Critical Role of the Phosphatidylinositol 3-Kinase/Akt/Glycogen Synthase Kinase-3β Signaling Pathway in Recovery from Anthrax Lethal Toxin-induced Cell Cycle Arrest and MEK Cleavage in Macrophages*

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Soon-Duck Ha†‡§$, Dennis Ng†#, Steven L. Pelech†, and Sung Ouk Kim†‡¶

From the 6Department of Microbiology and Immunology and 7Infectious Diseases Research Group, Siebens-Drake Research Institute, University of Western Ontario, London, Ontario N6G 2V4, 8Kinexus Bioinformatics Corporation, Vancouver, British Columbia V6T 1Z3, and the 9Department of Medicine, University of British Columbia, Vancouver, British Columbia V6T 2B5, Canada

Anthrax lethal toxin (LeTx) is a virulence factor causing immune suppression and toxic shock of Bacillus anthracis infected host. It inhibits cytokine production and cell proliferation/differentiation in various immune cells. This study showed that a brief exposure of LeTx caused a continual MEK1 cleavage and prevented tumor necrosis factor-α (TNF) production in response to lipopolysaccharide (LPS) in non-proliferating cells such as human peripheral blood mononuclear cells or mouse primary peritoneal macrophages. In human monocytic cell lines U-937 and THP-1, LeTx induced cell cycle arrest in G0-G1 phase by rapid down-regulation of cyclin D1/D2 and checkpoint kinase 1 through MEK1 inhibition. However, THP-1 cells adaptively adjusted to LeTx and overrode cell cycle arrest by activating the phosphatidylinositol 3-kinase/Akt signaling pathway. Inhibitory Ser-9 phosphorylation of glycogen synthase kinase 3β (GSK3β) by Akt prevented proteasome-mediated cyclin D1 degradation and induced cell cycle progress in LeTx-intoxicated THP-1 cells. Recovery from cell cycle arrest was required before recovering from on-going MEK1 cleavage and suppression of TNF production. Furthermore, pretreatment with LeTx or the GSK3-specific inhibitor SB-216763, or transfection with dominant active mutant Akt or degradation-defected mutant cyclin D1 protected cells from LeTx-induced cell cycle arrest, on-going MEK1 cleavage and suppression of TNF production. These results indicate that modulation of phosphatidylinositol 3-kinase/Akt/GSK3β signaling cascades can be beneficial for protecting or facilitating recovery from cellular LeTx intoxication in cells that depend on basal MEK1 activity for proliferation.

Bacillus anthracis is a Gram-positive spore forming bacterium and systemic infection of B. anthracis is often fatal when inhaled spores germinate inside the host (1). The virulence of B. anthracis is primarily attributed to two secreted exotoxins, lethal toxin (LeTx)3 and edema toxin, and an exterior capsule, encoded in two large plasmids (2, 3). The capsule comprises poly-α-glutamic acid, which protects bacteria from phagocytosis by the host immune cells and contributes to bacterial dissemination (4). LeTx and edema toxin are binary A:B toxins comprising protective antigen (PA) and lethal factor (LF) or edema factor (EF), respectively. PA is a molecular transporter allowing receptor-mediated entry and release of LF or EF into the cytosol. PA binds to either of two surface receptors: anthrax receptor 1 (also known as the tumor endothelial marker 8) and anthrax receptor 2 (also known as the capillary morphogenesis gene-2). Anthrax receptor 1 is expressed in high levels in macrophages, endothelial cells and several tumor cells (5–7); whereas, anthrax receptor 2 is widely distributed in human tissues (8). EF has adenylate cyclase activity (9), whereas LF is a zinc metalloprotease that cleaves and inactivates the N-terminal end of the mitogen-activated protein kinase (MAPK) kinases (MEK) 1 to 7, except MEK5, resulting in the inactivation of most their downstream signaling cascades. Both LF and EF cause severe defects in immune responses and contribute proliferation and dissemination of B. anthracis in the host (10, 11).

Monocytes and macrophages are key innate immune cells and likely the first immune cells encountering the spores and germinating bacteria (12–14). LeTx induces rapid necrosis-like cell death of macrophages prepared from certain strains of inbred mice (15–18). A genetic study investigating LeTx-sensitivity trait loci in different strains of mice identified the NACHT-leucine-rich repeat and pyrin domain-containing protein 1b (NALP1b) as a host factor that confers rapid LeTx cytotoxicity (19). Caspase-1−/− macrophages with LeTx-susceptible genetic backgrounds were resistance to LeTx. LeTx also rapidly induces the release of interleukin-1β and -18 in

3 The abbreviations used are: LeTx, lethal toxin; PA, protective antigen; LF, lethal factor; EF, edema factor; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; CFSE, carboxyfluorescein succinimidyl ester; NALP1b, NACHT-leucine-rich repeat and pyrin domain-containing protein 1b; Chk1, checkpoint kinase 1; TNF, tumor necrosis factor-α; LPS, lipopolysaccharide; P21K, phosphatidylinositol 3-kinase; GSK3β, glycogen synthase kinase 3β; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; PBMC, peripheral blood mononuclear cell; MOPS, 4-morpholinepropane-sulfonic acid; PI, propidium iodide; FACS, fluorescence-activated cell sorting; SB, SB202190; Ly, LY294002; Aktmyr, myristoylated Akt.
LeTx-susceptible macrophages (20), indicating that LeTx induces Nalp1b- and caspase-1-mediated cell death. However, human macrophages lack NALP1b and are resistant to rapid necrotic cell death by LeTx. Instead, LeTx was shown to cause delayed apoptotic cell death of differentiated macrophages and inhibit cell proliferation/differentiation, which are probably mediated through MAPK inhibition (18, 21, 22). Because sustained MAPK activation is required for cell cycle progress of many different cells and the prevalence in expression of PA receptors, it is expected that LeTx causes cell cycle arrest in many cells and tissues of infected hosts. In fact, LeTx was shown to inhibit cell proliferation/differentiation in other cell types, including melanocytes (23), B lymphocytes (24), and T lymphocytes (25–27), which contribute to the host immune paralysis. To date the signaling mechanism of cell proliferation inhibition by LeTx and its contribution in the pathogenesis of anthrax remains largely unknown.

This study examined further details on the effects of LeTx on cell proliferation. LeTx induced cell cycle arrest in G0/G1 phase via down-regulating key cell cycle progress proteins cyclin D1, D2, and checkpoint kinase 1 (Chk1) via MEK1 inhibition in human monocytic cell lines. LF incorporated inside cells had almost permanent MEK1-cleaving activity and completely blocked tumor necrosis factor-α (TNF) production in response to a bacterial component lipopolysaccharide (LPS). Recovery from the prolong MEK-cleaving LF activity and the TNF-suppressing effects required cell proliferation, which was mediated through adaptive response of THP-1 cells by inducing the phosphatidylinositol 3-kinase (PI3K)/Akt/GSK3β signaling pathway. Activation of PI3K/Akt by pretreating cells with LeTx or a chemical GSK3-inhibitor protected cell cycle arrest and facilitated recovery from cellular LeTx intoxication.

MATERIALS AND METHODS

Cell Culture and Reagents—The human monocytic leukemia cell line THP-1 and U937 cells were grown in complete RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (Sigma), 10 mM minimal essential 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 the humidified atmosphere of 5% CO2. LF and PA were prepared in the laboratory as previously described (17). Briefly, cells were lysed in ice-cold cell lysis buffer containing 20 mM MOPS, 15 mM EGTA, 2 mM EDTA, 1 mM Na3VO4, 1 mM dithiothreitol, 75 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 10 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1% Triton X-100 and then sonicated on ice. Cell extracts were obtained by centrifuging the homogenate at 18,000 × g for 10 min. These extracts were electrophoretically resolved in ready-made 10% SDS-PAGE gels (Bio-Rad), followed by transfer onto nitrocellulose membranes. Membranes were subsequently blocked with 5% skim milk for 30 min, immunoblotted with antibodies, and developed using an enhanced chemiluminescence detection system (ECL, Pierce Bioscience). Total cell lysates were obtained from THP-1 cells 48 h after LeTx (250 ng of LF and 500 ng of PA for 5 h) exposure and Kinetworks™ KCCP-1.0 Cell Cycle Protein multi-immunoblotting analysis was performed at Kinexus Bioinformatics Inc. (Vancouver, Canada) as described on the Kinexus website.

Quantitative Real-time PCR—mRNAs expression were quantified on the Rotor-Gene RG3000 quantitative multiplex PCR instrument using the Brilliant SYBR Green PCR Master Mix (Applied Biosystems). Total cellular RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. Briefly, 4 μg of total RNA was reverse transcribed by using oligo(dT) primers and the M-MuLV reverse transcriptase (New England Biotechnology) according to the manufacturer's recommendations. Oligonucleotide primers were the following: for cyclin D1, 5’-CCCTCGGTGTCCTACTTTAC-3’ (5’ primer) and 5’-AGGAAGCGGTTCCAGTAGTT-3’ (3’ primer); for cyclin D2, 5’-GTCCTCAAGCTTGCAGGAG-3’ (5’ primer) and 5’-ATATCCCCAGCTCTTTAGG-3’ (3’ primer); for cyclin B, 5’-CAAGCCCAATGGAAACATCT-3’ (5’ primer) and 5’-GGATCAGCTCACCCTTGCTG-3’ (3’ primer); for cyclin E, 5’-ATCCCTCAAATTTGCAGCAG-3’ (5’ primer) and 5’-AGGAGCTTTAAACGCGACCT-3’ (3’ primer); for CHK1, 5’-CTGAAGAAGCAGTCGCAGTG-3’ (5’ primer) and 5’-TTGGCCCTTCTCTCTGTGACC-3’ (3’ primer); for glyceraldehyde-3-phosphate dehydrogenase, 5’-ACCAC-TTCCCACTTGG-3’ (5’ primer) and 5’-CTCTTGTGCT-CTTGGTGG-3’ (3’ primer).
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**FIGURE 1. Anthrax lethal toxin (LeTx) induces cell cycle arrest in human monocytic cell lines.** Human monocytic cell lines THP-1 or U-937 cells were briefly exposed to LeTx (250 ng/ml LF and 500 ng/ml PA) for 5 h and re-plated with fresh culture medium after washing off residual LeTx by phosphate-buffered saline (10 μl). A, LeTx prevents cell proliferation in THP-1 and U-937 cells. Cells were stained with CFSE (0.5 μl) fluorescence in non-treated or LeTx-treated cells. Intensities of CFSE were measured each day starting 1 day after an LeTx treatment for the next 4 days. B, the percentage of cells at different cell cycle phase was analyzed using ModFit LT 3.0 software according to the PI content in cells 2 day after a LeTx exposure. A and B are representative data of similar results observed in three independent experiments.

**Cell Cycle and Proliferation Analysis**—Analyses of intracellular fluorescence by carboxyfluorescein succinimidyl ester (CFSE) and DNA content using propidium iodide (PI) were performed using CellQuest software on a FACSCalibur flow cytometer (Becton Dickinson). For cell proliferation studies by CFSE, cells were incubated with CFSE of 1 μM for 10 min at 37 °C, and reaction was quenched on an ice bath for 2 min. Cells were washed with 3 volumes of complete media and plated. Cells were harvested daily for FACS analysis.

For DNA content analysis by PI, 1.0 × 10^6 cells were harvested at the indicated time points, fixed by dropwise addition of ice-cold 70% ethanol after three times washing with 1× phosphate-buffered saline containing 0.1% glucose, and were stored at 4 °C. Subsequently, cell were pelleted by centrifugation and resuspended in staining solution containing 50 μg of PI/ml and 100 units of RNase A/ml. After 60 min of incubation at room temperature, the cell was loaded in a FACSCalibur flow cytometer. The data were analyzed using CellQuest and ModFit LT 3.0 software (BD Biosciences). For the purpose of analysis, acquired events were gated to eliminate cell aggregates and debris.

**Akt<sup>myr</sup> and Akt<sup>K179M</sup> Retroviruses Generation and Infection**—The virus preparation and infection were performed as described (31). Briefly, constitutively active myristoylated Akt (Akt<sup>myr</sup>) and dominant negative Akt (Akt<sup>K179M</sup>) retroviruses were generated in Phoenix Amphotropic producer cells using the calcium phosphate method. Empty retroviral vector was transfected by same procedure for control virus. Cells were cultured for 24 h at 37 °C and for another 24 h were incubated at 32 °C for collection of viruses. Virus was collected and filtered through a 0.45-m filter. THP-1 cells of 1 × 10^6 were suspended with viruses solution containing 4 μg of Polybrene per milliliter and plated in 6-well plates. The plates were centrifuged at 2,500 rpm for 45 min at 32 °C, and fresh RPMI 1640 media were added into the each well after 3-h incubation at 37 °C. Viruses expressing cells were selected by green fluorescent protein 48 h after infection with the LeTx for cell cycle analysis.

**pCDNA-Cyclin D1<sup>T286A</sup> Construction and Transfection**—pCDNA3 containing human mutant cyclin D1-T286A was made by digesting a pFlex-D1-T286A (32) with the EcoR1 restriction enzyme and ligated the resulting fragment into the pCDNA3.1 vector (Invitrogen). The construct was verified by DNA sequencing. The stable transfection of constructed plasmids was performed by electrophoresis using Gene Pulser Xcell (Bio-Rad) in THP-1 cell line, and mutant cyclin D1 expression cells were selected by G418 antibiotics.

**RESULTS**

**LeTx Induces G<sub>1</sub> Cell Cycle Arrest**—Previous studies have shown that LeTx inhibits proliferation and differentiation of immune cells (22, 24, 27). We further examined the mechanism of cell cycle arrest in the human monocytic cell lines U937 and THP-1. Proliferation of these cells after brief exposure of LeTx (250 ng/ml LF and 500 ng/ml PA for 5 h) was analyzed for 4 days by measuring the dilution of intracellular fluorescence dye CFSE in dividing cells. LeTx temporarily inhibited cell proliferation of both U937 and THP-1 cells for up to 2 and 3 days, respectively (Fig. 1A). Cell cycle analysis using PI chromosome staining showed that number of cells in G<sub>0</sub>-G<sub>1</sub> phase of cell cycle was increased, whereas numbers in S and G<sub>2</sub>-M phase were greatly diminished in LeTx-treated THP-1 and U937 cells (Fig. 1B).

**LeTx Causes a Rapid Down-regulation of Cyclin D1, Cyclin D2, and Chk1 mRNA Levels through MEK1 Inhibition**—To identify signaling molecules involved in the LeTx-induced cell cycle arrest, we examined 25 different cell cycle-related proteins using Kinetworks™ multi-immunoblotting analysis in LeTx-treated THP-1 cells. Among them, protein levels of cyclin D1 and Chk1 were most significantly diminished by LeTx (Fig. 2A). Both protein and mRNA levels of cyclin D1 rapidly dropped in 3 h and maximally diminished within 6 h; whereas, the decrease in protein and mRNA levels of Chk1 were less dramatic and maximally decreased 24–48 h after an LeTx treatment (Fig. 2B and C). The rapid decrease of cyclin D1 in mRNA and protein levels was likely due to inhibition by LF at the transcriptional levels and the short half-life of the mRNA and protein. It is unlikely that cyclin D1 was directly targeted by LF, because cells treated with the proteasome inhibitor MG132 (data not shown) or stably transfected with proteasome-mediated degradation-defected mutant cyclin D1 (cyclin D1<sup>T286A</sup>) were resistant to LeTx-induced cyclin D1 down-regulation (Fig. 2D).

Also cyclin D1 mRNA levels in cells treated with the transcription inhibitor actinomycin D were down-regulated with the same rate in both non-treated and LeTx-treated cells (data not shown).

Because LeTx cleaves the N-terminal end of MEKs and inhibits most downstream MAPKs, we examined whether inhibition of MAPKs could cause cell cycle arrest in THP-1 cells. Treatment of the MEK1 inhibitor U0126 (10 μM) for 24 h
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caused accumulation of cells at G₀-G₁ phase and prevented entering into S phase; whereas, the p38 MAPK inhibitor SB202190 (SB, 10 μM) had no effects on S phase progress, but it significantly prevented cells entering G₂ phase (Fig. 3A). Treatment of both U0126 and SB additively blocked cell cycle progression. A JNK inhibitor (10 μM) had no effect on cell cycle progression of THP-1 cells. These results indicate that LeTx prevents cells entering S phase and G₂ phase by inhibiting ERK1/2 and p38 MAPK, respectively. Similar results were detected in U937 cells. When THP-1 cells were treated with U0126, SB, or JNK inhibitor, only U0126 induced down-regulation of both cyclin D1 and Chk1, suggesting that the down-regulation of both cyclin D1 and Chk1 by LF was at least in part mediated through inhibiting MEK1 (Fig. 3B). In addition to cyclin D1, mRNA levels of cyclin D2 were similarly down-regulated by U0126 and LeTx, but not by SB or JNK inhibitor. LeTx had no effects on mRNA levels of cyclin B and cyclin E (Fig. 3C).

**Inhibition of MAPKs and TNF Production in Response to LPS by LeTx Were Permanent in Non-proliferating Cells**—We have previously shown that LF reside inside cells and continuously cleaves MEK1 for prolong periods (4–5 days) in murine macrophage cell line RAW264.7 cells (33). To examine the duration of LF activity in non-proliferating cells, PBMCs were briefly exposed to LeTx for 5 h, and cells were cultured in fresh media for up to 6 days. Each day, LeTx-exposed and non-exposed cells were treated with LPS (1 μg/ml) for 6 h, and TNF production was measured using a TNF bioassay as previously described (34) and MEK-1 N-terminal cleavage was examined using Western blots. Throughout the cell culture period, a brief exposure of LeTx continuously cleaved MEK1 (Fig. 4A, upper panel) and prevented TNF production in responses to LPS (Fig. 4A, middle panel). Because PBMCs are a heterogeneous population of cells, including monocytes, T cells, and B cells, we further characterized TNF-producing cells in PBMCs and found that almost all TNF-producing cells in day 1 were CD13⁺ myeloids and in day 6 were mostly CD3⁻ lymphocytes (data not shown). Most of the CD13⁻ cells were short-lived and did not survive after 24-h culture in both LeTx-treated and non-treated PBMCs (data not shown). To specifically examine in non-proliferating macrophages, mouse peritoneal macrophages were isolated from C57BL/6 mice harboring LeTx-resistant macrophages (17) and treated with LeTx. MEK1 was continuously cleaved by a brief exposure of LeTx for the next 6 days (Fig.

**FIGURE 2. Down-regulation of cyclin D1 and check point kinase 1 (Chk1) by LeTx through ERK1/2 in THP-1 cells.** A–C, LeTx induced pronounced down-regulation of Chk1 and cyclin D1 proteins and mRNAs. A, THP-1 cells were briefly exposed to LeTx (250 ng of LF and 500 ng of PA for 5 h), and protein levels of 25 cell cycle proteins were examined 48 h after the exposure using Kinetworks multi-immunoblotting analysis. The fold changes of protein levels in LeTx-treated cells compared with those of non-treated cells are shown. Data represent two separated experiments (mean ± S.D.). B and C, THP-1 cells were similarly treated with LeTx and protein levels of cyclin D1, Chk1, MEK1, and p38 MAPK (as a loading control) were analyzed at 0, 3, 6, 24, and 48 h after the treatments. C, similarly, mRNA levels of cyclin D1 and Chk1 were analyzed using real-time PCR analysis in LeTx-treated cells at various time periods as indicated in the panel. Data are expressed as mean ± S.D. (n = 3). D, cyclin D1 was not directly targeted by LF. Proteasome-mediated degradation-resistant mutant cyclin D1 (cyclin D1 T286A) or control vector was stably transfected in THP-1 cells. These cells were then exposed to LeTx (250 ng of LF and 500 ng of PA for 5 h), and the presence of cyclin D1 was examined 24 h after the treatment.

**FIGURE 3. LeTx induced cell cycle arrest by inhibiting ERK1/2 activity and down-regulation of cyclin D1, D2, and Chk1.** A, THP-1 cells were treated with different inhibitors for MEK1 (U0126, 10 μM), P38 MAPK (SB202190 (SB), 10 μM), and JNK (JNK inhibitor II (JNKi), 10 μM) for 2 days, and the cell cycle was analyzed using Modfit 3.0. Data are expressed as mean ± S.D. (n = 3). B and C, similarly, THP-1 cells were treated with the inhibitors or LeTx (250 ng of LF and 500 ng of PA for 5 h) and protein and/or mRNA levels of cyclin D1, and cyclin D2, Chk1, cyclin B, cyclin E were analyzed 24 h after the treatment using Western blotting or real-time PCR, respectively. B represents data of similar results observed in two independent experiments. C, data are expressed as mean ± S.D. (n = 3).
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4A, lower panel] by when cells started dying by senescence. TNF production in response to LPS was also abolished for the next 6 days (data not shown).

Cell Cycle Inhibition by LeTx Was Recovered in the Absence of ERK1/2 Activity in Monocytic Cells—The effect of LeTx is almost permanent in non-proliferating cells. If LeTx causes cell cycle arrest, the effect of LeTx should be permanent in these cells. However, when we examined MEK1 N-terminal cleavage and TNF production in response to LPS in LeTx-treated THP-1 cells, both MEK1 and TNF production were returned to normal levels in ~5 days after LeTx treatments (Fig. 4B). Based on Western blot analysis, LF inside cells was slowly degraded over ~3 days (Fig. 4C). LF was no longer detectable after 4 days of a LeTx treatment. We then examined whether cell cycle arrest by LeTx was alleviated before MEK1/ERKs activities were recovered. In fact, THP-1 cells resumed cell cycle before the third day even when LF was readily detectable by Western blots (Fig. 4C), and TNF production in response to LPS was completely blocked (Fig. 4B, lower panel). Cell cycle arrest was released after the second day and returned to almost normal levels after 3 days of a LeTx treatment (Fig. 4D). These results indicate that LeTx-exposed THP-1 cells underwent cell cycle progression in the absence of MAPK activities.

Cells Pre-exposed with LeTx Were Protected from Cell Cycle Arrest by Subsequent LeTx Treatment through Activation of PI3K—If cells adaptively adjusted themselves to LeTx and resumed cell cycle even in the absence of MEK activities, these cells should continue to proliferate even in the presence of further LeTx challenges. To examine whether these cells were indeed resistant to LeTx-induced cell cycle arrest, we briefly pre-treated cells for 5 h with LeTx and then treated them again 2 days after the pretreatment. Consistent with previous results, numbers of cells at S and G2-M phases were dramatically reduced by LeTx in 2 days, but LeTx-pretreated cells were resistant to cell cycle arrest by a subsequent LeTx-treatment (Fig. 5A, left panel). To examine whether the resistance to cell cycle arrest was due to inability of LeTx-exposed cells to incorporate LeTx into cells or MEK1-independent ERK1/2 activation, MEK-1 N-terminal cleavage and ERK1/2 phosphorylation were analyzed. Consistent with previous results in Fig. 4B, full-length MEK-1 and basal ERK1/2 phosphorylation were detected in 5 days after LeTx-exposure, which were cleaved and inhibited, respectively, by further LeTx treatment (Fig. 5A, right panel). These results indicate no defects of LeTx pre-exposed cells in incorporating LF into the cytosol and preventing ERK1/2 activation. Previous studies in hematopoietic cells have shown that PI3K activation rescues G1 cell cycle arrest (35). Thus, we examined whether PI3K inhibitors such as LY294002 (Ly) could prevent the adaptive response. Ly treatment alone in THP-1 cells had no effects on cell cycle progress. When LeTx-pretreated cells were treated with Ly (10 μM), the recovery from cell cycle entering S phase was completely prevented even after 4 days of LeTx treatment (Fig. 5B). Similar results were detected by another PI3K inhibitor wortmannin (data not shown). We then examine whether Akt, which is a downstream protein of PI3K signaling, was activated by LeTx. Indeed, LeTx induced Akt phosphorylation at the activating Ser-473 site, in 2 days of LeTx treatment, and it maximally phosphorylated Akt in day 3, which was gradually diminished over >6 days (Fig. 5B, upper panel). The Akt phosphorylation appeared to be mediated through PI3K, because Ly or wortmannin (Wort) was able to block the Akt activation (Fig. 5B, lower panel). To further confirm that Akt activation is involved in cell cycle re-initiation in the presence of LeTx, we analyzed cell cycle progress in THP-1 cells treated with LeTx for 2 days, followed by treating or not treating with the Akt inhibitor II (AktII) or in cells transfected with retrovirus vectors
Inhibition of GSK3β by Akt Stabilized Cyclin D1 Degradation and Protected from LeTx-induced Cell Cycle Arrest—Akt negatively regulates the GSK3α and -β isoforms by phosphorylating them on Ser-21 or Ser-9, respectively. We examined whether Akt activation by LeTx led phosphorylation of GSK3 and whether GSK3 inhibition protects LeTx-induced cell cycle arrest. Western blotting with a phosphospecific antibody for GSK3α/β readily detected only 46-kDa GSK3β in THP-1 cells, and the phosphorylation was further enhanced 2 days after LeTx treatment (Fig. 6A). The phosphorylation was diminished by Ly or Akt (Fig. 6A). Furthermore, THP-1 cells treated with LeTx together with the GSK3-specific inhibitor SB216763 were protected cells from cell cycle arrest induced by LeTx (Fig. 6B). GSK3β was shown to regulate cell cycle through cyclin D1 proteolysis (37).

Because GSK3β phosphorylates cyclin D1 on Thr-286 and causes proteasome-mediated degradation, we examine whether GSK3β inhibition by Akt stabilizes cyclin D1. THP-1 cells were non-treated or pretreated with LeTx for 24 h and then treated with the GSK3-specific inhibitor SB216763 for up to 16 h. Protein levels of cyclin D1 started to increase as early as 1 h of the treatment, whereas MEK1 was cleaved by LF (Fig. 6C, top panel). Cyclin D1 protein was accumulated with the similar rate by a proteasome inhibitor MG132 (Fig. 6C, bottom panel). However, cyclin D1 mRNA levels did not change by either treatment (data not shown). These results indicate that GSK3β inhibition by Akt at least in part stabilized cyclin D1 and overrode cell cycle arrest induced by LeTx in THP-1 cells.

Cell Cycle Progress Is Crucial for Recovery from Cellular LeTx Intoxication—LeTx intoxication was almost permanent in non-proliferating PBMCs and peritoneal macrophages (Fig. 4A). It took ~4–5 days for THP-1 cells to recover from LeTx intoxication (Fig. 4B), and their cell cycle resumed after 2 days of LeTx treatment. Therefore, it is expected that preventing or facilitating cell cycle recovery will result in slow or fast recovery from LeTx intoxication. LeTx-exposed THP-1 cells were treated by Ly 3 days after LeTx treatment to prevent cell cycle recovery (Fig. 5A), and recovery of MEK1 cleavage by LF was examined. Full-length MEK1 was readily detectible after 6 days of LeTx exposure, whereas Ly-treated LeTx-exposed cells failed to recover from MEK1 cleavage (Fig. 7A). Expression of cyclin containing inactive Akt mutant Akt (AktK179M), possessing an inhibitory mutation at the ATP-binding site) or membrane-targeted constitutively-active mutant Akt (Aktmyr, myristoylated Akt). The retroviral vectors also contained internal ribosome entry site sequences followed for green fluorescent protein, which allow sorting of only retrovirus-transfected cells (36). THP-1 cells treated with Akti or green fluorescent protein-positive AktK179M-transfected cells showed much slower recovery than non-treated or vector control-transfected cells; whereas, green fluorescent protein-positive cells transfected with Aktmyr recovered from cell cycle arrest much faster than vector control-transfected cells (Fig. 5C). Consistent with these results, protein levels of cyclin D1 and Chk1 were similarly recovered in 3 days after LeTx treatment, and Ly completely inhibited the spontaneous recovery of cyclin D1 and Chk1 in mRNA levels (data not shown) and protein levels (Fig. 5D). These results indicate that activation of PI3K and Akt in response to LeTx overrode cell cycle arrest induced by ERK1/2 MAPK inhibition, which is also required for recovery from cellular LeTx intoxication.
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A

B

C

D

FIGURE 6. Inactivation of GSK3β stabilizes cyclin D1 and prevents LeTx-induced cell cycle arrest. A, THP-1 cells were non-treated (CNT), treated with LeTx (250 ng of LF and 500 ng of PA for 5 h), or treated with LeTx plus Ly294002 (Ly, 10 μM) or Akt inhibitor (Akti, 5 μM). Two days later, phosphorylation of GSK3β on Ser-9 was analyzed using Western blotting. B, similarly, THP-1 cells were exposed to LeTx in the presence of absence of GSK3 inhibitor (GSK3i, 10 μM), and cell cycle progress was analyzed using Modfit 3.0 software according to the PI content in cells. C, similarly, THP-1 cells were exposed to LeTx and, 1 day later, cells were treated with GSK3 inhibitor (GSK3i, 10 μM) or proteasome inhibitor MG132 (10 μM) for different periods as indicated in the panel. N-terminal degradation of MEK1 or presence of cyclin D1 was analyzed using Western blotting. D, proteasome-mediated degradation-resistant mutant cyclin D1 (cyclin D1T286A) or control vector was stably transfected in THP-1 cells. These cells were then exposed to LeTx (250 ng of LF and 500 ng of PA for 5 h), and cell cycle progress was measured. Results shown in the figure are representative data of similar results observed in at least three independent experiments.

FIGURE 7. Recovery from LeTx intoxication requires a PI3K/Akt-mediated adaptive response. A, THP-1 cells were non-treated (CNT), treated with LeTx (250 ng of LF and 500 ng of PA for 5 h), and replated in fresh culture medium for the next 3 and 6 days. Ly294002 (10 μM) was added to the medium 3 days after initial LeTx treatment for day 6 plus Ly samples. N-terminal degradation of MEK1 was analyzed using Western blotting after 3 and 6 days of initial LeTx treatments. B, THP-1 cells stably transfected with proteasome-mediated degradation-resistant mutant cyclin D1 (cyclin D1T286A) or control vector were exposed to LeTx (250 ng of LF and 500 ng of PA for 5 h). Each day after treating LeTx, cells were harvest for MEK1 Western blotting (left panel) or treated with LPS (1 μg/ml) for 5 h, and production of TNF was measured using a TNF bioassay (right panel). Data show mean ± S.D. (n = 3). A and B, results shown in the figure are representative data of similar results observed in two independent experiments.

D1T286A prevented LeTx-induced cell cycle arrest (Fig. 6D) and induced faster recovery from LeTx intoxication. THP-1 cells stably transfected with cyclin D1T286A recovered from LeTx-induced MEK1 cleavage and suppression of TNF production in response to LPS much faster than vector transfected cells (Fig. 7B).

Inhibition of GSK3 Stabilized Cyclin D1 and Facilitated LeTx Recovery in Bone Marrow-derived Macrophages—We further examined whether cell cycle progress also facilitated recovery from LeTx intoxication in non-transformed cells. Bone marrow-derive murine macrophages proliferate in vitro for several weeks in the presence of macrophage-colony stimulating factor or IL-3, which requires ERK activities (38). LeTx also cleaved MEK1, down-regulated cyclin D1 (Fig. 8A), and induced cell cycle arrest in these cells (data not shown). After 4 days of a LeTx treatment, these cells also spontaneously recovered from MEK1 cleavage and cyclin D1 down-regulation. Such recoveries were not detected in cells treated with Ly, whereas cells treated with GSK3-specific inhibitor SB216763 increased cyclin D1 protein levels and facilitated recovery from MEK1 cleavage (Fig. 8A). In consistence, TNF production also returned to ~70% of normal levels after 4 days of a LeTx treatment (Fig. 8B, right panel). In cells treated with Ly, TNF production was still completely blocked even after 4 days of a LeTx treatment. In contrast, cells treated with SB216763 produced TNF up to ~50% of normal levels even in 2 days after a LeTx treatment (Fig. 8B, left panel). These results suggest that the PI3K/Akt/GSK3 signaling cascade is required for recovering from cellular LeTx intoxication and sustaining cell cycle progress by stabilizing cyclin D1 facilitated recovery from the intoxication.

DISCUSSION

LeTx was shown to inhibit cell cycle progress in monocytic cell lines (22), melanocytes (23), activated B cells (24), and activated T cells (25–27). This study further showed that LeTx prevented entry of S phase human monocytic cell lines through inhibition of ERK1/2 (Fig. 1). LeTx receptors have been shown to be widely expressed (8); therefore, cell cycle arrest by LeTx is likely to occur in a number of cell types that require basal ERK1/2 activ-
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Inhibition of GSK3 stabilizes cyclin D1 and facilitated LeTx recovery in bone marrow-derived macrophages. Bone marrow-derived macrophages from C57BL/6 mice were exposed to LeTx (250 ng of LF and 500 ng of PA for 5 h) in the presence or absence of GSK3 inhibitor (GSKi, 10 μM) or Ly294002 (Ly, 10uM). A, N-terminal degradation of MEK1 or presence of cyclin D1 was analyzed using Western blotting after 2 or 4 days of a LeTx treatment. Results shown in the figure are representative data of similar results observed in two independent experiments. B, production of TNF in response to LPS (1 μg/ml) was measured 5 h after treating LPS in these cells using TNF bioassay. Data show mean ± S.D. (n = 3).

Longevity for proliferation. ERK1/2 was shown to be required for cell cycle progression from G1 to S phase through multiple mechanisms (39). Using Kinetworks™ multi-immunoblotting analysis and real-time PCR analysis, we found that LeTx down-regulated cyclin D1 and Chk1 protein levels >10-fold (Fig. 2, A–C), likely through inhibiting transcriptions of their genes (Fig. 2, C and D). Because the cyclin D1 promoter contains a functional AP-1 binding site (40), it is possible that basal ERK1/2 activities were required for maintaining appropriate levels of cyclin D1 by phosphorylating AP-1 proteins such as c-Jun, JunB, JunD, and c-Fos in THP-1 cells. Indeed, the MEK1 inhibitor U0126, which specifically prevents ERK1/2 activation, also down-regulated mRNA and protein levels of cyclin D1 and Chk1 (Fig. 3, B and C). Regulation of cyclin D2 expression by ERK1/2 is less understood, but B cells were shown to require cyclin D2 accumulation through B-cell receptor-mediated ERK1/2 activation for proliferation (41). The essential role of cyclin D1 and D2 for escaping from G1 and initiation and completion of S phase through activating cyclin-dependent kinases 2, 4, and 6 has been documented as well (42). The down-regulation of Chk1 by LeTx was also likely mediated through inhibiting ERK1/2 activity (Fig. 3, B and C). Chk1 was shown to play a key role in the G2/M checkpoint in DNA damage response (43). Activation of Chk1 upon DNA damage prevents G2/M transition by inhibiting activation of Cdc25, a dual phosphatase, resulting in inhibition of Cdc2 kinases (44). Chk1 was also shown to be required for cell cycle progress for S and G2/M phases in normal cell growth (45, 46), but cellular requirement for Chk1 may be different at various developmental stages and cell types (47, 48). The Chk1 inhibitor SB218078 (1 μM) did not prevent cell cycle progression in THP-1 cells (data not shown), and expression of cyclin D1 (42) alone was able to override cell cycle arrest induced by LeTx (Fig. 6D), indicating that basal Chk1 activity is not required for normal cell cycle progress in THP-1 cells.

Previously we have shown that LF stays inside cell and continuously cleaves MEKs for 4–5 days after a brief exposure of LeTx in murine macrophage cell line RAW264.7 cells (33). When LeTx was briefly exposed to non-proliferating human PBMCs or murine peritoneal macrophages, the MEK1 cleavage and inhibition of TNF production in response to LPS lasted as long as the cells survived in culture (Fig. 4A). If LeTx causes cell cycle-arrest, it is expected that the LeTx effects are similarly prolonged as non-proliferating cells. However, intact MEK1 became visible, and TNF response to LPS was recovered in 4–5 days after LeTx treatment in THP-1 cells (Fig. 4B). In fact, cell cycle of LeTx-treated THP-1 cells progressed before day 3 of LeTx treatment, even before MEK1 cleavage was alleviated (Fig. 4D). Furthermore, THP-1 cells pre-exposed to THP-1 cells were resistant to cell cycle arrest by subsequent LeTx challenges (Fig. 5A). This phenomenon is a reminiscent of the adaptive response to LeTx-induced cytosis in murine macrophages (33, 49). Certain murine macrophage cell lines or primary macrophages originated from inbred mice undergo rapid NALP1b-dependent cytosis by LeTx (16, 17, 19). These macrophages adaptively respond to a low non-cytolytic dose of LeTx and become resistant to subsequent cytolytic doses of LeTx; this is termed toxin-induced resistance (33, 49). We have shown that toxin-induced resistance to cytosis is mediated through down-regulation of two mitochondrial cell death proteins, Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (Bnip3) and Bnip3-like (33). Human macrophages do not express NALP1b and are resistant to LeTx-induced cytosis (18, 21, 22). The adaptive response to LeTx-induced cell cycle arrest in THP-1 and U-937 cells was mediated through activating the PI3K/Akt signaling pathway (Fig. 5), and, more importantly, recovery from cell cycle arrest was a prerequisite for cellular recovery from the on-going MEK1 cleavage by LF (Figs. 5D and 7B). At this moment, it is unknown why LF can stay active for such a long period in the cytosol. A prolonged cellular intoxication of LF in non-proliferating cells indicates that the toxic effects of LeTx in a B. anthracis-infected host will last much longer than one may expect, considering that most tissues are terminally differentiated and not actively proliferating. One bolus administration of non-lethal dose of LeTx in mice was able to cleave MEK1 up to 7 days in various tissues such as heart, lung, liver, and kidney, although serum LeTx was cleared within 3 h after injection (data not shown).

Activation of the PI3K/Akt pathway was previously shown to regulate cell cycle progress through inducing cyclin D1 expression, cytoplasmic relocalization of the cyclin-dependent kinase inhibitor p27, and inhibiting transcriptional activation of a number of related forkhead transcriptional factors (FoxO1, FoxO3, and FoxO4). Several studies have also demonstrated that PI3K activation is sufficient to induce cyclin D1 expression in an ERK1/2-independent manner (50, 51) and override G1 arrest in hematopoietic cells (35). Given the importance of cyclin D1 in G1 to S phase cell cycle progress, it is not surprising that the adaptive response of THP-1 cells in cell cycle arrest was at least in part mediated through reinstating cyclin D1 protein via the PI3K/Akt/GSK3β pathway (Fig. 9). The half-life of
LeTx-induced Cell Cycle Arrest and Recovery

![Diagram of signaling pathways]

**FIGURE 9.** A diagram illustrating the LeTx-induced cell cycle arrest and recovery pathways. LeTx caused cell cycle arrest at least in part by inhibiting the MEK1 \(\rightarrow\) ERK1/2 \(\rightarrow\) cyclin D1 pathway, and possibly GSK3 activation, which is also catalyzed by MEK1/2. An adaptive response activating PI3K \(\rightarrow\) Akt stabilizes cyclin D1, overrides cell cycle arrest, and facilitates recovery from LeTx intoxication.

In proliferating cells, cyclin D1 turnover is due to ubiquitination and proteasome-mediated degradation of phosphorylated cyclin D1 (32). Cyclin D1 phosphorylation on Thr-286 was mediated by GSK3 (37), which is also catalyzed by MEK1/2. An adaptive response activating PI3K \(\rightarrow\) Akt stabilizes cyclin D1, overrides cell cycle arrest, and facilitates recovery from LeTx intoxication.

LeTx has been regarded as a key virulence factor in the pathogenesis of anthrax, causing immune paralysis, cell cycle arrest, and cell death in host immune cells. These effects could contribute survival and proliferation of *B. anthracis* within the host. Furthermore, the majority of the infected hosts eventually succumb despite bacterial clearance by antibiotics. The fatal effects are attributed to production and release of anthrax toxins. This study showed that LF resides inside cells and continues to cleave MEKs almost indefinitely in non-dividing cells, indicating an extensive period of LF intoxication in cells. The recovery from the effects of LeTx can be facilitated by activating PI3K/Akt/GSK3β signaling-mediated adaptive responses. Reagents activating the signaling pathways or inhibitors of GSK3β can be novel tools for treating for LeTx toxemia, whereas reagents that inhibit the adaptive signaling pathway can be detrimental to the host.

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