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Anthrax Lethal Toxin Enhances TNF-Induced Endothelial VCAM-1 Expression via an IFN Regulatory Factor-1-Dependent Mechanism

Jason M. Warfel*† and Felice D’Agnillo2*

Impaired host defenses and vascular dysfunction are hallmarks of the late, antibiotic-refractory stages of systemic anthrax infection. Anthrax lethal toxin (LT), a key virulence factor of *Bacillus anthracis*, was previously shown to enhance VCAM-1 expression on primary human endothelial cells suggesting a causative link between dysregulated adhesion molecule expression and the poor immune response and vasculitis associated with anthrax. In this study, we report that LT amplification of TNF-induced VCAM-1 expression is driven transcriptionally by the cooperative activation of NF-κB and IFN regulatory factor-1 (IRF-1). LT enhancement of NF-κB phosphorylation and nuclear translocation correlated temporally with a delayed reaccumulation of IκBα, while increased induction of IRF-1 was linked to STAT1 activation. LT failed to augment TNF-induced ICAM-1 or E-selectin expression, two adhesion molecules regulated by NF-κB, but not IRF-1. These results suggest that LT can differentially modulate NF-κB target genes and highlight the importance of IRF-1 in VCAM-1 enhancement. Altering the activity of key transcription factors involved in host response to infection may be a critical mechanism by which LT contributes to anthrax pathogenesis. *The Journal of Immunology*, 2008, 180: 7516–7524.

*Bacillus anthracis*, a spore-forming Gram-positive bacterium, is the causative agent of anthrax. An untreated systemic anthrax infection is usually fatal and mortality remains high even with aggressive antimicrobial therapy. This high mortality and morbidity is generally attributed to the actions of anthrax toxin, a key virulence factor of *B. anthracis*. The toxin consists of three proteins: protective Ag (PA), lethal factor (LF), and edema factor (EF). PA combines with LF to form lethal toxin (LT), while PA and EF form edema toxin (1, 2). PA translocates LF and EF into cells by receptor-mediated endocytosis involving at least two identified cell surface anthrax toxin receptors, ANTXR1 and ANTXR2 (3). LF is a protease that shuts down MAPK signaling by cleaving all of the upstream MEKs except MEK5 (4, 5). EF acts as a calcium/calmodulin-dependent adenylate cyclase that causes a dramatic increase in intracellular levels of cAMP (1, 2, 6). Accumulating evidence indicates that anthrax toxin disrupts innate and adaptive immune responses; however, the mechanisms are not well-defined (5, 7).

Vascular endothelium plays a critical role in innate and adaptive immune responses to infection. Many of the pathological features of anthrax such as vascular leakage, hemorrhages, vasculitis, and the poorly coordinated immune response implicate a disruption of vascular function and integrity during infection (7–9). Several of these vascular pathologies are reproduced in LT-challenged animals suggesting a possible direct interaction of LT with the endothelial lining (7, 10–14). Consistent with this idea, vascular endothelium expresses the highest levels of anthrax toxin receptors (15). Moreover, endothelial exposure to plasma levels of LF and PA during systemic infection can exceed 200 and 1000 ng/ml, respectively, based on animal and human data (16–18). Our laboratory previously reported that LT induces human endothelial barrier dysfunction consistent with the vascular permeability changes accompanying systemic anthrax infection (14). We also reported that LT enhances TNF-induced VCAM-1 expression which correlated with the increased adhesion of primary human monocytes to the endothelial monolayer (13). Similar enhancement of VCAM-1 has been noted with IL-1β and LPS stimulation (our unpublished observations). VCAM-1 plays a central role in directing leukocytes to underlying infected tissue. However, abnormal or dysregulated VCAM-1 activation is linked to vasculitis and inflammatory disorders (19–21). Vasculitis and defective host responses to infection have been linked to anthrax pathogenesis (8, 13, 22).

In this study, we examine the effects of LT on the transcriptional regulation of the VCAM1 gene which contains binding sites in its promoter region for NF-κB, IFN regulatory factor-1 (IRF-1), Sp1, GATA-2, and AP-1 (23–26). We show for the first time that LT enhances cytokine-induced activation of NF-κB and IRF-1, and identified them as key factors in the LT-mediated enhancement of TNF-induced VCAM-1 expression. Dysregulated activation of NF-κB and IRF-1 has been linked to many diseases and inflammatory disorders (27–30). These findings provide new mechanistic insight into how LT may alter immune and vascular function and contribute to the poor host response to anthrax infection.

*Laboratory of Biochemistry and Vascular Biology, Division of Hematology, Center for Biologies Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892; and †Department of Microbiology and Immunology, Georgetown University Medical Center, Washington, DC 20057

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2Address correspondence and reprint requests to Dr. Felice D’Agnillo, Center for Biologies Evaluation and Research, Food and Drug Administration, 29 Lincoln Drive, Building 29, Room 129, Bethesda, MD 20892. E-mail address: felice.dagnillo@fda.hhs.gov

3Abbreviations used in this paper: PA, protective Ag; LF, lethal factor; EF, edema factor; LT, lethal toxin; IRF, IFN regulatory factor; Cq, cycle threshold; ARE, AU-rich element; iNOS, inducible NO synthase.
Materials and Methods
Reagents
PBS, HBSS with calcium and magnesium (HBSS+), and Tris were obtained from Invitrogen. LF, PA, and mutant LF<sub>Em67C</sub> were provided by Dr. S. H. Leppala (National Institutes of Health, Bethesda, MD) (31, 32). Toxin proteins were diluted in sterile PBS before cell treatment. All other reagents were purchased from Sigma-Aldrich.

Antibodies
Mouse IgG1 mAbs to VCAM-1, ICAM-1, and E-selectin were purchased from BD Biosciences. Rabbit polyclonal Abs specific for IκBα, NF-κB, p65, p38, JNK, ERK, and the phosphorylated forms of p65 (pS536), p38 (pT180/Y182), JNK (pT183/Y185), and ERK (pT202/Y204) were purchased from Cell Signaling Technology. Mouse mAbs specific for phosphorylated STAT1 (pY701) and the total form of STAT1α were obtained from Invitrogen. All other rabbit IgG polyclonal Abs were purchased from Santa Cruz Biotechnology.

Endothelial cell culture and treatment
Primary human coronary artery endothelial cells were obtained from Cambrex and cultured as described previously (14). Cells were treated with 0.2 ng/ml TNF-α and/or 100 ng/ml LF, 500 ng/ml PA alone or in combination. For LF concentration-dependent studies, LF (1, 10, 100 ng/ml) were combined with a constant PA concentration (500 ng/ml). For mutant LT, cells were cotreated with 100 ng/ml LF<sub>Em67C</sub> and 500 ng/ml PA.

Real-time PCR
RNA was collected using the RNeasy Mini kit (Qiagen) and converted to cDNA using the TaqMan Fast Universal 2× PCR Master Mix (No AmpErase UNG) and TaqMan gene expression assays for GAPDH (Hs09999905_m1), ICAM1 (Hs00164932_m1), IRF1 (Hs00233698_m1), SELE (Hs00174057_m1), and VCAM1 (Hs00174239_m1) (Applied Biosystems). Reactions were performed in triplicate and run on the Applied Biosystems 7900HT real-time PCR system. Fold gene expression was calculated using the 2<sup>−ΔΔCT</sup> method using GAPDH as the reference gene (33), where Ct = the cycle threshold.

RNA stability
Cells were incubated with 0.2 μg/ml actinomycin D after a 12-h treatment with TNF alone or in combination with LT. RNA was collected just before actinomycin D addition and at 30, 60, 90, and 120 min thereafter. Real-time PCR was used to analyze mRNA content as described above. Data were normalized to GAPDH mRNA, and presented relative to the expression level at the initial time point.

Preparation of whole cell, nuclear, and cytoplasmic extracts
Cells were lysed in ice-cold RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Igepal-630, 0.5% deoxycholate, 1 mM EDTA) containing protease inhibitor mixture (Calbiochem), 1 mM NaF, and 1 mM sodium orthovanadate. Following centrifugation, whole cell supernatants were collected and stored at −80°C. Cytoplasmic and nuclear fractions were isolated using hypotonic buffer and high salt buffer, respectively, containing protease inhibitor mixture, 1 mM NaF, and 1 mM sodium orthovanadate as previously described (34). Protein concentrations were quantitated using the BCA (Pierce) or Quanti-IT protein assays (Invitrogen).

Western blotting
Reduced samples (4–6 μg) were run on NuPAGE 4–12% Bis-Tris gels in MOPS SDS running buffer. Proteins were transferred to polyvinylidene difluoride membranes and blocked for 1 h in TBS containing 0.1% Tween 20 (TBST) with 5% nonfat dry milk or 5% BSA for phosphospecific Abs. Membranes were then incubated in TBST containing 1% BSA with specific primary Ab followed by HRP-conjugated secondary Ab. Signal was detected on HyperECL film with the ECL Plus chemiluminescence kit (GE Healthcare). For phosphorylated proteins, blots were stripped and reprobed for total protein. Otherwise, blots were stripped and reprobed for loading controls using tubulin for whole cell and cytoplasmic extracts or lamin A/C for nuclear extracts. Densitometry analysis was performed using Image J software (National Institutes of Health, Bethesda, MD) and normalized to lamin A/C content.

EMSA
Nuclear extracts were prepared as described above. Unlabeled and 5′ biotinylated oligonucleotides were synthesized as follows to match the transcription factor binding sites for each gene (Food and Drug Administration Core Facility, Bethesda, MD): VCAM1 NF-κB-binding motif (κB): 5′-TGCCCTGGTTTCCCTGGAAGGATTTCCCTGCCG-3′; VCAM1 IRF-1 response element (5′-GCACAGACTTCTATTTACCTCC-3′); ICAM1 κB (5′-CTTTAGCTGGAAAAATCCGGAGCTGA-3′); and SELE κB site 3 (5′-AGGCCATGGGGATTCCTTATAGCG-3′). Transcription factor-binding sites are underlined for each probe. EMSAs were performed with the Lightshift Chemiluminescent EMSA kit (Pierce Biotechnology) according to the manufacturer’s instructions except for IRF-1 which used a different binding buffer described previously (35). For supershift reactions, lysates were preincubated with 2 μg of specific Ab for 10 min before addition of labeled probe.

Statistical analysis
Data are reported as means ± SE for replicate experiments. Statistical analysis was performed by ANOVA with the post-hoc Student t test using the JMP (version 5.1) software (SAS Institute). For real-time PCR experiments, the ΔC<sub>T</sub> value (C<sub>T</sub> of reference gene − C<sub>T</sub> of experimental gene) was used for statistical analysis. A value of p < 0.05 was considered statistically significant.

Results
LT enhances TNF-induced VCAM-1 mRNA and protein expression
To determine the effect of LT on VCAM-1 expression, primary human coronary artery endothelial cells were treated with LT alone or in combination with TNF and analyzed by cell-based ELISA and immunofluorescence. LT alone did not increase VCAM-1 expression at early time points and the apparent increase in VCAM-1 at 24 and 36 h did not reach statistical significance (Fig. 1A). TNF induced significant VCAM-1 by 4 h which peaked between 8 and 12 h and declined by 24 and 36 h. Consistent with our previous findings in human lung endothelial cultures (13), LT enhanced TNF-induced VCAM-1 expression by >10% at 8 h, >30% at 12 h, and >85% at 24 and 36 h. Importantly, the disappearance rate of surface-bound VCAM-1 from TNF-treated and LT-cotreated cells was similar between 12 and 36 h, suggesting that enhanced VCAM-1 is not mediated by LT inhibition of surface shedding. LT enhancement was LF-concentration dependent in the presence of 500 ng/ml PA, and treatment with the individual toxin components, LF or PA, did not augment VCAM-1 (Fig. 1B). Immunofluorescence analysis showed that TNF induced strong VCAM-1 expression on a subpopulation of the cells at 24 h, while cotreatment with LT produced a more intense and uniform expression (Fig. 1C).
VCAM1 transcription was LF concentration dependent in both nonstimulated and TNF-stimulated cells (Fig. 2B). RNA stability experiments with actinomycin D showed that VCAM1 mRNA is similarly stable in TNF-treated and LT-cotreated cells (Fig. 2C). Taken together, these results suggest that LT enhances VCAM-1 expression via a mechanism that augments VCAM1 gene transcription.

**LT increases TNF-induced NF-κB activation and nuclear translocation**

We next examined the transcriptional regulation of the VCAM1 gene by analyzing the activation of NF-κB as indicated by phosphorylation of the p65 subunit and nuclear localization (36). LT alone did not induce phosphorylation of p65 NF-κB (Fig. 3A). TNF-treated and LT-cotreated cells showed a similar degree of p65 phosphorylation at 10 min (data not shown). However, p65 phosphorylation was greater in LT-cotreated cells compared with TNF alone at 6 and 12 h (Fig. 3A). Fig. 3B shows that LT alone had no detectable effect on nuclear translocation of p65 NF-κB. TNF increased nuclear translocation of NF-κB at 6 and 12 h, while cotreatment with LT significantly augmented NF-κB translocation at both time points (Fig. 3B). By 18 h, there was no detectable difference between nuclear NF-κB of TNF-treated and LT-cotreated cells (data not shown). Immunofluorescence analysis corroborated the enhanced nuclear expression of p65 NF-κB in LT-cotreated cells compared with TNF alone (Fig. 3C). EMSA analysis of nuclear extracts showed increased binding of a single NF-κB complex to the VCAM1 promoter in TNF-treated cells, and marked enhancement of the NF-κB complex in LT-cotreated cells at 12 h (Fig. 3D). Consistent with previous reports that TNF induces specific binding of the p50/p65 heterodimer to the VCAM1 promoter (26, 37), the single band in the cotreated cells was supershifted by a p50 Ab while a p65 Ab reduced band intensity. This suggests that LT specifically enhances binding of p50/p65 to the VCAM1 promoter.

We also analyzed the expression of IkBα, a NF-κB-regulated protein that functions in a negative feedback loop to control the intensity and duration of NF-κB activation. Upon inflammatory stimulation, IkBα is rapidly degraded allowing NF-κB to translocate to the nucleus. As IkBα reaccumulates in the cell, NF-κB is gradually excluded from the nucleus. LT alone had no effect on
LT enhances TNF-induced IRF-1 expression

IRF-1 is an inducible transcription factor required for maximal stimulation of the VCAM1 gene (26). To determine whether IRF-1 participates in LT-mediated VCAM-1 enhancement, we examined the nuclear expression of IRF-1. Fig. 4A shows that LT alone induced a detectable increase in IRF-1 expression at 6 and 12 h, while increased IRF-1 expression in TNF-treated cells was markedly augmented by LT cotreatment. Immunofluorescence analysis corroborated the enhanced nuclear expression of IRF-1 in LT-cotreated cells compared with TNF alone (Fig. 4B). EMSA analysis of nuclear extracts showed marked enhancement of IRF-1 binding to the VCAM1 promoter in LT-cotreated cells at 12 h (Fig. 4C). The IRF-1 band disappeared when the binding reactions were preincubated with an IRF-1 Ab. Consistent with these data, LT alone produced a detectable increase in IRF1 mRNA at 12 h (p = 0.053) (Fig. 5, A and B). TNF stimulated significant increases in IRF1 transcript at 6 and 12 h that declined to near basal levels by 24 h. Notably, cotreatment with LT significantly enhanced IRF1 expression at 6 and 12 h (1.5- to 2-fold vs TNF) in a LT concentration-dependent manner (Fig. 5, A and B). LF or PA, or the inactive LT mutant in the presence or absence of TNF, did not affect IRF1 expression (Fig. 5B). RNA stability experiments showed that IRF1 mRNA is similarly stable in TNF-treated and LT-cotreated cells (Fig. 5C). These results reveal a close temporal and concentration-dependent correlation between the LT-mediated up-regulation of IRF-1 and the enhancement of VCAM-1.

To further investigate the mechanism underlying the up-regulation of IRF1, we examined the transcriptional regulation of the IRF-1 promoter which contains binding sites for NF-κB and STAT1 (38). Having already established that LT enhances NF-κB activation, we considered the possible involvement of STAT1 in IRF-1 induction. Western blot analysis of whole cell lysates showed that LT alone did not induce phosphorylation of STAT1 (pY701) (Fig. 5D). TNF induced detectable STAT1 phosphorylation at 6 and 12 h, while cotreatment with LT markedly augmented STAT1 activation. Importantly, activated STAT1 was preferentially localized to the nucleus (data not shown). These data support a role for STAT1 in the LT-mediated IRF-1 enhancement.

Materials and Methods

Cells were treated with medium alone, or medium containing LT, TNF, or both as described in Materials and Methods. Whole cell lysates were immunoblotted for p536 p65 NF-κB. Three blots were analyzed. B, Cytoplasmic and nuclear extracts were immunoblotted for p65 NF-κB. At least three blots were performed for each time point. Densitometry for nuclear p65 was normalized to lamin A/C content and presented as arbitrary units relative to 6-h LT-cotreated cells. C, Cells were grown on glass coverslips, treated for 12 h, and analyzed by p65 NF-κB immunofluorescence. Representative images are shown. D, NF-κB-binding activity was determined by EMSA after 12 h treatment as described in Materials and Methods. Abs used for supershift assays are indicated below the specific lane by p50 or p65. Three sets of binding reactions were performed. Arrowhead indicates level of the band supershifted by Ab to p50. E, Whole cell lysates were immunoblotted for IκBα. Two blots were analyzed; c, cytoplasmic; n, nuclear. **, p < 0.05 vs TNF alone.
LT does not enhance nuclear localization of Sp1, AP-1, or GATA-2

To determine whether other VCAM1-regulating transcription factors contribute to LT-induced VCAM-1 enhancement, we analyzed the nuclear expression of Sp1, GATA-2, and AP-1 by Western blot (23–25). LT or TNF alone or in combination did not alter the expression of Sp1 (Fig. 6A). LT slightly reduced GATA-2 expression in nonstimulated cells at 12 h, and suppressed GATA-2 expression in TNF-treated cells at 6 and 12 h. LT had no effect on the expression of c-Fos, a component of AP-1, in nonstimulated cells and did not alter TNF-induced c-Fos expression at 12 h. Notably, LT markedly suppressed nuclear c-Jun, the other component of AP-1, in nonstimulated and TNF-stimulated cells. We reasoned that LT suppression of c-Jun is likely caused by LT inhibition of JNK, which has previously been shown to stabilize c-Jun protein via phosphorylation (39). In support of this hypothesis, LT inhibited basal and TNF-induced activation of JNK (Fig. 6B). Fig. 6B also shows that LT shuts down ERK and p38, key activators of among others, the transcription factors c-Fos and ATF-2, respectively (40). Overall, the lack of a LT-mediated increase in Sp1, GATA-2, and AP-1 imply that these factors do not play a major role in VCAM-1 enhancement by LT.

LT does not enhance TNF-induced ICAM-1 or E-selectin expression

To distinguish between the roles of NF-κB and IRF-1 in VCAM-1 enhancement and to assess whether LT modulates the activation other adhesion molecules, we examined the effect of LT on the expression of ICAM-1 and E-selectin, two key endothelial adhesion molecules that are regulated by NF-κB, but not IRF-1. LT alone produced minor increases in ICAM1 transcription at 12 h and protein at 24 h that did not reach statistical significance (Fig. 7, A and B). TNF-induced ICAM1 transcription and protein expression were not significantly enhanced by LT cotreatment. For E-selectin, TNF induced significant SELECT transcription at 6 h which declined substantially by 12 h and reached basal levels by 24 h. In marked contrast to VCAM-1, LT suppressed TNF-induced SELECT transcription at each time point by >65% and reduced TNF-induced E-selectin protein expression at 6 h (Fig. 7, C and D).

To determine whether gene-specific differences in NF-κB-promoter interactions played a role in the lack of a LT-mediated increase of TNF-induced ICAM-1 and E-selectin expression, we analyzed NF-κB binding to the ICAM1 and SELE promoter sites. The ICAM1 promoter, which differs from the consensus p50/p65-binding consensus sequence, binds two separate NF-κB complexes (41, 42). The upper complex was detectable in control lysates and appeared enhanced by LT alone at 12 h (Fig. 7E). In TNF-treated cells, the upper complex was enhanced and the lower complex was induced. Interestingly, the lower complex was enhanced by LT cotreatment, while the upper complex was slightly decreased. The p50 Ab completely supershifted the lower band, while the upper band was still detectable. The p65 Ab disrupted the lower band completely and nearly completely disrupted the upper band (Fig. 7E). These data indicate that the lower complex was likely the p50/p65 heterodimer while the upper complex may consist of either a p65 homodimer or a p65/c-Rel heterodimer as previously reported (41, 42). With the SELE promoter, LT enhanced TNF-induced NF-κB-binding activity at 6 h (Fig. 7F). The band was supershifted by the p50 Ab and disappeared in the presence of the p65 Ab. These data indicate that, similar to the VCAM1 promoter, LT increases binding of the NF-κB p50/p65 heterodimer to ICAM1 and SELE promoters. These findings suggest that the inability of LT to augment TNF-induced ICAM-1 and E-selectin expression, despite increased NF-κB activity, may be driven by the inhibitory effect of LT on other ICAM1 or SELE-regulating transcription factors. Indeed, as shown earlier, LT down-regulates c-Jun (Fig. 6A), a critical component of AP-1 and c-Jun/ATF-2 which are necessary for the expression of ICAM-1 and E-selectin, respectively (43, 44).

Discussion

Many pathogenic bacteria produce toxins that amplify or suppress the host immune response by altering cell signaling or transcriptional responses. Evidence to date suggests that LT disrupts innate and adaptive immune responses during anthrax infection, in part, by down-regulating cytokine production through cleavage of MEK proteins and subsequent suppression of MAPK signaling (1, 2, 5, 7). This has generally promoted the idea that LT functions as a suppressor of inflammatory gene expression. However, this interpretation may need to be extended in light of our present findings that LT enhances proinflammatory activation of the VCAM1 gene.
We found that the combination of LT and TNF leads to cooperative activation of NF-κB and STAT1, promoting the increased expression of IRF-1, which in conjunction with NF-κB increases VCAM1 gene expression. To our knowledge, this is the first report that documents LT-mediated activation of NF-κB, STAT1, and IRF-1, three major regulators of innate and adaptive immune gene expression.

NF-κB is essential for the transcription of VCAM1, while IRF-1 is necessary for maximal induction of this gene (26). Previous studies have implicated the coactivation of NF-κB and IRF-1 in the synergistic expression of VCAM-1, CD40, IFN-β, MHC class I, and inducible NO synthase (iNOS) (35, 45–48). For some of these genes, including VCAM1 and the iNOS-encoding gene NOS2A, the mechanism for increased promoter activity appears to involve a physical interaction between IRF-1 and NF-κB (26, 45, 46, 48). Interestingly, iNOS was recently shown to be up-regulated in baboons exposed to B. anthracis spores supporting the hypothesis that these interactions may be relevant in vivo (49).

Dysregulated adhesion molecule expression is a feature of vasculitis and can lead to abnormal recruitment of leukocytes to the endothelium and disruption of host responses to infection (19–21). To our knowledge, the expression of endothelial adhesion molecules during an anthrax infection has not been investigated. However, vasculitis has been reported in clinical cases and experimental models of anthrax (8, 22, 50). Intense perivascular infiltrates and vasculitis were noted in cutaneous anthrax patients of the 2001 bioterrorism attack (22). Grinberg et al. (8) identified vasculitis in the victims of the Sverdlovsk inhalational anthrax outbreak and hypothesized that vasculitis may contribute to the extensive hemorrhage that is a hallmark of disseminated anthrax infections. Our present findings may provide insight on the possible role of LT in the vasculitic pathology of anthrax.

Our data show that LT enhanced the nuclear expression of IRF-1 which correlated with increased binding of this factor to its VCAM1 promoter site. Consistent with IRF-1 being a prerequisite for LT enhancement of VCAM1, we found that 1) up-regulation of IRF-1 by LT cotreatment at 6 h preceded significant enhancement of VCAM1 transcription observed at 12 h, 2) the magnitude
of increased IRF-1 synthesis correlated with the level of enhancement of VCAM-1, and 3) TNF-induced E-selectin and ICAM-1 expression, two molecules regulated by NF-κB, but not IRF-1, were not augmented by LT. We attributed the enhanced induction of IRF1 to the increased activation of NF-κB and STAT1 which regulate the IRF1 promoter via a κB and a composite GAS/κB-binding site (38). Our finding that TNF alone or in combination with LT induced STAT1 phosphorylation was surprising given that STAT1 is typically activated by JAKs upon binding of IFN family molecules to cell surface receptors (51). However, some studies have reported that STAT1 phosphorylation can be induced by TNF within 5–15 min by direct interaction of TNFR-1 and Jak2, or alternatively after several hours by a mechanism involving autocrine stimulation of IFN receptors by increased production of endogenous IFN-γ or IFN-β (52–54). The latter mechanism may explain the minor TNF-mediated activation of STAT1 in our system given that STAT1 phosphorylation was observed at 6 and 12 h (Fig. 5D), but not after 10 min (data not shown). At both of the later time points, LT markedly increased STAT1 phosphorylation, but whether this enhancement is due to amplification of the same pathway activated by TNF or via a separate mechanism is not yet known.

Despite the increased activation and binding of NF-κB to SELE and ICAM1 promoters, we did not observe an increase in SELE or ICAM1 transcription suggesting that inhibition of other transcription factors may account for the differential regulation of these genes. In accordance, we found that LT down-regulates the expression of c-Jun, a key component of both AP-1 and c-Jun/ATF-2 transcription factors, which play essential roles in the maximal expression of ICAM-1 and E-selectin (43, 44). Though the VCAM1 promoter also contains an AP-1-binding site, it is not required for efficient VCAM1 expression (23, 37). We attributed the down-regulation of c-Jun to the LT-mediated inactivation of JNK signaling, which plays a central role in stabilizing c-Jun (39). These findings suggest that the differential effect of LT on inflammatory gene expression may depend on the specific set of transcription factors that regulate a particular gene and the resulting balance between the activation or inhibition of these factors.

The MAPK-signaling pathways play an essential role in the induction of many genes involved in inflammation via their involvement in: 1) direct activation of transcription factors (40), and 2) regulating mRNA stability of intrinsically unstable inducible genes which possess sensitive AU-rich elements (AREs) in the 3′ untranslated regions of their mRNA (55). In this regard, LT inhibition of MAPK signaling was previously implicated in the destabilization of IL-8 mRNA and reduction in endothelial IL-8 production (56). In contrast to those findings, LT did not alter the stability of the ARE-containing VCAM1 and IRF1 transcripts in our system. However, these differences may not be completely surprising given the varied regulation of ARE-containing transcripts (55, 57). In regard to the significant debate about whether MAPK activation is required for VCAM1 induction (13, 43, 58–61), we provide clear evidence that LT enhanced TNF-induced VCAM1 despite complete suppression of functional MAPK signaling. In a previous study, we showed that MAPK inhibitors did not replicate the effect of LT on TNF-induced VCAM1 expression (13). Together, these observations suggest that functional MAPK signaling is not required for VCAM1 enhancement.

The present findings are important in the context of B. anthracis infections given that spore or bacterial challenge studies in nonhuman primates and rodents have shown increased induction of proinflammatory cytokines including TNF, IL-1β, and IL-6 (49, 62–64). However, it should be noted that intravascular injections of purified LT are lethal in certain murine and rat strains via processes that are independent of proinflammatory cytokine production suggesting that LT itself can trigger some of the pathologies of anthrax (10, 12). The present study provides evidence that LT alone can increase VCAM1 transcription albeit on a much lower scale and with delayed kinetics compared with TNF, suggesting that LT can impact transcriptional pathways independent of TNF. Along these lines, we previously reported that LT alone induces endothelial barrier dysfunction (14), and recently found this effect can be significantly accelerated in the presence of proinflammatory...
cytokines (our unpublished data). The present study did not identify any new LT cleavage target that could account for the series of molecular events described. To date, the only known substrates of LF are the members of the MEK family (MEK1–7, except MEK5). It is conceivable that the LT-induced events described in this study are caused by disruption of MEK functions that are unrelated to the downstream activation of MAPKs. However, our previous findings would argue against a direct role for MEK1 or MEK2 in these events given that a specific MEK1/2 inhibitor, U0126, was unable to reproduce VCAM-1 enhancement (13). A more complete understanding of both the early and delayed effects of LT will help explain the impaired immune responses and vascular pathologies observed during anthrax infection and possibly lead to more effective treatment strategies.

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Disclosures
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References


