets murine B cells in vivo at sublethal doses

We next investigated the effects of anthrax LT on B cells in vivo, using the BALB/c mouse strain, a well characterized animal model for anthrax infection and LT activity (35, 36). Pairs of BALB/c mice were treated with varying amounts of LF and PA as indicated (Fig. 4*A*). Following 3 h of treatment, the mice were sacrificed and B cells isolated from each of the mice. Western blot analysis of B cell protein lysates demonstrated cleavage of MEK-1 and MEK-2 at a dose as low as 8 μ g LF and 20 μ g PA.

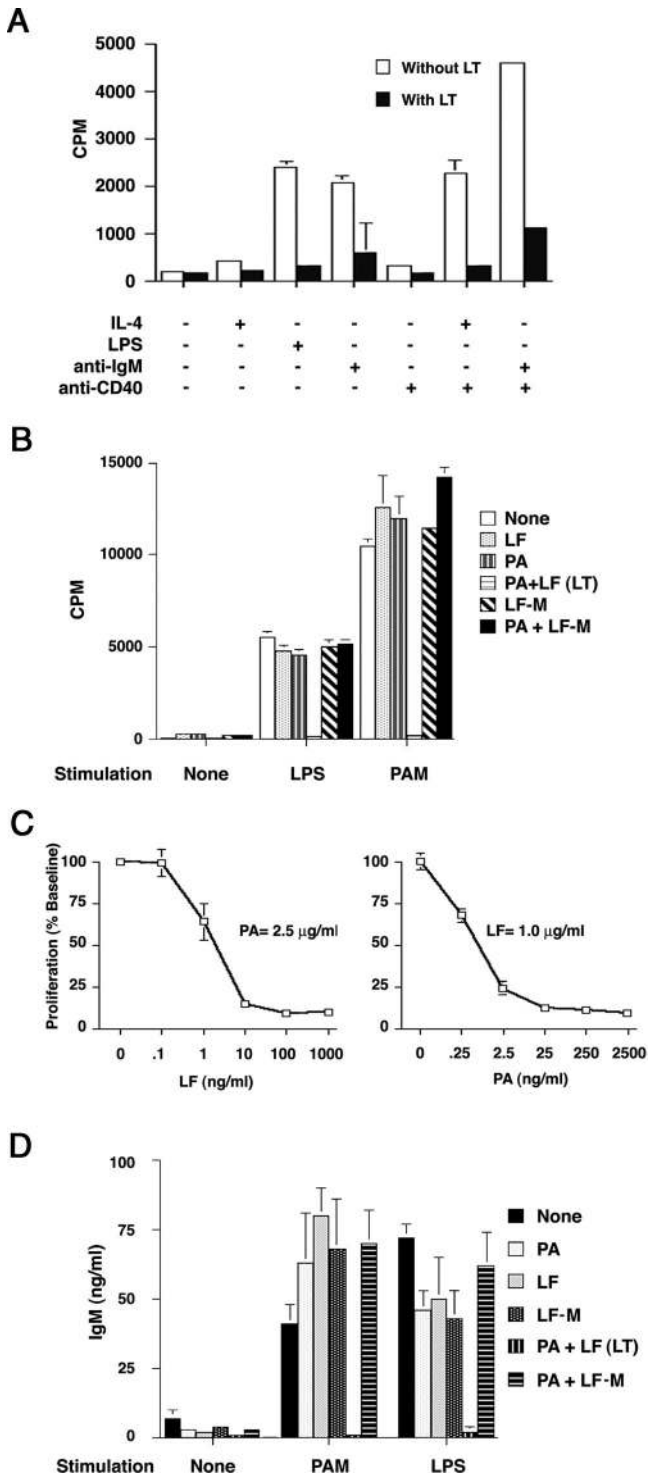


FIGURE 3. Anthrax LT blocks B cell proliferation and IgM production. *A*, Purified murine B cells were cultured with anthrax LT (1 μ g/ml LF, 2.5 μ g/ml PA), IL-4 (5 ng/ml), LPS (25 μ g/ml), anti-IgM (5 μ g/ml), and/or anti-CD40 (5 μ g/ml) as shown. Cellular proliferation was determined using tritiated thymidine assays as described (cpm). *B*, Murine B cells obtained from BALB/c control mice (also used for experiment depicted in Fig. 4*B*) were cultured for 24 h with or without LPS or Pam₃-Cys-Ser-(Lys)₄ (PAM) in the presence or absence of anthrax PA (2.5 μ g/ml), LF (1 μ g/ml), or proteolytically inactive LF mutant (LF-M, 1 μ g/ml) as indicated. Cellular proliferation was determined as described. *C*, Murine B cells were cultured for 24 h with Pam₃-Cys-Ser-(Lys)₄ (PAM) in the presence or absence of varying amounts of LF (PA at fixed, saturating concentrations, 2.5 μ g/ml) or PA (LF at fixed, saturating concentrations, 1 μ g/ml). *D*, Murine B cells were cultured for 7 days with or without LPS or Pam₃-Cys-Ser-(Lys)₄

B cells were subsequently analyzed for proliferation and IgM production from animals treated with the highest dose anthrax LT tested (40 μ g LF, 100 μ g PA). Even when cultured in anthrax LT-free conditions ex vivo, B cells isolated from anthrax LT-treated animals had a severe blockade in TLR-dependent proliferation and IgM production. Whereas B cells isolated from untreated mice proliferated robustly following stimulation with LPS or Pam₃-Cys-Ser-(Lys)₄, TLR-dependent proliferative responses of B cells isolated from anthrax LT-treated mice were almost entirely blocked (Fig. 4*B*). Furthermore, the induction of IgM production by either LPS or Pam₃-Cys-Ser-(Lys)₄ stimulation was completely blocked by prior in vivo treatment with anthrax LT as well (Fig. 4*C*).

We next investigated whether lower, sublethal doses of anthrax LT would suppress B cell function as well. Pairs of BALB/c mice were treated with varying amounts of PA, LF, or LF-M as indicated (Figs. 4, *D* and *E*), and B cells were isolated following 3 h of treatment. Even at treatment doses as low as 1 μ g of LF/2.5 μ g PA, a marked inhibitory effect was observed on ex vivo proliferation (40,092 cpm vs 9,838 cpm). In vivo LT treatment had a slightly less potent effect on B cell IgM production, where substantial effects were first observed at a dose of 8 μ g of LF/20 μ g of PA (69 ng/ml in B cells from PBS-treated animals vs 1 ng/ml in B cells from LT-treated animals). Nevertheless, this dose was still well below the LD₅₀ for anthrax LT administered i.p (35). No effects on ex vivo proliferation and IgM production were observed in B cells obtained from mice treated with combined anthrax PA and LF-M, indicating that these effects of anthrax LT required its protease activity. Taken together, these results demonstrated that anthrax LT has rapid and specific activity in vivo, even at sublethal doses, causing sustained inhibitory effects on B cell function that can be detected in ex vivo cultures.

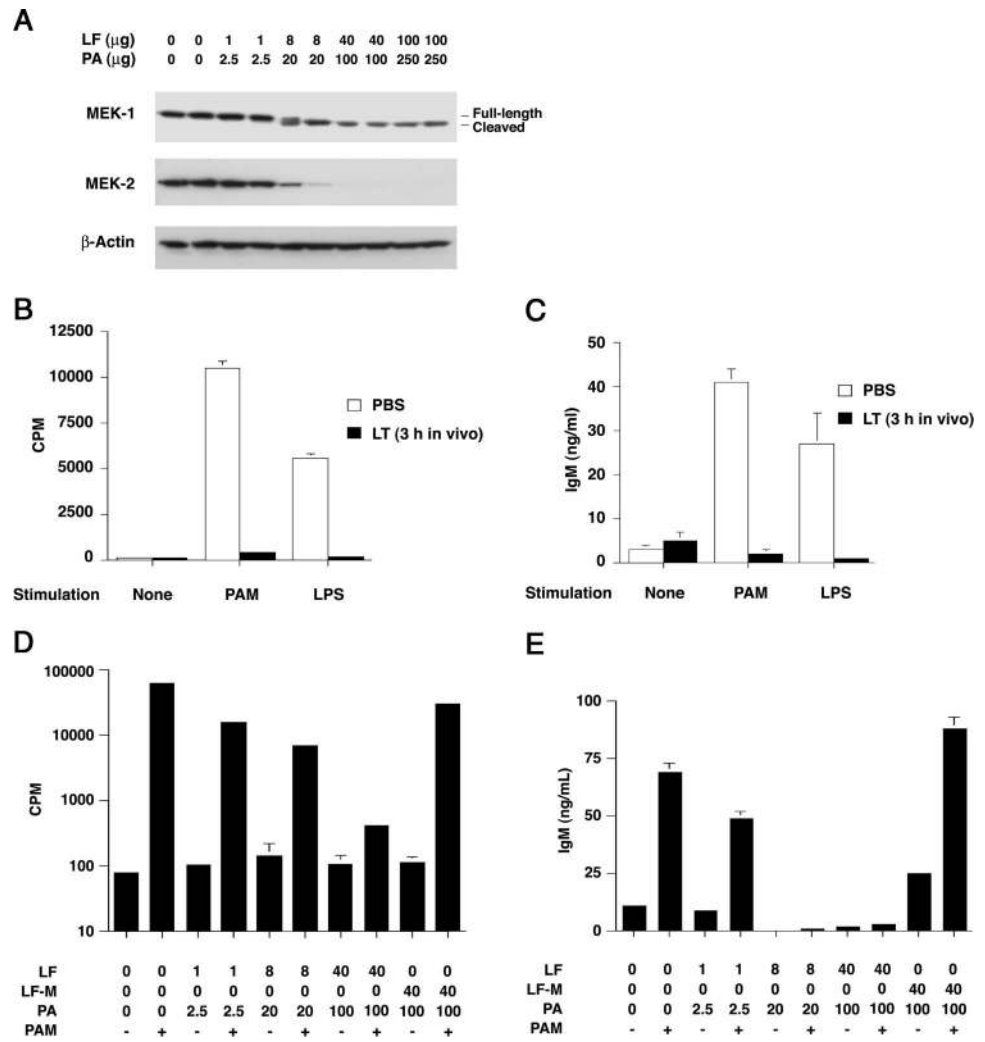
Discussion

In addition to its direct effect on macrophages, the immune cell population that phagocytoses and kills bacterial pathogens such as *B. anthracis*, anthrax LT acts to modulate adaptive immune responses as well. Some of these actions are indirect, resulting from defective costimulatory function of APCs exposed to anthrax LT (11). However, anthrax LT also has direct effects on lymphocytes, the key effector cells of adaptive immunity. In this regard, we were among the first to show that anthrax LT blocks MAPKK-dependent cytokine production in CD4⁺ T cells (19, 20). We herein report that anthrax LT also has direct effects on B cells, where it severely disrupts proliferation and Ig production, critical B cell functions that are associated with host protection during infection.

Primary B cells from both mice and humans bind anthrax PA, allowing intracellular transport of anthrax LF, which then cleaves and inactivates MAPKKs. Because MAPKK-dependent signal transduction pathways are activated by a variety of stimuli (e.g., Ag recognition, cytokines, and TLR ligands), blockade of MAPKK-dependent signaling by anthrax LT leads to pleiotropic effects on B cell function. For example, MAPKKs are known to have a direct role in regulating proliferative responses to B cell

(PAM) in the presence or absence of PA (2.5 μ g/ml), LF (1 μ g/ml), and/or LF-M, 1 μ g/ml. IgM concentrations in cell-free supernatants from these cultures were determined by ELISA. Representative experiments from three (*A* and *C*) or two (*B* and *D*) independent experiments. Error values shown in *A*, *B*, *C* and *D* represent intraassay SD generated from triplicate samples. In each independent experiment, high-dose anthrax LT treatment (1 μ g/ml LF, 2.5 μ g/ml PA) led to at least 80% blockade of LPS- and/or PAM-stimulated proliferation and IgM production.

FIGURE 4. Anthrax LT targets murine B cells in vivo. **A**, Pairs of BALB/c mice were treated with varying amounts of anthrax LF and PA as indicated. As a negative control, two BALB/c mice were treated with vehicle alone (PBS). Following 3 h of treatment, spleens were harvested from sacrificed mice. Purified splenic B cells were then analyzed by Western blotting for MEK-1, MEK-2, and β -actin protein levels. **B** and **C**, B cells isolated from BALB/c mice treated i.p. for 3 h with either anthrax LT (40 μ g LF, 100 μ g PA) or PBS control were cultured in the presence or absence of Pam₃-Cys-Ser-(Lys)₄ (PAM) or LPS. Proliferation was assessed by 24-h tritiated thymidine incorporation (**B**), and IgM production in 7-day cultures was assessed by ELISA (**C**). **D**, In separate experiments, B cells were isolated from pairs of BALB/c mice treated as above (**A**). Purified B cells pooled from pairs of mice from each treatment condition were cultured in the presence or absence of Pam₃-Cys-Ser-(Lys)₄ (PAM). Proliferation was assessed by 24-h tritiated thymidine incorporation (**D**), and IgM production in 7-day cultures was assessed by ELISA. **E**, Representative experiments are shown from at least two independent experiments that showed the identical proportional trend. In each independent quantitative experiment (**B–E**), high-dose anthrax LT led to least 90% blockade of LPS- and/or PAM-stimulated proliferation. Error values shown in **B–E** represent intraassay SD generated from triplicate samples.



(37, 38). Supporting this critical role for MAPKKs in B cell proliferative responses, anthrax LT markedly reduces proliferative responses to a variety of stimulation pathways that would likely be triggered during anthrax infection, including the TLR-2, TLR-4, BCR, and CD40 pathways. Moreover, blockade of TLR-stimulated production of IgM by anthrax LT is consistent with a vital role for MAPKK-dependent signaling in Ig production by naive B cells as well. These observations, along with data demonstrating that anthrax LT has activity against B cells at pM concentrations in vitro and sublethal concentrations in vivo, suggest that this toxin could suppress MAPKK-dependent humoral responses during the early stages of infection.

Important questions remain regarding the role of the direct effects of anthrax LT on B cells. Whether the reduction in Ig production results from a direct effect of anthrax LT on Ig-producing cells or, instead, is a secondary effect reflecting decreased numbers of Ig-producing cells (due to decreased B cell proliferation) is yet to be determined. In addition, it will be informative to perform adoptive transfer experiments to delineate the impact of direct effects on B cells from indirect effects mediated by cellular targets of anthrax LT that provide accessory function for B cells (e.g., T cells or dendritic cells). Moreover, investigations are warranted that address whether alternative stimuli exist that can overcome the blockade on B cell functions caused by anthrax LT. In this regard, it is intriguing to consider the possibility that B cell stimulators that do not require MAPKK-dependent signal transduction could

be used as adjunctive therapies to stimulate endogenous humoral responses.

However, in the near term, our findings have a more immediate relevance to the development of mAbs, polyclonal Abs and other protein and small molecule inhibitors of the anthrax PA toxin component that are currently being studied as therapeutics for anthrax infection (39–47). Despite the great interest in the potential utility of toxin blockade in anthrax therapy, identification of the critical cellular targets for anthrax LT is still an active area of investigation. Our findings demonstrate that B cells are direct targets for anthrax LT, thereby introducing the possibility that therapeutic targeting of anthrax LT could act to clear infection by reversing the blockade on endogenous B cell responses. Because the development of toxin-neutralizing Abs is one of the most important factors associated with host immunity to anthrax infection of humans and other mammals (22, 24–27), this mechanism of action could be critical for the efficacy of potential therapeutics.

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Disclosures

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