Mitochondrial Proteins Bnip3 and Bnip3L Are Involved in Anthrax Lethal Toxin-induced Macrophage Cell Death*

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Anthrax lethal toxin (LeTx) induces rapid cell death of RAW264.7 macrophages. We recently found that a small population of these macrophages is spontaneously and temporally refractory to LeTx-induced cytoxicity. Analysis of genomewide transcripts of a resistant clone before and after regaining LeTx sensitivity revealed that a reduction of two closely related mitochondrial proteins, Bcl-2/adenovirus E1B 19-kDa interacting protein 3 (Bnip3) and Bnip3-like (Bnip3L), correlates with LeTx resistance. Down-regulation of Bnip3 and Bnip3L was also found in “toxin-induced resistance” whereby sublethal doses of LeTx induce resistance to subsequent exposure to cytolytic toxin doses. The role of Bnip3 and Bnip3L in LeTx-induced cell death was confirmed by showing that overexpression of either Bnip3 or Bnip3L rendered the resistant cells susceptible to LeTx, whereas down-regulation of Bnip3 and Bnip3L in wild-type macrophages conferred resistance. The down-regulation of Bnip3 and Bnip3L mRNAs by LeTx occurred at both transcriptional and mRNA stability levels. Inhibition of the p38 pathway by lethal factor was responsible for the destabilization of Bnip3/Bnip3L mRNAs as confirmed by showing that p38 inhibitors stabilized Bnip3 and Bnip3L mRNAs and conferred resistance to LeTx cytoxicity. Therefore, Bnip3/Bnip3L play a crucial role in LeTx-induced cytotoxicity, and down-regulation of Bnip3/Bnip3L is a mechanism of spontaneous or toxin-induced resistance of macrophages.

Anthrax is often a fatal bacterial infection that occurs after inhalation of endospores of Bacillus anthracis, a Gram-positive soil bacterium (1). Endospores germinate within phagocytes becoming metabolically active vegetative bacteria (2, 3) that are released from the cells, multiply in the lymphatic system, and enter the bloodstream, causing severe septicemia and toxemia (1, 4). Several virulence factors are encoded in B. anthracis plasmids including the anthrax lethal toxin (LeTx) and edema toxin, which contribute to the survival of vegetative bacteria and toxemia (5–7). LeTx and edema toxin are binary A-B toxins comprising protective antigen (PA) and lethal factor (LF) or edema factor, respectively. PA is a molecular transporter allowing receptor-mediated entry and release of LF or edema factor into the cytosol. Edema factor has adenylate cyclase activity (8), whereas LF is a zinc metalloprotease that cleaves the N-terminal end of Mek1 to -7, except Mek5, resulting in the inactivation of these kinases. LF also rapidly induces the release of interleukin-1β and interleukin-18 likely through activation of caspase-1 (9). LeTx and edema toxin by themselves are toxic to animals, but both together further enhance toxin lethality (6, 7, 10).

Macrophages are the first immune cells encountering the spores (11) and key innate immune cells defending against the infection (12–14). However, LeTx secreted from germinating bacteria induces necrotic (15–18) and/or apoptotic (19) cell death of macrophages, which can contribute to the survival of the vegetative bacteria (3, 20–22). LeTx also mediates animal death after bacterial clearance by antibiotics in experimental infections with spores or live bacteria (23). Although the cytotoxic effects of LeTx on macrophages are not the only mediator for the LeTx toxemia (24, 25), susceptibility of macrophages to LeTx contributes to the overall sensitivity to LeTx in vitro (26, 27) and in vivo (28).

Macrophages from different strains of inbred mouse exhibit striking differences in the susceptibility and cell death characteristics. For example, LeTx causes rapid necrotic cytolyis in macrophages originated from 129s/1, C3H/HeJ, and BALB/c, whereas slow apoptosis-like cell death is found in macrophages from DBA/2, AKR, and C57BL/6 mice (18, 29, 30). The mechanism of rapid cytolyis in susceptible macrophages has been enigmatic. Because efficient Mek cleavage by LeTx is present in LeTx cytolyis-resistant macrophages and many other cell types without apparent cell death (18, 31, 32), the physiological importance of the Mek proteolysis relative to macrophage cell death by LeTx cannot be conclusively established. A recent

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4 The abbreviations used are: LeTx, anthrax lethal toxin; PA, protective antigen; LF, lethal factor; TIR, toxin-induced resistance; Bnip3, Bcl-2/adenovirus E1B 19-kDa interacting protein 3; Bnip3L, Bnip3-like; Jnk, c-Jun N-terminal kinase; Erk, extracellular signal-regulated kinase; Mek, mitogen-activated protein kinase/extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MOPS, 4-morpholinepropanesulfonic acid; siRNA, small interfering RNA; GFP, green fluorescent protein; SB, SB202190; BH3, Bcl-2 homology 3; Bnip3, Bcl-2/adenovirus E1B 19-kDa interacting protein 3; Bnip3L, Bnip3-like; Jnk, c-Jun N-terminal kinase; Erk, extracellular signal-regulated kinase; Mek, mitogen-activated protein kinase/extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MOPS, 4-morpholinepropanesulfonic acid; siRNA, small interfering RNA; GFP, green fluorescent protein; SB, SB202190; BH3, Bcl-2 homology 3; ARE, AU-rich element.
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genetic study investigating LeTx sensitivity trait loci in different strains of mice identified Nalp1b as a host factor that confers rapid LeTx cytotoxicity (33). The gene product, a NACHT-LRR and pyrin domain-containing protein (NALP1), is a member of the nucleotide-binding oligomerization domain-like receptor family proteins involved in innate immunity and inflammation (34). NALP1 induces caspase-1 activation by forming the NALP1 inflammasome that consists of NALP1, the adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain, caspase-1, and caspase-11. It was suggested that NALP1b activation by LF induces caspase-1 and subsequently causes rapid macrophage cell death (9, 33). However, the mechanism by which caspase-1 induces rapid macrophage cell death still remains to be defined. Several features manifested in LeTx cytotoxic cells include early mitochondrial dysfunction (35), Ca2+ influx, and generation of reactive oxygen species (36–39). Based on inhibitor studies, several molecules including the proteasome (40) are involved in LeTx cytotoxicity, but definitive molecular and signaling mechanisms of LeTx cytotoxicity are unknown.

Macrophages can adaptively adjust to LeTx cytotoxicity, a phenomenon termed toxin-induced resistance (TIR). Pretreatment of macrophages with low doses of LeTx induces a physiological state in which macrophages become refractory to challenge with high cytolytic doses of LeTx (41). It has been speculated that the mechanism for TIR involves either adaptive Erk activation in the presence of LeTx or decreased proteasome activity in TIR cells (41). Although the majority of TIR cells are protected from LeTx cytotoxicity for 3–4 days, we observed that a small fraction of RAW264.7 cells (~2%) remain resistant to LeTx cytotoxicity, and about 1% of the surviving cells retained resistance for an extended time period of up to 5–6 weeks. The prolonged LeTx resistance of the clones allowed us to recover enough cells from a single resistant clone for microarray analysis, thus eliminating background noise between clones. Analysis of more than 39,000 transcripts from this single clone during and after LeTx resistance revealed only 15 genes that displayed up to a 5-fold reduction in their expression levels. Among these genes, we found two closely related mitochondrial genes: Bcl-2 adenovirus EIB 19-kDa interacting protein 3 (Bnip3) and Bnip3-like (Bnip3L; also known as Nip3-like protein X). Here we show that Bnip3 and Bnip3L play a key role in LeTx-induced cytotoxicity in macrophages and that down-regulation of these gene products is a mechanism for TIR, which at least in part is also mediated through continual inhibition of the p38 mitogen-activated protein kinase pathway by LF inside cells. This study provides the first mechanistic description of TIR and Bnip3/Bnip3L-mediated LeTx cytotoxicity.

EXPERIMENTAL PROCEDURES

Materials—LF and PA were prepared in the laboratory as described previously (39). SB203580, SB202190, U0126, Jnk inhibitor II, and actinomycin D were purchased from Calbiochem (EMD Biosciences). Antibodies toward p38, Akt, Mek1, and Jnk inhibitor II, and actinomycin D were purchased from Cell Signaling Technologies (Pickering, Ontario, Canada). Antibodies raised against the N terminus of Mek1 and Bnip3L were from QED Bioscience Inc. and eBioscience, respectively.

Macrophages, Cell Culture, and Screening of TIR Clones—RAW264.7 murine macrophages and bone marrow-derived immortalized macrophages from a C57BL/6 mouse (obtained from Dr. B. Aggarwal, Houston, TX) were cultured in Dulbecco’s modified Eagle’s medium or medium containing 10% heat-inactivated fetal bovine serum (Sigma). 10 mm minimum Eagle’s medium non-essential amino acids solution, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 1 mm sodium pyruvate. Cells were grown at 37 °C in a humidified atmosphere of 5% CO2. Primary peritoneal macrophages were isolated from 129s/j mice as described previously (18). TIR cells were obtained by treating RAW264.7 macrophages with LeTx (10 ng/ml LF and 200 ng/ml PA) for 5 h, and surviving cells were plated in a fresh tissue culture dish. To screen and select spontaneous LeTx resistance clones, RAW264.7 cells were plated on a 10-cm dish and exposed to a cytolytic dose of LeTx (500 ng/ml LF and 1 μg/ml PA) for 5 h (Fig. 1). Two weeks later surviving clones were individually picked and plated on a 96-well plate. Each clone was duplicated, and one of them was tested for LeTx sensitivity. LeTx-resistant clones were then passaged every 3–4 days with fresh medium, and LeTx susceptibility was tested at every passage.

Microtiter Tetrazolium (MTT) Assay—RAW264.7 macrophages were cultured with or without LeTx in 96-well plates for 5 h, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was then added at a final concentration of 1 mg/ml. After incubating at 37 °C for an additional 4 h, culture medium was carefully aspirated, and 100 μl of dimethyl sulfoxide was added to dissolve crystals. Optical densities of the wells were analyzed using an automatic enzyme-linked immunosorbent assay plate reader (UV MAX, Molecular Devices, Sunnyvale, CA) at 590 nm. The ratio of cell survival was estimated based on the optical density of wells by comparison with non-treated cells as 100% survival.

Total Cell Lysate Preparation and Western Blot Analysis—Cells were lysed in ice-cold lysis buffer (20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 1 mM Na2VO4, 40 mM β-glycerophosphate, 30 mM sodium fluoride, and 20 mM sodium pyrophosphate, pH 7.2) containing 1% Triton X-100 and protease inhibitor mixtures (Roche Applied Science). Cell lysates were incubated on ice for 10 min and centrifuged at 12,500 rpm for 15 min at 4 °C. Cell extracts were electrophoretically resolved in SDS-polyacrylamide gels followed by transfer onto nitrocellulose membranes. The membranes were blocked at room temperature for 1 h with 5% (w/v) skim milk and then incubated overnight at room temperature with primary antibodies. The membranes were washed and developed using an enhanced chemiluminescence detection system (Pierce).

Promoter-Reporter Assay—Wild-type or lethal toxin-exposed RAW264.7 macrophages were transfected with 1.6 μg of Bnip3L promoter-reporter plasmids (42) and 0.4 μg of β-galactosidase-encoding plasmids using the Nucleofector™ II kit (Amaxa Biosystems). After 24 h, transfected cells were harvested and washed with phosphate-buffered saline and resuspended in cell lysis buffer (BioVision) followed by incubation on ice for 5 min. Cell lysates were centrifuged at 12,000 rpm for
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FIGURE 1. Down-regulation of Bnip3 and Bnip3L confers resistance to LeTx-induced cell death. A, RAW264.7 cells were treated with a cytolytic dose of LeTx (500 ng/ml LF and 1 μg/ml PA) for 5 h, and surviving cells were further cultured to form colonies in a culture dish. Each colony was transferred to a 96-well plate, and sensitivity to LeTx challenged at the same cytolytic dose was examined using microtiter tetrazolium (MTT) assay. About 1% of the surviving colonies maintained LeTx resistance for up to 42 days after the first LeTx challenge. Results obtained from one of the clones are shown as a percentage of survival compared with non-treated wild-type cells. C, H9262 cells were further cultured to form colonies in a culture dish. Each colony was transferred to a 96-well plate, and sensitivity to LeTx (500 ng/ml LF and 1 μg/ml PA) for 5 h, and N-terminal cleavage of Mek1 and phosphorylation of Erk were examined using Western blots. C, amounts of Bnip3 and Bnip3L mRNAs in the LeTx-resistant colony before and after regaining LeTx sensitivity were measured by using real time PCR analysis. D, the LeTx resistance cells were transfected with empty vector (VC) or GFP-Bnip3L, and susceptibility to LeTx (500 ng/ml LF and 1 μg/ml PA) was measured after 5 h using MTT assay. E, Bnip3 and/or Bnip3L were knocked down by using siRNAs in RAW264.7 cells, and mRNA levels and protein levels were measured by using real time PCR analysis (upper panel) and by Western blots (lower panel), respectively. Western blot for p38 was used for loading control. After 48 h of siRNA transfection, the cells were treated with LeTx (500 ng/ml LF and 1 μg/ml PA) for 5 h, and cell survival was measured using MTT assay. A, C, and D, data are expressed as mean ± S.D. (n = 3). pErs, phospho-Erk.

10 min. The supernatant was divided into two equal aliquots. One aliquot was assayed for luciferase activity in a Berthold luminometer (Sirius, Montreal Biotech Inc.) using a luciferase reporter assay kit (BioVision). The other aliquot was used for a luminometer (Sirius, Montreal Biotech Inc.) using a luciferase assay. The relative activity was normalized on the basis of β-galactosidase activity.

Metabolic Radiolabeling and Immunoblotting—At 48 h post-LeTx exposure, LeTx-treated or untreated control cells were placed in methionine-free medium for 60 min. The cells were then labeled for 1 h with 200 μCi/ml [35S]methionine (PerkinElmer Life Sciences). The labeled cells were lysed, and proteins were separated on SDS-polyacrylamide gels and visualized by autoradiography. Immunoblotting analysis of labeled proteins for Mek1-NT or Mek1-CT was performed as described above.

Quantitative Real Time PCR—mRNA expression of Bnip3 and Bnip3L in macrophages was quantified on a Rotor-Gene RG3000 quantitative multiplex PCR instrument using Brilliant SYBR Green PCR Master Mix (Applied Biosystems). Total cellular RNA was isolated using TRizol (Invitrogen) according to the manufacturer’s instructions. Four micrograms of total RNA was reverse transcribed by using oligo(dT) primers and the Superscript II reverse transcriptase system (Invitrogen) according to the manufacturer’s recommendations. Oligonucleotide primers were the following: for Bnip3, 5′-GCTCCAGACACCACAAGAT-3′ (5′ primer) and 5′-TGAGAGTATGCTGTCCGC-3′ (3′ primer); for Bnip3L, 5′-CCTGCTCTCCATCAGATT-3′ (5′ primer) and 5′-GTCCTCTGTATCATCT-3′ (3′ primer); for glyceraldehyde-3-phosphate dehydrogenase, 5′-GCAATGCTGCCTGAGG-3′ (5′ primer) and 5′-TTGCTTTGAAAGTGCCGAG-3′ (3′ primer).

Small Interfering RNA—Small interfering RNA (siRNA) oligonucleotides directed against mouse Bnip3 and Bnip3L were purchased from Dharmacon (Lafayette, CO). Transfection of RAW264.7 cell with siRNAs was performed with the Nucleofector II kit (Amaza Biosystems) according to the manufacturer's instructions. Cells were subcultured 1 day before nucleasefection. Cells were harvested, resuspended at room temperature in Nucleofector solution V to a final concentration of 2.0×10^6 cells/100 μl, and mixed with 2 μg of siRNA. The mixtures were used for nucleasefection using Nucleofector program D-32 in the Nucleofector I device (Amaza Biosystems).

Adenoviral-p38 Transfection—A recombinant, replication-deficient adenovirus vector encoding the human wild-type p38 and an identical construct lacking the insert (Ad0) were purified and transactivated as described previously (43). Briefly RAW264.7 cells were transiently infected with adenovirus at 15 multiplicity of infection/cell for 1 h in serum-free medium. Cells were then washed and cultured in growth medium for 24 h.

pEGFP-Bnip3L Construction—Mouse Bnip3L cDNAs were prepared from total RNAs of RAW264.7 macrophages. The total RNAs were reverse transcribed, and the target gene for Bnip3L was amplified by PCR using gene-specific primers (forward, 5′-ATTAGATTTTCTTCTACTAGTTGCCGCGC-3′; reverse, 5′-TTAGAATTTCTACGTAGGTCGTGCAGG-3′). The amplified fragment was digested with BglII and EcoRI and cloned into pEGFP-C1 vector (Invitrogen). Transfection of recombinant plasmids was performed using the same method as for siRNA transfection.

RESULTS

Bnip3 and Bnip3L Are Involved in LeTx-induced Cytotoxicity of Macrophages—A small population (~2%) of RAW264.7 macrophages is resistant to cytolytic doses of LeTx, but when individually cloned and restested for LeTx susceptibility, most of the resistant cells become sensitive to LeTx in about 4 days (41). However, about ~2% of these cells remained LeTx resistant for up to 6 weeks (Fig. 1A). Cells that originated from one LeTx-resistant clone before (post-LeTx treatment day 35) and after (post-LeTx treatment day 49) regaining LeTx sensitivity...
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**FIGURE 2. Down-regulation of Bnip3 and Bnip3L is involved in the TIR in macrophages.** TIR cells were obtained by pretreating RAW264.7 macrophages with a sublethal dose of LeTx (10 ng/ml LF and 100 ng/ml PA) for 5 h. A, TIR cells were treated with a cytolytic dose of LeTx (500 ng/ml LF and 1 μg/ml PA) for 3 h on different days after inducing TIR, and LeTx susceptibility was measured using MTT assay. Results are shown as percent survival compared with non-treated wild-type cells. B, amounts of Bnip3 and Bnip3L mRNAs and proteins were measured by using real time PCR (upper panel) and Western blot analysis (lower panel), respectively, in RAW264.7 cells (CNT) and the TIR cells post-LeTx treatment days 1–6. Western blot for Akt was used for loading controls. C, TIR cells were transfected with empty vector (VC), GFP-Bnip3, or GFP-Bnip3L, and susceptibility to LeTx (500 ng/ml LF and 1 μg/ml PA) was measured after 5 h using microtiter tetrazolium (MTT) assay. D, regulation of Bnip3L expression in TIR cells. Left panel, basal Bnip3L promoter (−5362 to −1)-luciferase reporter activities in wild-type and TIR cells. Wild-type or TIR RAW264.7 macrophages were transfected with 1.6 μg of −5362 bp Bnip3L promoter-luciferase vector and 0.4 μg of β-galactosidase-encoding plasmids. The β-galactosidase vector containing β-galactosidase under the control of the cytomegalovirus immediate early promoter was co-transfected to normalize transfection efficiency. After 24 h of transfection, luciferase activity was measured as described under "Experimental Procedures." Results are shown as luciferase activity as a percentage of control (wild type (WT)) cells. Right panel, RAW264.7 cells were pretreated with actinomycin D (3 μg/ml) for 1 h and then treated with a sublethal dose of LeTx (10 ng/ml LF and 100 ng/ml PA). Levels of Bnip3L mRNA were analyzed by real time PCR analysis. Results are shown as fold decrease compared with actinomycin D only-treated cells. Data are expressed as mean ± S.D. (n ≥ 3).

showed no differences in MeK1 N terminus cleavage and Erk1/2 phosphorylation inhibition by LeTx (Fig. 1B). Therefore, the long term resistance phenotype was not due to defects in toxin uptake and release into cytosol of resistant cells. To identify genes involved in the resistance phenotype, a genome-wide transcript analysis using the murine 430A array (Affymetrix) was carried out comparing transcripts from cells before (post-LeTx treatment day 35) and after (post-LeTx treatment day 49) regaining LeTx sensitivity. Using cells derived from the same clone allowed us to eliminate most of the background noise between clones. From 39,000 transcripts examined, we detected 14 transcripts with more than a 5-fold change in their levels of expression. Two of them encoded closely related mitochondrial proteins: Bnip3 and Bnip3L (or Nip3-like protein X protein). The more than 5-fold reduction of Bnip3 and Bnip3L transcript levels in the resistant cells (post-LeTx treatment day 35) was further verified by real time PCR (Fig. 1C). The expression of known cell death-related genes in the resistant cells including Bad, Bax, Noxa, Bid, and Puma or antiapoptotic genes such as Bcl-2, Bcl-XL, and Mcl-1 did not significantly change by more than 2-fold relative to wild-type cells (data not shown). To confirm whether the down-regulation of Bnip3 and/or Bnip3L is associated to the resistance phenotype, N-terminal GFP-conjugated Bnip3 and/or Bnip3L were overexpressed in the resistant cells, and these cells were also exposed to a cytotoxic dose of LeTx (500 ng/ml LF and 1 μg/ml PA). N-terminal GFP-conjugated Bnip3 had the same effect as wild-type Bnip3 (37), and GFP+ cells were visualized using a fluorescence microscope to confirm >50% transfection efficiency (data not shown). Under these conditions, overexpression of Bnip3L alone induced about 25% cell death in resistant macrophages (Fig. 1D). However, Bnip3 overexpression in combination with a cytolytic LeTx dose induced high levels of cell death (~75%) in the resistant cells (Fig. 1D). To confirm whether Bnip3 and Bnip3L are indeed required for LeTx cytotoxicity, we silenced the expression of Bnip3 and/or Bnip3L in RAW264.7 cells using siRNAs. Both Bnip3 and Bnip3L mRNA levels were reduced at least 4-fold in the siRNA-treated cells (Fig. 1E, left upper panel). Western blots against Bnip3L further showed that Bnip3L protein levels in Bnip3L or Bnip3 + Bnip3L siRNA-treated cells were also greatly diminished (left lower panel). Down-regulation of the Bnip3L expression prevented LeTx cytotoxicity in about 50% of the cells, whereas down-regulation of Bnip3 had a smaller effect (~15%) (Fig. 1E, right panel). Additive protection was detected when both genes were down-regulated (~65%) (Fig. 1E, lower panel). Together these results support the notion that both Bnip3 and Bnip3L are required for LeTx cytotoxicity of macrophages.

**TIR Is Mediated by Down-regulation of Bnip3 and Bnip3L—**

TIR is an adaptive phenomenon by which macrophages exposed to sublethal doses of LeTx become refractory to subsequent challenge with high cytolytic doses of LeTx (41). To examine whether Bnip3 or Bnip3L is also involved in TIR, RAW264.7 macrophages were treated with a sublethal dose of LeTx (10 ng/ml LF and 100 ng/ml PA) that causes about 15% cell death. Consistent with a previous study (41), TIR lasted for up to 3–4 days (Fig. 2A). By day 5, an intermediate TIR phenotype was observed, whereas by day 6 cells were as sensitive to high doses of LeTx as the untreated controls. Transcript levels of Bnip3 and Bnip3L were dramatically reduced (>10-fold) after 12 h of LeTx treatment and remained low for at least 3–4 days (Fig. 2B, top panel). Bnip3L protein levels were also reduced in TIR cells; this correlated well with mRNA levels (Fig. 2B, lower panel). By day 5, transcript and protein levels increased, and at day 6 they were identical to those found in...
untreated cells. The temporal variation in Bnip3L transcript and protein levels correlated with the duration of the TIR state (Fig. 2, compare A and B). To confirm whether the down-regulation of Bnip3 or Bnip3L is responsible for TIR, we expressed N-terminal GFP-conjugated Bnip3 or Bnip3L in TIR cells and challenging them with a cytolytic dose of LeTx (500 ng/ml LF and 1 μg/ml PA). Fig. 2C shows that overexpression of either Bnip3 or Bnip3L sensitized TIR cells to LeTx, suggesting that down-regulation of Bnip3 and Bnip3L is required for TIR. We further determined whether the down-regulation of Bnip3L in TIR cells was due to a decreased transcription rate or reduced mRNA stability. We used a mouse Bnip3L promoter sequence (−5362 bp upstream of ATG start codon of mouse Bnip3L)-fused luciferase reporter system to examine the basal transcription activity of Bnip3L in wild-type and TIR cells. Fig. 2D (left panel) shows at least 3-fold lower reporter activity in TIR cells relative to wild-type cells. To examine the stability of Bnip3L mRNA, wild-type RAW264.7 or TIR cells were pretreated with a broad spectrum transcription inhibitor, actinomycin D (3 μg/ml), for 1 h, and mRNA levels of Bnip3L were analyzed by real-time PCR. Fig. 2D (right panel) shows an enhanced degradation rate for Bnip3L mRNA in TIR cells. Similar results were obtained for the Bnip3 mRNA levels (data not shown).

Together these results indicate that Bnip3 and Bnip3L are involved in TIR, and down-regulation of Bnip3L in TIR cells is due to reduced basal transcription activity and mRNA stability.

**Mek Cleavage Correlates with LeTx Resistance in TIR Cells**—LF targets Meks inhibiting most MAPK-mediated signaling cascades (44, 45). To examine whether TIR is associated with inhibition of MAPKs, RAW264.7 cells were exposed to a sublethal dose of LeTx (10 ng/ml LF and 100 ng/ml PA) for 5 h, and cleavage of Mek1 and phosphorylation of Erks, Jnks, and p38 were monitored for 6 days. The results show that in the same time period (3–4 days) after LeTx treatment Mek1 remained cleaved at the N terminus for up to 4 days, and phosphorylation of Erk, p38, and Jnk was undetectable (Fig. 3A). Because the total immunoreactivity for Mek1 appeared to be lower in TIR cells, we further examined whether LeTx also induces Mek1 down-regulation by interfering with Mek1 expression. We found no changes in Mek1 mRNA levels between wild-type and TIR cells (data not shown) and no reduction in Mek1 protein production by TIR cells as assessed by radiolabeling of newly synthesized Mek1 (Fig. 3B, bottom panel). A much lower level of total Mek1 protein was immunoprecipitated by the Mek1 C-terminal antibody in TIR cells (Fig. 3B, top panel); this may also suggest an enhanced rate of Mek1 degradation in TIR cells.

To examine whether the continuous cleavage of the Mek1 N terminus was due to residual LF activity, TIR cells were treated with the membrane-permeable LF protease inhibitor III (10 μM; Calbiochem) at various time points, and Mek1 cleavage and susceptibility to LeTx were measured. Intact Mek1 was gradually detectable at 6–9 h after treatment (Fig. 3C). Furthermore TIR cells treated with LF inhibitor for 16 h became susceptible to LeTx (Fig. 3D). These results suggest that continual Mek1 N-terminal cleavage depends on residual LeTx and is required for sustaining TIR.

**p38 Inhibition Confers Resistance to LeTx in Macrophages**—Phosphorylation of the three MAPKs was undetectable in TIR cells (Fig. 3A). If inhibition of MAPK by LeTx is responsible for inducing TIR, inhibitors of MAPKs should be able to mimic the TIR effects in macrophages. RAW264.7 cells were pretreated with various concentrations of U0126 (Erk inhibitor), SB202190 (SB; p38 inhibitor), or Jnk inhibitor II (Jnk inhibitor) for 16 h, and susceptibility to a cytolytic dose of LeTx (500 ng/ml LF and 1 μg/ml PA) was analyzed. As shown in Fig. 4A, only pretreatment with SB caused a dose-related protection of macrophages from LeTx cytotoxicity. We also performed these experiments with another p38 inhibitor, SB203580, obtaining identical results (data not shown). As in the case of the TIR phenotype resulting from LeTx induction, cells had to be pretreated with the p38 inhibitor for at least 6 h before RAW264.7 macrophages became resistant to LeTx cytotoxicity (Fig. 4B).

**LeTx-induced Inhibition of p38 Is Responsible for the Down-regulation of Bnip3 and Bnip3L**—To examine whether p38 inhibitor-induced LeTx resistance is also mediated through down-regulation of Bnip3 and/or Bnip3L mRNAs, RAW264.7 macrophages were treated with SB (25 μM) or other MAPK inhibitors (U0126 or Jnk inhibitor; 25 μM each) for 16 h, and mRNA levels of Bnip3 and Bnip3L were analyzed using real-time PCR. As shown in Fig. 5A, both Bnip3 and Bnip3L mRNAs were significantly reduced only in SB-treated but not in U0126-
The promoter activity of Bnip3L in SB-treated cells remained at the same level as that of wild-type cells (Fig. 5B, lower panel). Promoter activities in TIR cells and the spontaneous LeTx resistance clone were significantly lower than that of wild type. These results suggest that reduction of Bnip3 and Bnip3L levels by SB was due to an increased rate of Bnip3 and Bnip3L mRNA degradation. It is conceivable that resistance to LeTx in TIR cells was at least in part mediated through inhibition of p38.

Therefore, we next examined whether overexpression of p38 in TIR cells induces higher expression of Bnip3 and Bnip3L and renders susceptibility to LeTx. As shown in Fig. 5C, overexpression of p38 in TIR cells, using an adenoviral vector, increased Bnip3 and Bnip3L mRNA levels (upper panel) and rendered TIR cells susceptible to LeTx (lower panel). The adenoviral vector alone (Ad0) had no effect on LeTx sensitivity in TIR cells.

TIR and Role of Bnip3/Bnip3L in Primary Peritoneal Macrophages—To examine the effects of TIR and the role of Bnip3 and Bnip3L in LeTx-induced cytolysis in other macrophages, primary peritoneal macrophages isolated from 129s/j mice were challenged with a cytolytic dose of LeTx (500 ng/ml LF and 1 μg/ml PA) with or without pretreatment with a sublethal dose of LeTx (10 ng/ml LF and 100 ng/ml PA), various doses of p38 inhibitor SB202190, or siRNAs. As in RAW264.7 macrophages, a sublethal dose of LeTx or the p38 inhibitor conferred LeTx resistance in the primary cells (Fig. 6A, left and middle panels). Treatment with siRNAs against Bnip3 and Bnip3L caused an approximately 70% decrease in mRNA levels (data not shown) and protected the primary cells from LeTx-induced cytolysis (Fig. 6A, right panel). These results suggest that TIR and involvement of Bnip3 and Bnip3L in the cytolysis are phenomena not only limited to RAW246.7 macrophages.

LeTx-induced Cytosolysis-resistant Macrophages Express Significantly Lower Levels of Bnip3 and Bnip3L than Susceptible Macrophages—The susceptibility of macrophage cytolysis induced by LeTx is mouse strain-dependent. The RAW264.7 macrophage cell line was established from bone marrow cells of a BALB/c mouse harboring LeTx-sensitive macrophages. To examine the level of Bnip3 and Bnip3L expression in LeTx-resistant macrophages, macrophage cell lines (C57) that originated from bone marrow cells of a C57BL/6 mouse harboring LeTx-resistant macrophages were used. C57 macrophages were as efficient as RAW264.7 macrophages in taking up LeTx and cleaving Mek1 but completely resistant to LeTx-induced cytolysis (data not shown). When the basal levels of Bnip3 and Bnip3L were compared using real-time PCR and Western blots, both Bnip3 and Bnip3L mRNA levels were much lower (>5-fold) in C57 cells (Fig. 6B, left panel), and Bnip3L protein was almost undetectable in C57 macrophages (Fig. 6B, right panel). LeTx did not cause a further decrease in Bnip3 and Bnip3L mRNA levels (Fig. 6B, left panel).

**DISCUSSION**

The mechanism of macrophage cytolysis induced by LeTx is not completely understood. Here we report that mitochondrial proteins Bnip3 and Bnip3L are required for the rapid cell death process. TIR is an adaptive response of macrophages leading to resistance to cytolytic doses of LeTx by a sublethal
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We demonstrated that reduced expression of Bnip3L and Bnip3 genes was associated to LeTx resistance in a small population of macrophages (Fig. 1) and in toxin-induced resistance cells (Fig. 2). However, the mechanism of Bnip3 and Bnip3L down-regulation still remains to be elucidated. Although pretreatment of macrophages with a sublethal dose of LeTx induced 10–15% cell death (data not shown), cleavage of Mek1 was almost complete in all cells (Fig. 3A). This indicates that Mek cleavage is not the cause of macrophage cell death, which is consistent with previous studies (18, 31, 33). A proposed mechanism for TIR involves adaptive Erk activation in the presence of LeTx or decreased proteasome activity in TIR cells (41). However, we found that LeTx induces reduction of Bnip3 and Bnip3L mRNA levels by both inhibiting transcription and enhancing degradation of these gene products (Fig. 2D), conferring resistance to LeTx. The Mek1 N-terminal cleavage and lack of phosphorylated MAPKs were well correlated with the mechanism of Bnip3 and Bnip3L down-regulation still remains to be elucidated. Although pretreatment of macrophages with a sublethal dose of LeTx induced 10–15% cell death (data not shown), cleavage of Mek1 was almost complete in all cells (Fig. 3A). This indicates that Mek cleavage is not the cause of macrophage cell death, which is consistent with previous studies (18, 31, 33). A proposed mechanism for TIR involves adaptive Erk activation in the presence of LeTx or decreased proteasome activity in TIR cells (41). However, we found that LeTx induces reduction of Bnip3 and Bnip3L mRNA levels by both inhibiting transcription and enhancing degradation of these gene products (Fig. 2D), conferring resistance to LeTx. The Mek1 N-terminal cleavage and lack of phosphorylated MAPKs were well correlated with the duration of TIR of ~4 days (Fig. 3). Phosphorylation of Erk and Jnk was restored by day 4, which is earlier than that of Mek1 (restored at approximately day 5). However, the early returning Erk and Jnk phosphorylations were not due to an adaptive Mek-independent activation in TIR cells because phosphorylation of Erk and Jnk was further blocked upon re-exposure to LeTx (data not shown). We further confirmed that the lack of MAPK phosphorylation was not due to LeTx-induced down-regulation of Mek1 transcription or translation but rather due to the direct proteolytic activity of LF (Fig. 3, B and C).

Using specific inhibitors for MAPK isoforms, we found that only p38 inhibitors SB202190 or SB203580, but not Erk or Jnk inhibitors, down-regulated both Bnip3 and Bnip3L mRNA levels (Fig. 5A) and conferred resistance (Fig. 4A). Inhibition of p38 enhanced Bnip3 and Bnip3L mRNA degradation rates without affecting basal Bnip3L promoter activity (Fig. 5B). The role of p38 in the regulation of mRNA stability of a number of 3' untranslated region AU-rich element (ARE) gene products can occur in multiple pathways, including direct phosphorylation and regulation of ARE binding proteins such as HuR and tristetraprolin (51). LeTx destabilizes interleukin-8 mRNA through AREs in the 3'-untranslated region via inhibiting all three MAPKs (52). Unlike interleukin-8, we have shown that destabilization of Bnip3 and Bnip3L mRNAs was specifically
mediated through inhibition of p38 (Fig. 5A). It is now recognized that, in addition to cytokines, various genes (as much as 8% of mRNAs transcribed in humans) with diverse functions contain AREs (53). 3' Untranslated regions of mouse Bnip3 (NCBI accession number NM_009760) and Bnip3L (NCBI accession number NM_009761) contain two and one ARE conserved AUUUA pentamer motif (54), respectively, suggesting a possible regulation of ARE-mediated mRNA stability by p38.

Although p38 inhibitors enhanced the mRNA degradation as effectively as LeTx, the degree of mRNA down-regulation induced by p38 inhibitors (~50%) even at the highest concentration without toxicity never reached the level induced by LeTx (~80%) (Figs. 2B and 5A). These results suggest that a p38-independent pathway further down-regulates Bnip3 and Bnip3L mRNA levels in TIR cells. In fact, Bnip3L promoter activity was significantly inhibited in TIR (Fig. 2D) but not in SB-treated cells (Fig. 5B). The prolonged LeTx resistance in a small population of RAW264.7 macrophages cannot be attributed to the inhibition of p38 phosphorylation and subsequent destabilization of Bnip3 and Bnip3L mRNAs because Mek1 and phosphorylation of Erk (Fig. 1B) or p38 (data not shown) were normal. Down-regulation of these genes in the LeTx resistance clone is likely to be mediated through inhibition of transcription activities. Both Bnip3 and Bnip3L share similarities in their promoter regions including binding sites for hypoxia-inducible factor, p53, and Sp1 (42, 55). In the heart, however, Bnip3 and Bnip3L were shown to be regulated by different mechanisms: Bnip3 is hypoxia-inducible, whereas Bnip3L is induced through Go<sub>q</sub>-mediated Sp1 site (42). Bnip3 expression was also shown to be constitutively suppressed by p65-NF-κB-mediated histone deacetylase 1 in the heart (56). In RAW264.7 macrophages, histone deacetylase 1 is involved in the suppression of lipopolysaccharide-induced tumor necrosis factor α expression (57). Whether LeTx resistance cells have high p65 or histone deacetylase 1 activity warrants further investigation.

Unlike RAW264.7 macrophages, C57 macrophages were resistant to LeTx (data not shown) and expressed much lower basal levels of Bnip3 and Bnip3L (Fig. 6B). In fact, C57 macrophages share common characteristics with LeTx-resistant clones from RAW264.7 cells (Fig. 1) in that both cells express low levels of Bnip3 and Bnip3L without defects in expression and basal phosphorylation of p38. Whether C57 macrophages and LeTx-resistant clones share a common signaling mechanism in suppressing Bnip3 and Bnip3L expression remains to be investigated.

To date the signaling mechanism for the activation of Bnip3 and Bnip3L and the molecular mechanism of Bnip3 and Bnip3L induction of mitochondrial membrane permeabilization and cell death are largely unknown. Both Bnip3 and Bnip3L contain N-terminal PEST sequences, a BH3 domain, and C-terminal transmembrane domains (46, 49). PEST sequences facilitate degradation of proteins by proteasome, and lactacystin-sensitive proteasome mediates degradation of Bnip3, leaving C-terminal fragments of undefined function (46). At present it is unknown whether N-terminal cleavage of Bnip3 by proteasome is a prerequisite step for mitochondrial membrane permeabilization. LeTx induces a rapid reduction in ubiquitin-modified proteins before cell death in LeTx-sensitive macrophages, whereas TIR cells maintain normal levels of ubiquitylated proteins (41), suggesting that LeTx enhances proteasome activity in sensitive cells. If a rapid cleavage of Bnip3 N terminus by proteasome induces mitochondrial permeabilization, an alteration in proteasome activity in TIR cells may also contribute to the resistance through inhibiting the Bnip3 and Bnip3L process. Proteasome inhibitors have also been shown to protect macrophages from LeTx-induced cytolysis (40). The transmembrane domain and C terminus of Bnip3 and Bnip3L are required for mitochondrial localization, homo- or heterodimerization, and cell death. Dimerization of Bnip3 is dispensable for cell death induction because substitution of alanine for histidine at position 173 within the transmembrane domain prevented homodimerization but did not inhibit cell death (58). Unlike classical BH3-only domain proteins, the BH3 domain can be spared for the cell death inducing activity of Bnip3 and Bnip3L (49). In cardiomyocytes, Bnip3 induced by hypoxia is loosely associated with mitochondria and does not cause cell death, but acidosis induced by reperfusion enhances hydrophobicity of Bnip3 and causes tight association with mitochondria, coinciding with mitochondrial membrane permeabilization and cell death (58). RAW264.7 macrophages (Figs. 1 and 2) and primary peritoneal macrophages from 129s/j mice (data not shown) express readily detectible levels of Bnip3 and Bnip3L either by reverse transcription-PCR or Western blots. Whether LeTx induces acidosis in sensitive cells or increases hydrophobicity of Bnip3 and Bnip3L through a protein modification remains to be examined. Macrophages are professional phagocytes that reside in almost all tissues and are a crucial component of innate immunity. LeTx causes defects in atherogenesis and stability of acute arterial plaques and LeTx toxemia (26–28). Targeting and killing macrophages through the release of LeTx could be an important tactic for survival and proliferation of B. anthracis within the host. Current therapy for anthrax relies on antibiotic treatments. However, such treatments do not typically alter the outcome of disease especially if administered after the onset of symptoms (61). Failure of antibiotic treatment to prevent death despite bacterial clearance in experimental animals is attributed to production and release of anthrax toxins (23). This study has determined the crucial role of Bnip3 and Bnip3L in LeTx cytotoxicity and TIR. The novel function of p38 inhibitors in mimicking TIR effects suggests a possible use of the inhibitor for adjunctive therapy for LeTx toxemia.

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