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Critical role of PI3-K/Akt/GSK-3β signaling pathway in recovery from anthrax lethal toxin-induced cell cycle arrest and MEK cleavage in macrophages

Dennis Y.M. Ng

Critical role of PI3-K/Akt/GSK-3β signaling pathway in recovery from anthrax lethal toxin-induced cell cycle arrest and MEK cleavage in macrophages

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By

Dennis Y.M. Ng

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Faculty of Graduate Studies
The University of Western Ontario
London, Ontario

ABSTRACT

Lethal toxin (LeTx) is a critical virulence factor of *B. anthracis* causing immune suppression and toxic shock of the infected host. It inhibits cytokine production and cell proliferation/differentiation in various immune cells. In the present study, I demonstrated that a brief exposure to LeTx caused a continual MEK1 cleavage and prevented TNF-α production 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 G_0-G_1 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 PI3K/Akt signaling pathway. Activated Akt inhibited GSK3 and prevented proteasome-mediated cyclin D1 degradation in LeTx-intoxicated THP-1 cells. Recovery from cell cycle arrest was required before recovering from on-going MEK1 cleavage and suppression of TNF-α production. This study demonstrated that modulation of PI3K/Akt/GSK3 signaling cascades can be beneficial for protecting or facilitating recovery from cellular LeTx intoxication in cells that depend on basal MEK1 activity for proliferation.

Keywords

Bacillus anthracis, lethal toxin, Akt, phosphatidylinositol 3-Kinase, macrophage, THP-1, cell cycle, cyclin D1, glycogen synthase kinase- 3

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ABBREVIATIONS

Akt	protein kinase B
Akti	Akt Inhibitor II
AP	activator protein
APC	antigen presenting cell
B. anthracis	*Bacillus anthracis*
B. megaterium	*Bacillus megaterium*
BMDM	bone marrow derived macrophage
CDK	Cyclin-dependent kinase
CFSE	carboxyfluorescein succinimidyl ester
CHK1	checkpoint protein kinase-1
CMG2	cellular receptors capillary morphogenesis gene 2
CNT	Control
CRM1	chromosome region maintenance protein
CSF-1	colony stimulating factor-1
CT	carboxyl-terminus (C-terminus)
DC	dendritic cell
E. coli	*Escherichia coli*
EdTx	Edema toxin
EF	Edema factor
ERK	extracellular signal regulated kinase
FBS	fetal bovine serum

FPLC	fast performance liquid chromatography
g-CSF	granulocyte-colony stimulating factor
GI	Gastrointestinal
GSK3	glycogen synthase kinase 3
GSKi	SB216763 (GSK3 inhibitor)
hPBMC	human peripheral blood mononuclear cell
IFN-	interferon-
IL	Interleukin
IP	Intraperitoneal
ITAM	immunoreceptor tyrosine-based activation motif
JNK	c-Jun amino-terminal kinase
JNKi	JNK inhibitor II
LeTx	lethal toxin
LF	lethal factor
Ly	Ly294002 (PI3K inhibitor)
MAPK	mitogen-activated protein kinase
m-CSF	macrophage colony stimulating factor
MEK	mitogen-activated protein kinase kinase
MEKK	mitogen-activated protein kinase kinase kinase
MVB	multivesicular body
MWCO	molecular weight cutoff
Nalp1b	NACHT-leucine rich repeat pyrin domain containing protein 1b
NLR	NOD-like receptor

ND	not detected
NT	amino-terminus (N-terminus)
PBS	phosphate buffered saline
PDK1	3-phosphoinositide-dependent protein kinase-1
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol 4,5-bisphophate
PIP3	phsophatidylinositol 3,4,5-triphsphate
PMA	phorbol myristate acetate
pRb	Retinoblastoma
PTEN	Phosphatase and tensin homolog
PTK	protein tyrosine kinase
qPCR	Quantitative polymerase chain reaction
RTK	tyrosine kinase receptor
SAPK	stress-activated protein kinase
SB	SB202190 (p38 inhibitor)
SHIP	SH2-containing inositol phosphatase
SOS	son of sevenless
TCR	T cell receptor
TEM8	tumor endothelial marker 8
TH-1	type 1 T-helper cells
TNF-α	tumor necrosis factor- α
TRE	12-O-tetradecanoylphorbol-13-acetate response element

UO126	MEK1 and MEK2 inhibitor
VC	vector control

CHAPTER 1 - INTRODUCTION

1.1. Anthrax

Bacillus anthracis, the causative agent of anthrax, is a Gram-positive, spore forming bacterium found ubiquitously in the soil. Distinct from other strains of bacteria within the *Bacillus* family, *B. anthracis* is encapsulated by a poly-D-glutamic acid layer and is capable of secreting potent cytotoxic toxins known as anthrax lethal toxin (LeTx) and edema toxin (EdTx) [1]. Anthrax is a zoonotic infectious disease that has plagued animals and humans for centuries. The incidences of anthrax outbreaks are generally higher in developing countries with poor or ineffective public health systems. According to a report published by the Centers for Disease Control and Prevention, areas currently ranked with high risks of anthrax include Asia, Africa, the Caribbean, the Middle East, South and Central America, Southern and Eastern Europe [2]. However, anthrax outbreaks do occur in developed countries as well. In 2006, Canada had a major anthrax outbreak in Saskatchewan where dozens of farms were under quarantine and over 200 animals were affected by the disease in the province.

Natural occurrence of anthrax in humans is rare mainly because the bacterium or spores are normally found in soil, but herbivores commonly contract the disease by ingesting the organism through grazing. Human can be infected through contacts with diseased animals or by consuming uncooked and infected meat. Generally, anthrax can be divided into three types based on the routes of entry: cutaneous, gastrointestinal and inhalation anthrax [1-4].

1.1.1. Cutaneous anthrax

Cutaneous anthrax is the most common form of the disease, and it occurs following deposition of spores through abrasions on the skin. After spores germinate in skin tissues, local edema causes the formation of a papule that resembles a pimple. Within a few days, the papules develop into fluid filled vesicles and when ruptured they form a painless, depressed, black ulcer termed an eschar. Eschar formation gave rise to the name *anthracis*, derived from the word "*anthrakis*" meaning coal in Greek to describe the black ulcer characteristic of cutaneous anthrax infection. All of these symptoms allow clear diagnosis of cutaneous anthrax, and treatment with various antibiotics, although they do not change the course of eschar formation and healing, can generally ensure complete recovery. If left untreated, however, infection can spread through the lymphatic system causing septicemia, with a mortality rate of up to 20% [1, 5].

1.1.2. Gastrointestinal anthrax

Gastrointestinal (GI) anthrax occurs frequently after ingestion of uncooked meat from infected animals [6]. It is generally a result of pre-existing lesions in the upper or lower gastrointestinal tract, where entries of spores or a large quantity of vegetative bacteria are possible. Symptoms of an upper GI infection can include oral or esophageal ulceration leading to the development of regional lymphadenopathy, edema, and sepsis [7, 8]. Infection in the lower gastrointestinal tract can cause nausea, vomiting, acute abdominal pain, bloody diarrhea, and sepsis. These symptoms resemble many other GI diseases making early diagnosis difficult and leading to a relatively high mortality rate [1, 9].

1.1.3. Inhalation anthrax

Inhalation anthrax is considered to be the most deadly form of anthrax among the three routes of infection. *B. anthracis* can produce spores under unfavorable conditions and these spores can survive in extreme environments for extended periods of time [9, 10]. Inhaled anthrax spores enter the lung and deposit into the alveolar space. The spores are then phagocytosed by macrophages, some of which are lysed and destroyed while surviving spores are transported to mediastinal lymph nodes via the lymphatic system [4, 11]. Spores can remain dormant for variable amounts time up to 60 days, and the condition or factors that initiate spores' germination to vegetative cells is not known. Once germination occurs, clinical symptoms follow rapidly with the secretion of exotoxins, leading to hemorrhage, necrosis, and edema [1, 12, 13]. Studies have shown that once toxin production reaches a critical threshold, death occurs even though the bloodstream has been cleared of bacteria with antibiotics [14]. Initial symptoms of inhalation anthrax are mild and flu-like, but abrupt progression of the disease leads to hypotension and toxemia. Diagnosis of inhalation anthrax is difficult, and treatment must be administered early to prevent progression of the disease [9]. The rapid disease progression along with the ability for spores to survive in harsh environment make anthrax a good candidate to be developed into a biological weapon. Eleven cases of inhalation anthrax were reported in the 2001 terrorist attack in the US where anthrax spores were sent out through the postal service. Despite extensive antibiotic treatment, five people were killed in the incident which initiated an immediate renewed interest into the study of anthrax [15].

1.2. *B. anthracis* virulence factors

There are two major virulence factors that contribute to *B. anthracis* pathogenicity: a poly-D-glutamic acid capsule and anthrax exotoxins. These factors are encoded by two different plasmids (pXO1 and pXO2) [16]. The poly-D-glutamic acid capsule encoded in pXO2 is composed of negatively charged molecules, providing the vegetative *B. anthracis* protection against phagocytosis by the host phagocytes [16, 17]. The two exotoxins, known as anthrax lethal toxin (LeTx) and edema toxin (EdTx), expressed by pXO1, are secreted during infection and have been shown to be critical in leading to the host demise during anthrax infection [9, 16, 18]. Each toxin is comprised of two proteins where protective antigen (PA) and edema factor (EF) act together to form EdTx, while PA and lethal factor (LF) form LeTx. Both toxins additively cause toxic effects to the host, causing extensive tissue damage and systemic immune impairment [19].

1.2.1. Anthrax toxins

PA is a 83kDa protein, and its main function is to facilitate the transport of EF and LF inside mammalian cells. To date, two host receptors, the tumor endothelial marker 8 (TEM8) and the cellular receptor capillary morphogenesis gene 2 (CMG2), have been identified to bind PA [20]. They are type 1 transmembrane proteins with an extracellular region that can bind directly to PA. CMG2 was shown to be expressed ubiquitously in many different cell types, whereas TEM8 expression is limited to macrophages, endothelial and cancer cells [21]. The current model of toxin entry shows that full length PA (PA83) binds to TEM8 or CMG2 and is cleaved by a furin-like protease. Processed PA (PA63) spontaneously heptamerizes to form a prepore structure on the plasma membrane where 3 moieties of LF or /EF can bind to the PA heptamer.

The complex is taken up through clathrin-mediated endocytosis, where the toxin-receptor complex is trafficked as an intraluminal vesicle and fuses with early endosomes. Recent studies have suggested that the vesicles continue to traffic through the multivesicular bodies and progress into late endosomes where they can back fuse with endosomal-limiting membrane to release toxin molecules into the cytoplasm [14, 20, 22].

1.2.2. Anthrax edema toxin (EdTx)

Edema factor is a calcium- and calmodulin-dependent adenylate cyclase that utilizes the host calmodulin and calcium to catalyze the formation of cyclic AMP from ATP. Historic studies have shown that direct subcutaneous injection of edema toxin in mice is non-lethal, but could induce localized edema and tissue damage [16, 23]. However, more recent studies utilizing highly purified EF have shown that EdTx alone could cause widespread tissue damage, multi-organ failure and even death in BALB/cJ mice.

1.2.3. Anthrax lethal toxin (LeTx)

LeTx is believed to be the fatal factor in systemic anthrax because injection of LeTx alone can mimic many symptoms exhibited in an actual infection [24-26]. In fact, antitoxins such as chemical LF inhibitors or neutralizing antibodies against PA have been developed as treatments for anthrax, and the efficacy of such treatments strongly supports the notion that LeTx is a major contributor of anthrax fatality [27]. LF is a zinc-dependent metalloproteinase that had been shown to cleave mitogen-activated protein kinase (MAPK) kinases (MEK) 1-4, 6 and 7 of the MAPK signaling pathway [19]. LF is composed of an N-terminal PA-binding domain, a central substrate binding domain and a

C-terminal catalytic domain. The catalytic domain has common metalloproteinases His-Glu-X-X-His (X represents any amino acid) amino acid sequence that constitutes the zinc binding and catalytic activity [18, 27, 28]. Previous studies have shown that mutations of the catalytic residues eliminate the toxicity of LF, suggesting that the proteinase activity is critical for its toxic effects [29-31]. To date, MEKs are the only identified direct targets of LeTx which cleaves several N-terminal amino acid residues of MEKs. The N-terminus of MEKs stabilizes the association between their substrates and the activation loop of MEKs, promoting the phosphotransferase activity of MEKs [32]. Therefore, LeTx inhibits MEK activity, subsequently blocking activation of downstream MAPKs. The cleavage motif recognized by LF lies within the MAPK docking site (D-site) on MEKs, consisting of a basic amino acid center flanked by hydrophobic residues on either side. Even though MAPKs also possess similar LF cleavage sequence, LeTx does not cleave MAPKs because a functionally conserved Carboxyl-terminal region of MEKs has been shown to be required for LF activity [27, 33].

1.3. MAPK intracellular signaling pathway

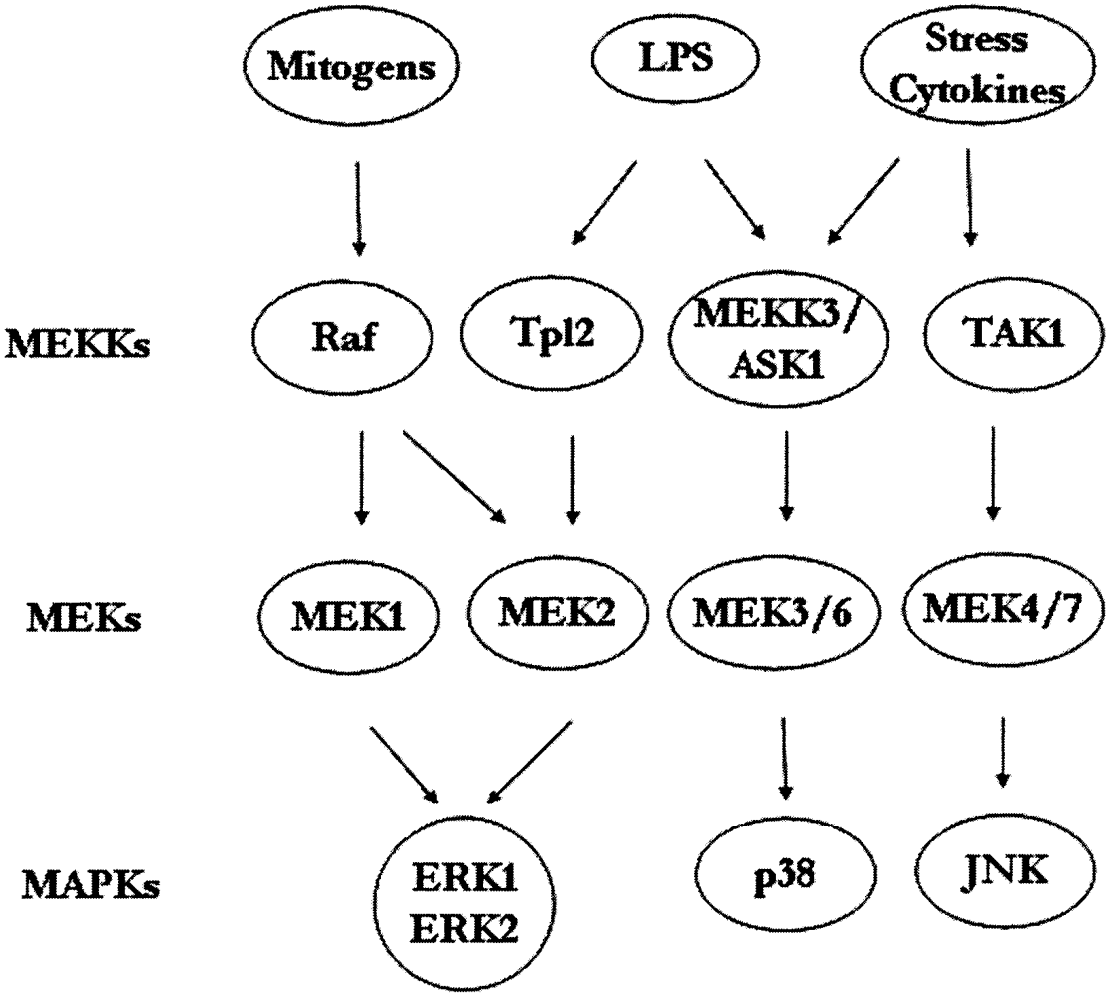
MAPK pathways process extracellular signals, such as mitogens, environmental stress or various cytokines, triggerd by extracellular receptors, to various cellular effector molecules [34-37]. The signaling events, in a simplified model, initiate when activation of a cell surface receptor leads to phosphorylation of MAPK kinase kinases (MEKK). Activated MEKKs then phosphorylate downstream MAPK kinases (MEK) and in turn, leads to the activation of various MAPKs, ultimately resulting in the transcriptional up-regulation of many critical genes of the immune system and in cell activation (Figure

1.1). In mammals, five distinct groups of MAPKs have been characterized, and, among them, the extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK 1 and 2), p38 MAPK isoforms and c-Jun amino-terminal kinases (JNKs) have been most extensively studied [34-37].

1.3.1. ERK1 and ERK 2

In most cell types, growth factors induce mitogenic signals from the cytoplasm into the nucleus through the nuclear translocation of ERK 1 and 2, also known as p44 and p42 MAPK, respectively [38]. Activation of ERKs is typically mediated through receptor tyrosine kinases (RTKs) where activating signals are transmitted through various membrane associated proteins. Mitogens stimulate auto-phosphorylation of RTKs and lead to the recruitment of SOS (son of sevenless). SOS is a guanine nucleotide exchange factor and stimulates a small guanosine triphosphate (GTP)-binding protein, Ras, to promote the conversion of GDP into GTP (GTP-Ras) [39]. GTP-Ras interacts with downstream serine/threonine kinase Raf (MEKK). Activated Raf binds to and phosphorylates MEK1 and MEK2 to promote the activation of ERK1 and 2 [38, 39]. ERK1 and 2 are expressed ubiquitously, and can phosphorylate numerous substrates involved in a number of biological functions including cell differentiation [40], smooth muscle cells contraction [41], tissue repair [42], and regulation of different early response genes through direct activation of various transcription factors [35]. ERK1 and 2 have also been implicated to play major roles in cell proliferation through interaction with various cell cycle associated proteins [43, 44]. In terms of immune function, ERK activation is required

Figure 1.1 . Mitogen Activated Protein Kinase Signaling Pathway. MAPK pathway is used by cells to recognize extracellular stimuli and produce various physiological responses. Activation of cell surface receptors leads to phosphorylation of MEKKs which leads to phosphorylation of MEKs and activation of MAPKs.


Mitogens
LPS
Stress Cytokines
MEKKs
Raf
Tpl2
MEKK3/ ASK1
TAK1
MEKs
MEK1
MEK2
MEK3/6
MEK4/7
MAPKs
ERK1 ERK2
p38
JNK

for differentiation of monocytes into macrophages [40], cytokine secretion in dendritic cells [45], and T cells selection and maturation process [46].

1.3.2. p38 MAPK

In contrast to the ERK1 and 2 signaling pathway, the p38 MAPK pathway is strongly activated by environmental stresses such as ischemia, hypoxia or inflammatory cytokines such as interleukin (IL) - 1 and tumor necrosis factor-α (TNF-α) [47, 48]. Four isoforms of p38 MAPK have been identified (α, , and ϵ), where p38α and p38 MAPKs are ubiquitously expressed in many different cell types. p38α MAPK is predominantly expressed in monocytes and macrophages [35]. Activation of MEKKs such as MEKK1-4, MLK-2 and -3 by extracellular stimuli induces phosphorylation of MEK3 and MEK6. Activation of MEK6 phosphorylates all isoforms of p38 MAPKs, while MEK3 selectively activates p38α and ß MAPK isoforms [34, 35, 49]. Activated p38 MAPKs can affect numerous substrates to induce biological responses. In macrophages and neutrophils, p38 MAPK participates in respiratory burst activity, chemotaxis, granular exocytosis, adherence and apoptosis [35]. In T cells, p38 MAPK activation mediates differentiation and regulation of interferon- (IFN-) induced apoptosis. Therefore, p38 MAPK activity is critical for regulation of normal immune and inflammatory responses [49, 50].

1.3.3. JNK

Since JNK can be activated by many stress related stimuli, JNK has also been referred to as stress-activated protein kinases [51]. However, more recent studies suggested

that JNK could also be activated by growth factors and various cytokines, and it has been shown that JNK could directly phosphorylate various transcription factors and regulate numerous non-nuclear proteins; therefore JNK signaling has much more diverse biological functions than previously thought [37, 47]. In mammalian cells, there are three JNK genes (*Jnk1, Jnk2* and *Jnk3*) with 10 or more different splicing variants. Phosphorylation of JNK requires activation of MEK4 and MEK7, which are themselves activated by several MEKKs. JNK signaling has been known to be required for induction of apoptosis in T cells and also participates in type 1 T-helper (TH1) cell differentiation [52]. Other studies have also found that JNK activation is required for differentiation, growth and survival of macrophages in response to stimulation by colony stimulating factor-1 [53].

1.4. Effects of LeTx on immunity

MAPK signaling cascades are involved in virtually all immune cells functions. Since LeTx receptors are ubiquitously expressed, LeTx can target and inactivate MAPK signaling cascades in almost all immune cells, causing general immune paralysis of infected hosts.

1.4.1. Effects of LeTx on the innate immunity

Neutrophils, macrophages and dendritic cells are phagocytic cells of the innate immune system and are likely the first immune cells to encounter spores and germinating bacteria. Neutrophils circulate through the vascular system in large numbers, representing approximately 70% of peripheral blood leukocytes in humans [54]. Upon

detection of an infection, neutrophils immediately exit the vasculature and migrate toward the site of infection through a process known as chemotaxis [54-56]. They engulf foreign materials that are encountered, and internalized microbes are destroyed through production of superoxide. The action of chemotaxis in neutrophils requires assembly of filamentous actins, mediated through the activation of ERKs and p38 MAPKs [56]. Furthermore, the activation of the NADPH oxidase complex, required for superoxide production, also depends on MAPK signaling. Therefore, inactivation of MEKs by LeTx impairs both chemotaxis and superoxide production, resulting in great defects in the bactericidal activities of neutrophils [57].

Macrophages are important inflammatory mediators capable of producing pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 in response to infection. Like neutrophils, macrophages are phagocytes and can engulf bacteria, virus-infected cells and tumor cells [54]. Macrophages express high levels of cell-surface toll-like receptors (TLRs), allowing them to readily recognize microbial components such as lipopolysacharide (LPS) from Gram negative bacteria, lipoteichoic acids from Gram positive bacteria and viral particles [58, 59]. TLRs activate MAPKs to promote the production and release of pro-inflammatory cytokines [60]. LeTx inhibits MAPK signaling and thereby suppresses the induction of numerous pro-inflammatory cytokines, chemoattractants (e.g. IL-8 and RANTES) and inflammatory mediators [e.g. inducible nitric oxide synthase (iNOS)] [61-63]. Incapacitated macrophages allow anthrax spores to germinate within the phagosomes, and the vegetative bacteria is released to flourish under a severely impaired immune system [62].

Dendritic cells (DCs) bridge innate immunity to the adaptive immune system [54]. Even though macrophages are also capable of ingesting foreign antigenic materials for processing and presenting on the cell surface to other immune cells, DCs are the primary antigen presenting cells (APCs) because of their potency in activating T cells and B cells [64, 65]. Like macrophages, DCs also express many TLRs to detect various microbial components. Detection and ingestion of pathogens promote DCs into a maturation state where engulfed microbes are disintegrated and processed into protein fragments for presentation on the cell surface through MHC II molecules [65, 66]. Studies have shown that LeTx exerts profound inhibitory effects on DCs by causing impairment of the MAPK pathways [67, 68]. Furthermore, up-regulation of costimulatory molecules such as CD40, CD80 and CD86, which are critical for induction of adaptive immunity are also severely impaired [68].

1.4.2. Effects of LeTx on the adaptive immunity

Cells within the adaptive immune system also depend on MAPK pathways for proper function and, therefore, are affected by LeTx as well. The adaptive immune system is mainly comprised of two types of specialized leukocytes: B lymphocytes (B-cells) and T lymphocytes (T cells). They circulate through the blood stream but are concentrated in the spleen, lymph nodes, and other lymphatic tissues [54]. Activation of the adaptive immunity requires signals from the innate immune system to provide the information on a particular antigen in order to generate efficient and selective immune responses.

The primary function of B cells is production of antibodies against specific antigens. Induction of B lymphocyte differentiation from naïve cells into antibodies producing plasma cells begins when antigen binding to the B cell receptor occurs on the cell surface. Recruitment of protein tyrosine kinases (PTK) to activated B-cell receptors allows phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) within each Igα and Ig cytoplasmic tail, leading to the production of diacylglycerol and inositol-3,4,5-phosphate through activating phospholipase C and the activation of MAPK through indirectly activating MEKK 3 and 6 [69, 70]. The effects of LeTx on B-cells have not been fully explored, but it has been demonstrated that LeTx inhibits LPS-induced proliferation and IgM production in murine splenic B-cells [71]. Given that B-cell activation requires MAPK activation, LeTx potentially inhibits B-cell differentiation and compromises humoral immunity.

T cells are an important subset of lymphocytes effective against host cells that have been infected with viruses or parasites [54]. T cell receptors (TCRs) found on the surface of T cells mediate the recognition of antigen fragments that are bound to MHC molecules on APCs. Stimulated T cells further expand and differentiate into effector T cells mounting an immune response known as cell-mediated immunity [54]. It has been shown that activation of ERK1 and 2 is important for T cell proliferation [72], while p38 MAPKs promote TH1 cells differentiation and IFN- production [49]. Several studies examining LeTx effects on T cells have indeed reported impairments of T cell proliferation [73] and inhibition of CD69 (early T cells activation marker) and CD25 (IL2 receptor) expression [74]. Cytokine production including TNF-α, IL-2, IL-5 and IFN- in activated T cells is also prevented by LeTx [74]. T-cells are derived from hematopoietic

stem cells in the bone marrow and mature in the thymus through a series of selection mechanisms to ensure proper TCR rearrangement and reactivity. Although there is no direct evidence suggesting that LeTx impairs T cell maturation, MAPKs are involved in T cell selection process that eliminates thymocytes harboring non-functional or auto-reactive TCRs [46, 75]. LeTx is likely to affect T cell development through MAPKs inhibition.

1.5. Mechanism of LeTx-induced cytotoxicity

1.5.1. LeTx-induced macrophage cell death

Among the studies of anthrax and host immunity, LeTx killing of murine macrophages has received the most attention and controversy [76-79]. In the early years, macrophages had been thought of as the primary target of LeTx mainly because macrophages from certain strains of mice have shown remarkable sensitivity toward LeTx [80, 81]. Macrophages isolated from sensitive mouse strains exhibit necrotic cell death within 5hrs after LeTx exposure, in contrast to apoptotic cell death observed in the resistant strains [82]. This striking difference in sensitivity had been one of the main focuses of anthrax research for decades. Recently, positional cloning between sensitive and resistant strains of mice identified that highly polymorphic NACHT-leucine rich repeat and pyrin domain containing protein 1b (*Nalp1b)* allele which confers susceptibility phenotypes to LeTx in murine macrophages [83]. The *NALP* genes encode members of the NOD-like receptor (NLR) family involved in caspase-1 activation and rapid necrosis-like cell death. NALP1b is an intracellular innate immune receptor that recognizes microbial components. Activation of NALP1b induces formation of a multi-

protein complex containing NALP1b and caspase-1, termed the inflammasome. Caspase-1 recruited to the inflammasome is autocatalycally activated and cleaves pro-IL-1ß and pro-IL-18 to mature IL-1ß and IL-18, respectively [84, 85]. Human macrophages do not exhibit necrotic cell death when exposed to LeTx, but they have been shown to undergo programmed cell death under activating conditions [62, 86]. Therefore in human infection, LeTx may promote immune impairment in macrophages to allow *B. anthracis* spores to germinate within.

1.5.2. LeTx-induced cell cycle arrest

Aside from immune impairment, LeTx has also been shown to inhibit proliferation of different cells including melanocytes [87], B lymphocytes [71] and T lymphocytes [73, 88], which is likely mediated by targeting MAPK signaling cascades. To date, the detailed mechanisms of cell cycle arrest induced by LeTx and the impact of cell cyle arrest in the pathogenesis of anthrax are largely unknown.

1.5.2.1. Role of cyclins in cell cycle progression

Cell cycle progression is generally divided into four phases: G_0/G_1, S G_2 and M phases. In G_0-phase, cells are in a quiescent state where cells are non-dividing. Once a growth signal has been received, cells prepare for replication by manufacturing the required protein machinery during the G_1-phase. When preparations are ready, cells move into the S-phase to begin DNA replication. In G_2-phase, cells focus on protein synthesis such as the production of microtubules required for division. Finally in the M-phase cells divide into two daughter cells and return to the G_1 or quiescent state (57).

Progression of each phase is tightly coordinated by numerous cell cycle associated proteins including cyclins and cyclin-dependent kinases (CDKs). Cyclin proteins are transiently expressed to bind and regulate corresponding CDKs. During G_1 phase, D-type cyclins (D1, D2 and D3) are especially important; in particular cyclin D1, whose expression is governed by a variety of mitogen-activated signals, binds to CDK4 or CDK6 to facilitate phosphorylations of the tumor suppressor retinoblastoma protein (pRb) (92, 93). There are 16 possible phosphorylation sites found on pRb, but CDK4 and CDK6 have been shown to preferentially phosphorylate Thr821 and Thr826 [89]. In G_0 or early G_1-phase, pRb remains unphosphorylated and binds to transcription factors such as E2F to prevent transcriptional activation of genes involved in nucleotide metabolism and DNA synthesis [90]. Phosphorylation of pRb by CyclinD-CDK4 or CDK6 complex inhibits its repressing activity and induces E2F transcriptional actvivity. Among the E2F-regulated genes, Cyclin E and Cyclin A2 are key effectors of the G_1 to S-phase transition. Cyclin E binds CDK2 to facilitate subsequent pRb phosphorylation in place of CyclinD-CDK 4 or 6, while cyclin A associates with CDK2 to regulate DNA synthesis [91]. During the S phase, cyclin B accumulates and interacts with cell cycle regulator CDK1 to promote mitosis and rapid ubiquitin-mediated degradation of cyclin B leads to the exit from the M phase and completion of the cell cycle [92].

1.5.2.3. Role of MAPKs in cell cycle progression

The Ras/Raf/MEK/ERK pathway has a well established role regulating G_1 to S-phase cell cycle progression in response to mitogenic stimulation. Studies have shown that ERK1 and 2 phosphorylation leads to induction of cyclin D1 protein and regulates

G1-phase progression [43, 93]. ERK1 and 2 signaling mediates the activation of multiple transcription factors including Elk1, c-Jun, c-Myc and c-Fos. These transcription factors control the expression of genes critical to cell cycle progression. Specifically c-Jun and c-Fos genes encode proteins that can heterodimeize to form members of the activator protein (AP)-1 family. AP-1 is a transcription factor that regulates many early response genes and has been described as the modulator of proliferation, differentiation and apoptosis. The AP-1 complex binds to a palindromic 12-O-tetradecanoylphorbol-13-acetate response element, which is found in the promoter region of many genes, including cyclin D1, and regulates their expression [93]. Furthermore, ERK 1 and 2 signaling can phosphorylate and inactivate a cyclin D1 transcriptional co-repressor (Tob) [94]. Tob binds the promoter of cyclin D1 and recruits histone deacetylase to inhibit transcription activity [95]. Thus, ERK 1 and 2 activation can strongly influence cyclin D1 level required for progression of G_1 to S-phase. Other than cyclin D1 expression, activation of ERK 1 and 2 also greatly enhances the stability of transcription factor c-Myc through direct phosphorylation. Similar to AP-1, c-Myc also plays a central role in cell cycle progression and apoptosis. c-Myc activates various genes encoding ribosomal units and translation factors, required for cell cycle progress. Together as a heterodimer with Max protein, they can directly activate various cell cycle targets including Cyclin D2 [96], CDK4 [97] and cell regulator p21 [98]. p21 was originally thought to inhibit proliferation, but further studies have shown that p21 can induce or repress the activity of different cyclin/CDK complexes according to the phase of the cell cycle [98].

1.5.2.4. PI3K/Akt/GSK3β regulates cell cycle progression

The phosphatidylinositol 3-kinase (PI3K)/Akt/Glycogen synthase kinase 3β (GSK3β) signaling pathway is involved in various cellular functions including proliferation, differentiation and motility [99-103]. PI3Ks are a family of enzymes that catalyse the phosphorylation of the 3-position of the inositol ring in phosphoinositide substrates [104]. On the basis of structural and functional homologies, PI3Ks have been classified into three major classes: I, II, and III [100]. Discussed here, class I PI3Ks represent the major signal transduction pathway downstream of cell-surface receptors, which can be activated by growth factors such as insulin or IL-3 [105]. PI3K phosphorylates its substrate phosphatidylinositol 4,5-bisphophate (PIP_2), converting it into phosphatidylinositol 3,4,5-triphsphate (PIP_3). PIP_3 is an important intracellular lipid secondary messenger, and can interact with the pleckstrin homology domain of Akt (also known as protein kinase B) recruiting Akt to the plasma membrane. Akt binds to PIP_3 and undergoes a conformational change to allow 3-phosphoinositide-dependent protein kinase-1 (PDK1) to phosphorylate threonine Thr-308 and PDK2 to phosphorylate serine Ser-437 on Akt [99, 106]. This pathway can be inactivated by SH2-containing inositol phosphatase (SHIP) which dephophorylates PIP_3 at the 5-position to produce phosphatidylinositol 3,4-bisphosphate, and by tumor suppressor phosphatase and tensin homolog (PTEN) at the 3-position to produce phosphatidylinositol 4,5-bisphosphate [100]. Since the PI3K/Akt pathway plays critical roles in the regulation of cell cycle progression, overactivation of PI3K or constitutive activation of Akt is frequently observed in a wide range of human tumor cells [107].

Akt is the primary mediator of PI3K signaling through interaction with numerous effectors to promote a variety of functions [100, 108]. GSK3β is one of the major

downstream components of Akt mediating cell cycle progress [109]. Akt activation directly phosphorylates the Ser-9 residue on GSK3 and inhibit its activity [109]. Normally, active GSK3 phosphorylates cyclin D1 on Thr-286 to promote recruitment of a nuclear exporter chromosome region maintenance protein-1 (CRM1) [110]. CRM1-mediated export of cyclin D1 to the cytoplasm leads to ubiquitin-mediated proteolytic degradation of cyclin D1 [110]. Thus, Akt indirectly facilitates the stability of cyclin D1 through inactivation of GSK3ß. In addition, Akt has been shown to inactivate the CDK2 inhibitor p27 through direct phosphosphorylation on Thr157 to promote its nuclear export [100, 111-114]. While CDK2 is required to drive the S and M phase of the cell cycle, Akt-induced p27 nuclear export prevents CDK2 inhibition and cell cycle arrest. Lastly, the PI3K/Akt pathway has also been demonstrated to inhibit transcriptional activities of a number of related forkhead transcriptional factors (FoxO1, FoxO3, and FoxO4) through direct phosphorylation on multiple Ser and Thr residues [115]. Forkhead transcriptional factors induce expression of proapoptotic genes including p27 and Fas in mammalian cells, thereby promoting cell cycle arrest or cell death [116].

1.6. Hypothesis and rationale

LeTx is a major virulence factor of *B. anthracis* causing immune paralysis and toxemia of the infected host. LeTx targets and inactivates MEKKs which are critical signaling molecules involved in various cellular activities, particularly in cell cycle progression. Previous studies have shown that LeTx causes cell cycle arrest in certain cell types including monocytes, melanocytes, B lymphocytes and T lymphocytes. However, the detailed mechanisms of cell cycle arrest induced by LeTx have not yet been

addressed. I **hypothesized that LeTx induces cell cycle arrest in proliferating cells through MAPK inhibition, and activation of the PI3K/Akt/GSK3β signaling should promote cell cycle re-initiation after LeTx exposure.** In order to address the hypothesis, I first assessed the duration of LeTx cytotoxicity by measuring MEK1 cleavage and examined cell cycle progression in different immortalized immune cells and primary macrophages. In addition, I have also characterized LeTx-induced cell cycle arrest in macrophages and attempted to dissect the mechanism of inhibited proliferation by LeTx. Secondly, I have examined whether activation of PI3K/Akt /GSK3 pathway was involved in the spontaneous recovery of LeTx-induced cell cycle arrest, and if artificial activation or inhibition of the PI3K/Akt/GSK3 pathway could manipulate the outcome of the recovery from LeTx-induced cytotoxicity in macrophages.

CHAPTER 2 – MATERIALS AND METHODS

2.1. Cell Culture and Reagents

The human monocytic leukemia cell lines THP-1 and U937 cells were grown in complete RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (Sigma), 10mM minimal essential medium non-essential amino acids solution, 100 units/ml penicillin G sodium, 100 g/ml streptomycin sulfate, and 1 mM sodium pyruvate. Cells were grown at 37 °C in the humidified atmosphere of 5% CO_2. LY294002, Akt inhibitor II, SB202190, U0126, and JNK inhibitor I were purchased from Calbiochem (EMD Biosciences, La Jolla, CA). Chk1 inhibitor (SB218078) and GSK3 inhibitor (SB216763) was obtained from Sigma. Pan and phospho-site specific antibodies toward Akt, p38 MAPK, ERK 1 and 2, MEK1 (COOH terminus of MEK1), and GSK3a/β were obtained from Cell Signaling Technologies (Pickering, Ontario, Canada). The antibody raised against the N-terminus of MEK1 was from QED Bioscience Inc. MEK3 and Chk1 antibodies were purchased form Santa Cruz Biotechnology, and cyclin D1 and LF antibodies were from Neo Markers (Fremont, CA) and List Biological Laboratories (Campbell, CA), respectively.

2.2. Preparation of anthrax lethal toxin

Protective antigen (PA) and lethal factor (LF) were purified as described [117, 118]. PA was purified from the *E. coli* expression strain BL21 (DE3) bearing the appropriate plasmid construct (pET22B-PA). Overnight cultures were added to Luria broth containing 50 g/mL ampicillin. Cultures were grown with shaking at 37°C until an OD_{600} of 1.0 was

reached. IPTG was added to 0.5mM, and incubated with shaking at 30°C for an additional 3 - 4 h. Cells were harvested by centrifugation, resuspended in a hypertonic solution with 30mM Tris-HCl pH8.0, 20% sucrose, 1mM EDTA incubated at room temperature for 10 min. Cells were pelleted again and resuspended in an isotonic solution with 20mM of $MgSO_4$ and incubated on ice for 30min. LF was purified from *Bacillus megaterium* transformed with LF expressing plasmid (pWH1520-LF). Overnight cultures were added to Luria broth containing 10ug/mL tetracycline. Cultures were grown with shaking at 37°C until an OD_{600} of 0.3 was reached. Expression of LF was induced by adding 0.5% xylose into the culture and grown for an additional 8h. PA containing cell lysate and LF containing culture media were precipitated with 80% $(NH_4)_2SO_4$, and the precipitated proteins were pelleted by centrifugation. The pellets were resuspended in 20mM Tris-HCl with 1mM PMSF, and dialyzed using dialysis tubing 30,000MWCO (Sigma). Samples were loaded onto a Q-Sepharose anion exchange column (Amersham) and proteins were isolated with an AKTA FPLC chromatographic system. Active fractions were combined and dialyzed with PBS.

2.3. Preparation of Human and Murine Macrophages

2.3.1. Human peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from whole blood using Ficoll-Paque Plus (Amersham Biosciences). Blood obtained from volunteers was diluted with 3 volumes of phosphate-buffered saline and layered on top of the Ficoll at a 3 to 1 ratio. Blood cells were separated into different strata, and the lymphocyte layer was extracted. Isolated PBMCs

were washed three times with phosphate-buffered saline and plated with complete RPMI 1640 media.

2.3.2. Mouse peritoneal macrophages

Mice received intraperitoneal injections of 3% thioglycollate 4 days before the preparation of peritoneal macrophages. Macrophages were harvested from mice by injecting 5 ml of saline into the peritoneal cavity and re-extracted by withdrawal with a syringe. Cells were put through a cell strainer (40 m, BD Biosciences) and incubated at 37 °C overnight in a humidified atmosphere of 5% CO_2.

2.3.3. Bone marrow-derived macrophages

Bone marrow cells were obtained from C57BL/6 mice as previously described [119]. In brief, the femurs and tibiae were isolated from the animal and cleared of tissue. Bone marrow cells were flushed out with DMEM. Cells were put through a cell strainer (40 m, BD Biosciences). Bone marrow cells were propagated in complete DMEM medium containing 10% heat-inactivated fetal bovine serum (Sigma), 10mM minimal essential medium non-essential amino acids solution, 100 units/ml penicillin G sodium, 100 g/ml streptomycin sulfate, and 1 mM sodium pyruvate, supplemented with 1nM IL-3 and 0.22nM recombinant murine CSF-1 (eBioscience) for 7 days. Fibroblast and mature macrophages were removed by subtractive adherence to the plates. For the maturation process, IL-3 and the recombinant CSF-1 were replaced with 20% L929-conditioned medium as the source of CSF-1. L929-conditioned medium was prepared from confluent plates of L929 fibroblasts split 1:10 and cultured in complete RPMI with 20% FBS for

10-14 days. The medium was removed and filtered through a 0.2μm membrane (Nalgene).

2.4. Total Cell Lysate Preparation and Western Immunoblot Analysis

Total cell lysate extraction and Western blotting analysis were performed as previously described [120]. Briefly, cells were lysed in ice-cold cell lysis buffer containing 20 mM MOPS, 15mM EGTA, 2mM EDTA, 1mM Na3VO4, 1 mM dithiothreitol, 75 mM -glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 g/ml aprotinin, 10 g/ml pepstatin A, 1 g/ml leupeptin, and 1% Triton X-100 and then sonicated on ice. Cell extracts were obtained by centrifuging the homogenate at 18,000 x 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).

2.4.1. Kinetworks™ multi-immunoblotting

Protein multi-immunoblotting analysis was performed at Kinexus Bioinformatics Inc. (Vancouver, Canada) as described on the Kinexus website. Kinetworks™ is a multi-immunoblotting technology developed by Kinexus Bioinformatics Corporation (Vancouver, Canada). It relies on sodium dodecyl sulphate (SDS)-polyacrylamide minigel electrophoresis and multilane immunoblotting to permit the specific and quantitative detection of many proteins with one given sample. In this study we used

Kinetworks™ Cell Cycle Protein 1.0 Screen to simultaneously monitor the protein expression of 25 cell cycle-regulated proteins.

2.5. Quantitative Real-time PCR

Expression of mRNAs was 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 g of total RNA was reverse transcribed by using oligo (dT) primers and the M-MuLV reverse transcriptase (NewEngland Biotechnology) according to the manufacturer's recommendations.

2.5.1. Oligonucleotide primers for real-time PCR:

cyclin D1

5'-CCCTCGGTGTCCTACTTCAA-3' (5'primer) and

5'-AGGAAGCGGTCCAGGTAGTT-3' (3' primer);

cyclin D2

5'-GTCTCAAAGCTTGCCAGGAG-3' (5' primer) and

5'-ATATCCCGCACGTCTGTAGG-3' (3' primer);

CHK1

5'-CTGAAGAAGCAGTCGCAGTG-3' (5' primer) and

5'-TTGCCTTCTCTCCTGTGACC-3' (3' primer);

2.7. 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 (1 M) for 10 min at 37 °C, and the 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 X 10^6 cells were harvested at the indicated time points, fixed by dropwise addition of ice-cold 70% ethanol after three times washing with 1X phosphate-buffered saline containing 0.1% glucose, and were stored at 4 °C. Subsequently, cells were pelleted by centrifugation and resuspended in staining solution containing 50 g of PI/ml and 100 units of RNase A/ml. After 60 min of incubation at room temperature, cells were loaded in a FACSCalibur flow cytometer. To determine in which phase of cell cycle LeTx inhibited proliferation, cell cycle profiling was performed 2 days after brief exposure of LeTx in U937 and THP-1 cells. During the G_0/G_1 phase of the cell cycle, only a single pair of chromosomes is present giving a peak with low PI intensity. In S phase, chromosomes are being replicated; hence the PI signal is spread out due to the variable amount of DNA present in the cells. Finally cells in G_2/M phase have 2 sets of chromosomes show up as a peak with PI intensity twice of that in G_0/G_1 phase. 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.

2.8. Akt^{myr} and Akt^{K179M} Retroviruses Generation and Infection

Constitutively active myristoylated Akt (Akt^{myr}) and dominant negative Akt (Akt^{K179M}) retrovirus expression vectors were kindly provided by Dr. Michael Croft (La Jolla Instititue for Allergy and Immunology, San Diego, CA)[121]. The virus preparation and infection were performed as described [122]. Briefly, retroviruses were generated in Phoenix Amphotropic producer cells using the calcium phosphate method. Empty retroviral vector was infected 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. $1x10^6$ THP-1 cells were suspended with virus solution containing Polybrene 4 g/mL 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 was added into the each well after 3-h incubation at 37 °C.

2.9. TNF Bioassay

TNF-α was measured in the supernantant by a L929 cytotoxicity bioassay. This assay is based upon quantification of the cytotoxic activity of TNF-α on L929 cells in the presence of cycloheximide. Live cells adhered to the 96-well plates and were stained with crystal violet as an indicator of viability. L929 cells were seeded at a density of 4×10^4 cells per well into a 96-well microtiter plate and cultured for overnight in RPMI complete medium. For quantification of TNF-α production, a standard curve was prepared by the addition of recombinant TNF-α 0.75 to 50 pM to the cells. The plates were then incubated with 200 g/mL cycloheximide for an additional 24 h at 37°C.

2.10. *In vivo* LeTx challenge

Specific pathogen-free male and female C57BL6/J mice 4 to 10 weeks old were purchased from the Jackson Laboratories (Bar Harbor, Maine). In one set of experiment, 27 animals were injected through intraperitoneal injection with LeTx (PA 100ug and LF 75ug) for 1, 3, 5, 7 and 10 days. Mice were anesthetized with a mixture of ketamine 100mg/mL and xylxene 100mg/mL through IP injection and sacrificed by cervical dislocation. Liver, spleen, thymus, heart, lung, bone marrow and peripheral blood were harvested. Tissues were weighed and homogenized with a sonicator in lysis buffer containing 20 mM MOPS, 15mM EGTA, 2mM EDTA, 1mM Na3VO4, 1 mM dithiothreitol, 75 mM -glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 g/ml aprotinin, 10 g/ml pepstatin A, 1 g/ml leupeptin, and 1% Triton X-100. Protein levels were analyzed by Bradford Assay and normalized before immunoblotting.

CHAPTER 3 – RESULTS

3.1. Cytotoxic effects of LeTx in proliferating and non-proliferating macrophages.

3.1.1. LeTx inhibits TNF-α production in different subsets of hPBMC.

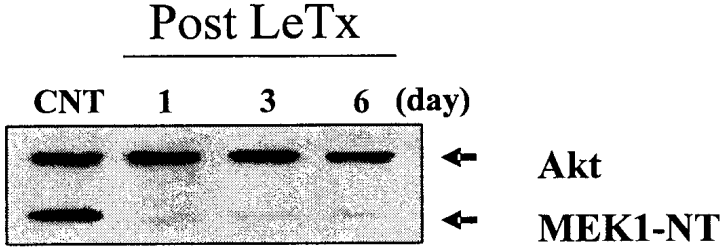
Freshly isolated human peripheral blood mononuclear cells (hPBMC) were briefly treated with LeTx (250 ng/ml LF and 500 ng/ml PA for 5 h) and the status of MEK1 cleavage using Western blots against the amino-terminus of MEK and the amount of TNF-α production in response to LPS were monitored for 6 days after the treatment. LeTx cleaved MEK1 (Figure 3.1 A) and prevented the production of TNF-α in response to LPS (Figure 3.1 B) throughout the duration of the experiment. Since hPBMC are heterogeneous in cell types, I further examined which types of cells were affected by LeTx in the production of TNF-α induced by LPS. Cells were double labeled for either CD13 or CD3 and TNF-α using fluorescently-conjugated antibodies. FACS analysis of the cells showed that major TNF-α producing cells in 1 day cultured hBPMC were $CD13^+$ (likely monocytes), whereas, in 6 day cultured cells, almost all TNF-α producing cells were $CD3^+$ (likely T cells) (Figure 3.2). A single brief treatment of LeTx was able to prevent TNF-α production in both cell populations.

3.1.2. Prolonged LeTx cytotoxicity in primary peritoneal macrophages.

Since monocytes in hPBMC survive only for a short time (~ 1 day) in culture medium [123], I used primary peritoneal macrophages isolated from mice to examine the duration of LeTx effects in macrophages. These macrophages survive up to 7 days in culture medium before undergoing apoptosis. Similar to what was observed in hPBMCs, a single brief LeTx treatment (PA 500 ng/ml and LF 250 ng/ml for 5 h) caused prolonged

Figure 3.1. Persistent effects of LeTx in hPBMC. A) Human PBMCs were treated with LeTx (PA 500ng + LF 250ng) for 5 hr and cultured in fresh medium for 6 days. MEK-1 N-terminal cleavage was examined at different time points as indicated and total Akt was used as loading control. B) Production of TNF-α was measured 5 h after LPS (1 μg) treatment following 1, 3, 6 days after brief initial LeTx exposure. The presence of TNF-α in the culture medium was measured using bioassays as previously described in materials and methods. Data show mean ± S.D. (n = 3)

A)


Post LeTx
CNT
1
3
6
(day)
Akt
MEK1-NT

B)

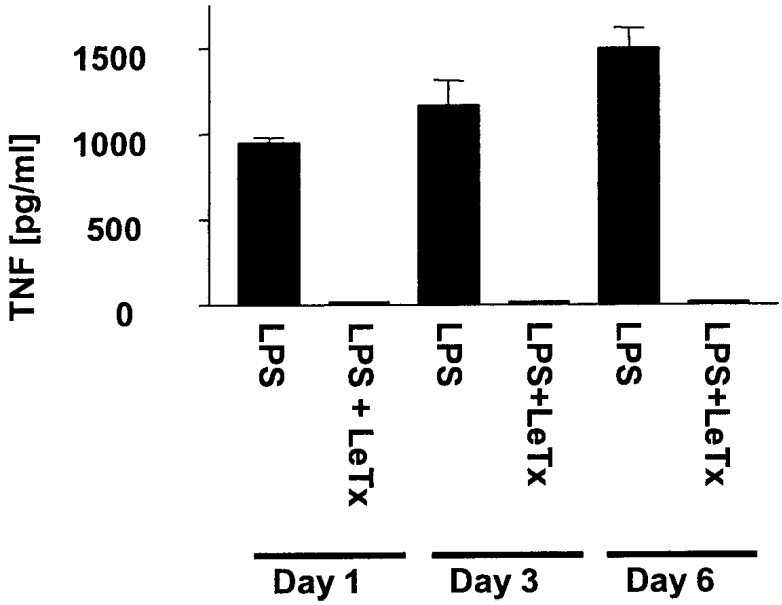
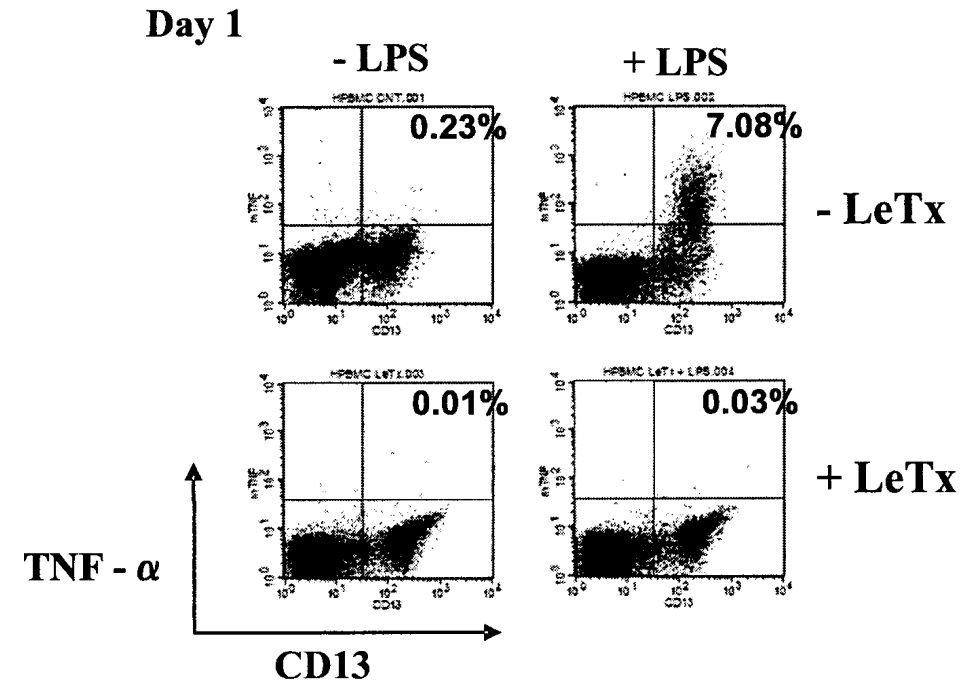
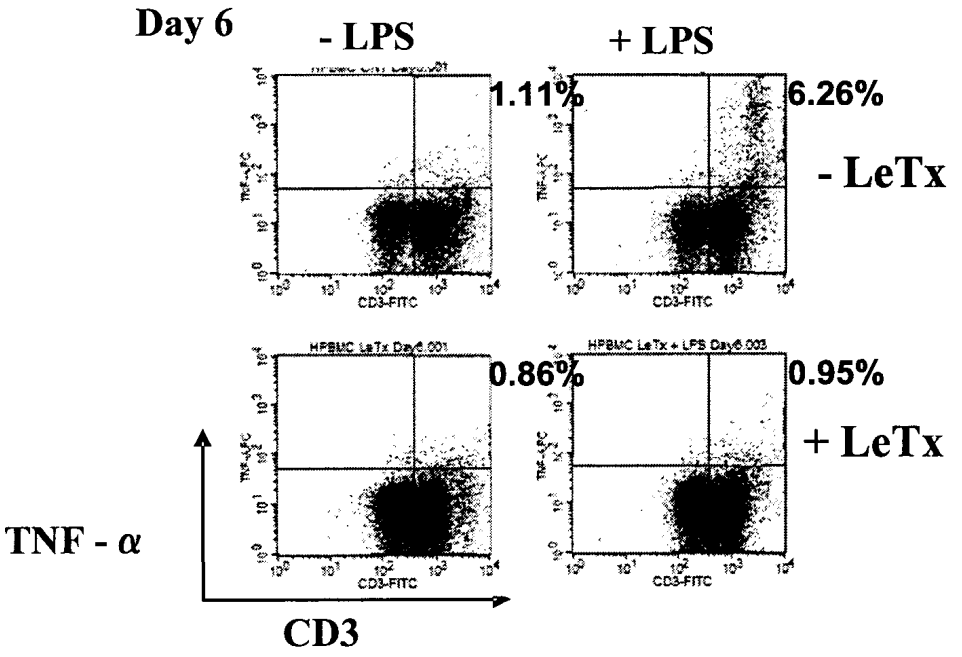

TNF [pg/ml]
1500
1000
500
0
LPS
LPS + LeTx
LPS
LPS+LeTx
LPS
LPS+LeTx
Day 1
Day 3
Day 6

Figure 3.2. LeTx inhibits TNF-α production in different subsets of hPBMC. Cells were briefly treated with LeTx (PA 500ng and LF 250ng 5h) and cultured in fresh medium for 6 days. Cells were treated with LPS (1ug) for 5h to stimulate TNF-α production on 1 and 6 days after LeTx. Cells were incubated with fluorescence antibodies against TNF-α, CD3 (T cells) and CD13 (myeloid cells). Samples were analyzed by flow cytometry.


Day 1
- LPS
+ LPS
0.23%
7.08%
- LeTx
0.01%
0.03%
+ LeTx
TNF - α
CD13


Day 6
- LPS
+ LPS
1.11%
6.26%
- LeTx
0.86%
0.95%
+ LeTx
TNF - α
CD3

MEK1 cleavage and defects in producing TNF-α in response to LPS for 6 days (Figure 3.3 A&B).

3.1.3. Transient LeTx cytotoxicity in proliferating murine BMDM and THP-1 cells.

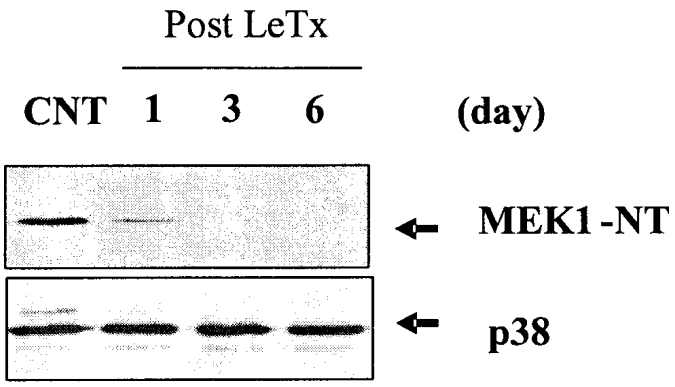
To examine the effect of LeTx on macrophage proliferation, bone marrow derived murine macrophages (BMDM) and the human monocytic cell line THP-1 cells were used. BMDM were derived from bone marrow cells isolated from C57BL/6J mice and cultured in the presence of IL-3 (100 U/mL) and GM-CSF (200 U/mL). These cells carry the LeTx-resistant NALP1b allele, which is similar to human macrophages in LeTx cell death sensitivity are more suitable for our study, can proliferate for about 3 weeks after isolation. These cells were briefly exposed to LeTx (250 ng/ml LF and 500 ng/ml PA) for 5 h, and cultured on fresh culture dishes and medium for 6 days. THP-1 cells phenotypcally resemble human mature monocytes [124]. Unlike non-proliferating cells, LeTx-induced MEK1 cleavage was transient and intact MEK1 was detected as early as 3 days after the LeTx treatment (Figure 3.4A). TNF-α production in response to LPS was also restored to normal levels as early as 4 days after the LeTx treatment in both BMDM and THP-1 cells (Figure 3.4B).

3.1.4. Effects of LeTx on different mouse tissues *in vivo*.

To further examine the effects of LeTx in mice, C57BL/6J mice were injected intraperitoneally with a sub-lethal dose of LeTx (PA 100ug and LF 75ug). Mice were monitored daily for signs of abnormal behavior or symptoms. Although all of the mice injected with LeTx or PBS survived without obvious abnormal behaviors,

Figure 3.3. Persistent effects of LeTx in Murine macrophages. A) Peritoneal macrophages were isolated from C57BL/6J mice as described in materials and methods. Cells were treated with LeTx (PA 500ng and LF 250ng) for 5 h and cultured for 6 days. MEK-1 N-terminal cleavage was examined for different time points as indicated. Western blot for p38 MAPK was used as a loading control. B) Production of TNF-α in response to LPS (1μg) was measured 5 h after LPS treatment of cells for 5 days after initial LeTx exposures. The presence of TNF-α in the culture medium was measured using bioassays as previously described in materials and methods. Data show mean ± S.D. (n = 3)

A)


Post LeTx
CNT 1 3 6 (day)
MEK1-NT
p38

B)

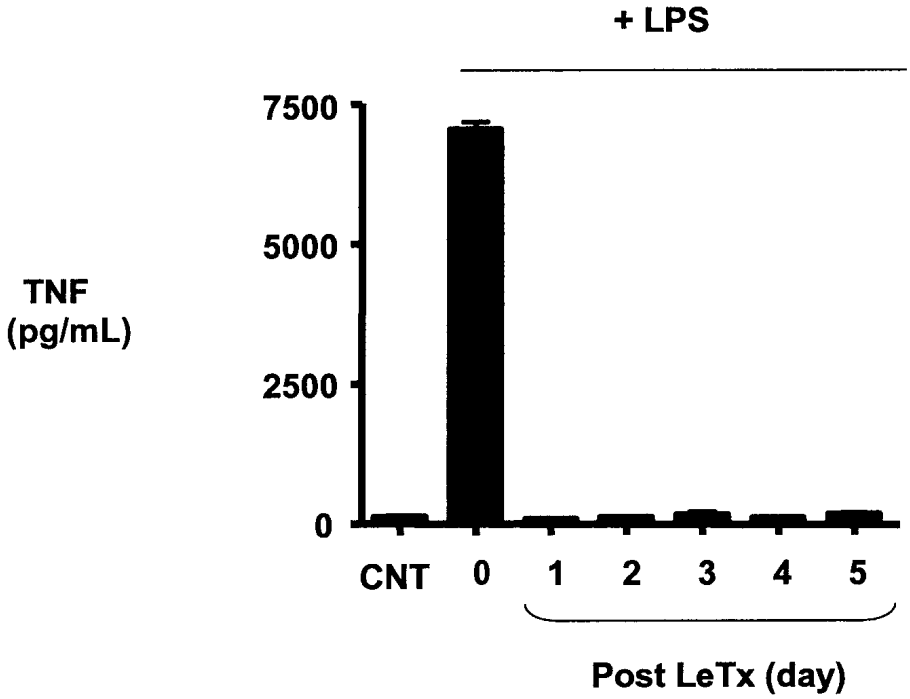
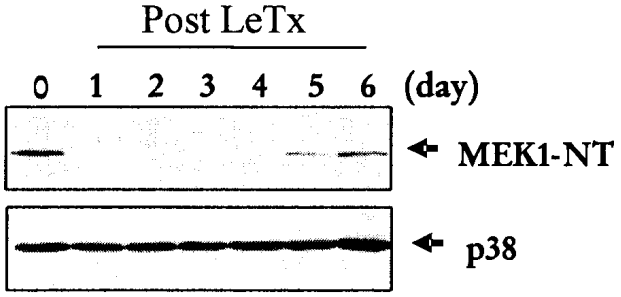
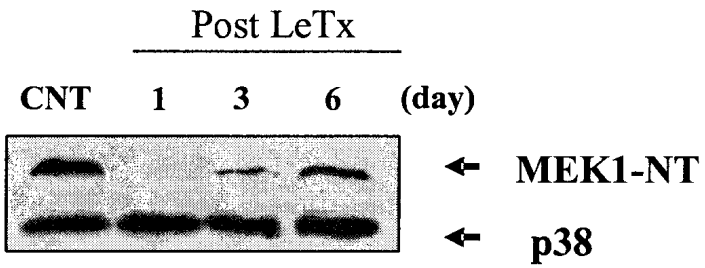

+ LPS
TNF (pg/mL)
7500
5000
2500
0
CNT 0 1 2 3 4 5
Post LeTx (day)

Figure 3.4. Transient effects of LeTx in THP-1 and murine BMDM. Human monocytic THP-1 cells (Panel A) and murine BMDM (Panel B) were treated with LeTx (PA 500ng + LF 250ng) for 5 hr and cultured in fresh medium for 6 days. MEK-1 N-terminal cleavage was examined for different time point as indicated. Western blot for p38 MAPK was used as a loading control. C) THP-1 TNF-α production in response to LPS (1μg) was measured 5 h after LPS treatment of cells for 5 days after brief initial LeTx exposures. The presence of TNF-α in the culture medium was measured using bioassays as previously described in materials and methods. Data show mean ± S.D. (*n* = 3)

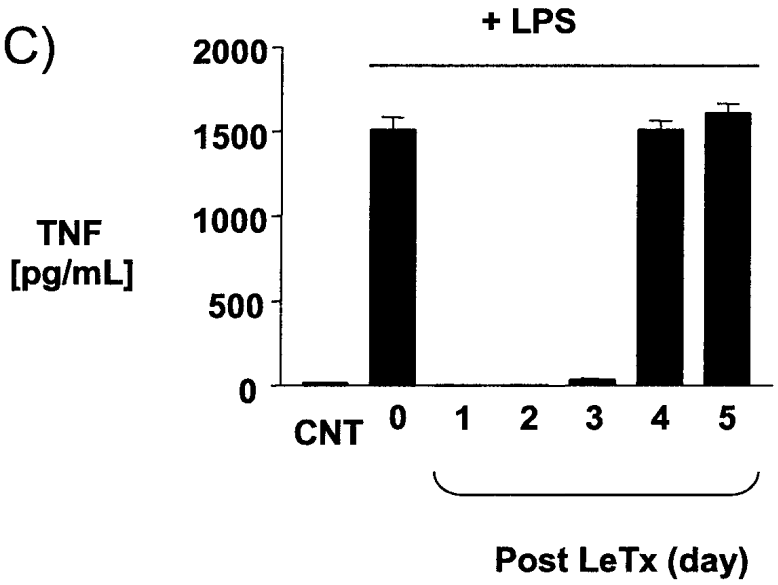
A)


Post LeTx
0 1 2 3 4 5 6 (day)
MEK1-NT
p38

B)


Post LeTx
CNT 1 3 6 (day)
MEK1-NT
p38

C)


+ LPS
2000
1500
1000
500
0
TNF
[pg/mL]
CNT 0 1 2 3 4 5
Post LeTx (day)

the animals showed signs of hepatomegaly and hypovolemia (based on difficulties in drawing blood). LeTx was shown to cause leaky endothelium leading to exudation of circulating blood into the peritoneal or thoracic cavities [125]. Mice were sacrificed 1, 3, 5, 7 and 10 days after LeTx injection, and the heart, lung, spleen, thymus, bone marrow and peripheral blood were harvested to examine MEK1 cleavage. A bolus intraperitoneal LeTx injection was able to cause cleavage of MEK1 in all organs or cells examined (Figure 3.5). However, the time required for detecting intact MEK1 differed between organs or cells. Intact MEK1 was detected as early as 3 days after LeTx injection in bone marrow cells, after 7 days in thymus and after 10 days in spleen (Figure 3.5). Heart and lung tissues also required approximately 10 days to recover (Figure 3.6).

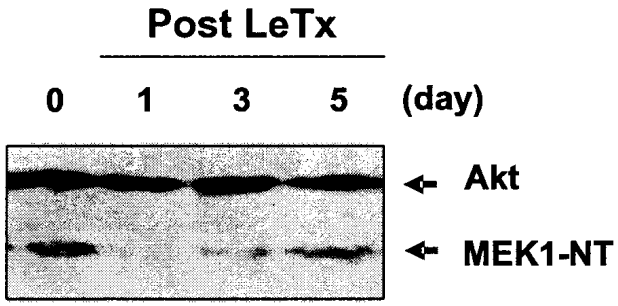
3.2. Mechanisms of LeTx-induced Cell cycle arrest in macrophages

3.2.1. LeTx causes G_0-G_1 arrest in immortalized macrophages.

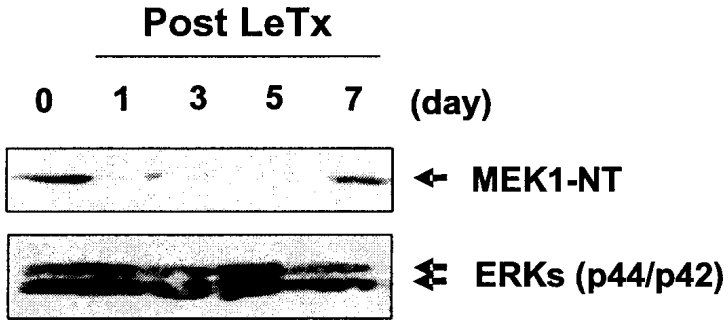
Previous studies have shown that LeTx can inhibit proliferation in several cell types [71, 73, 87, 88]. However, the molecular mechanisms by which LeTx induces cell cycle arrest remained to be investigated. I first examined whether LeTx induced cell cycle arrest in different immortalized immune cells including Jurkat (T cells), Lg2.1 (B cells), U937 and THP-1 cells. Cells were labeled with the intracellular fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) (10mM) and cultured for 4 days with daily analyses of fluorescence intensities in cells using FACS. As cells divide and multiply, the fluorescent dye within the cells is diluted leading to a decrease in fluorescence intensity. Jurkat cells had no apparent defects in proliferation after LeTx treatment, but proliferation of Lg2.1, U937 and THP-1 cells was delayed (Figure

Figure 3.5. Persistent in vivo activity of LeTx in lymphoid tissues. In C57BL6 mice were injected with a sub-lethal dose of LeTx (PA 100ug and LF 75ug). Mice were sacrificed 1, 3, 5, 7 and 10 days after LeTx challenge. Bone marrow cells, thymus and spleen were harvested and prepared for western blot analysis using antibody against MEK-1 N-terminus and regular Akt or Erk (p42/p44) as a loading control.

Bone marrow



Thymus



Spleen

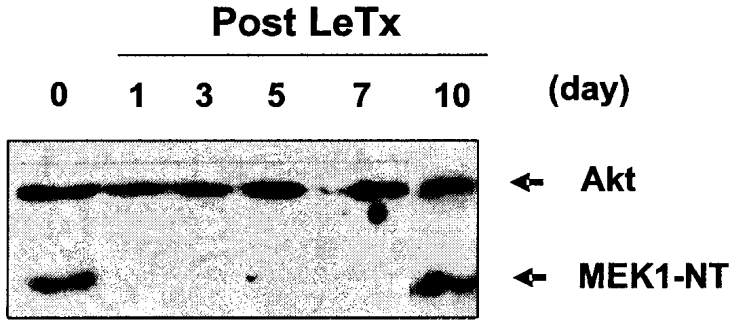
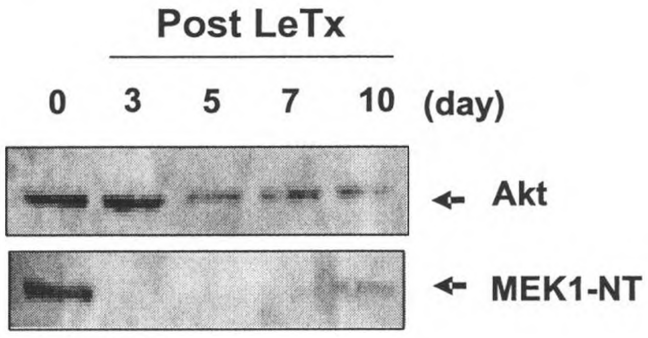
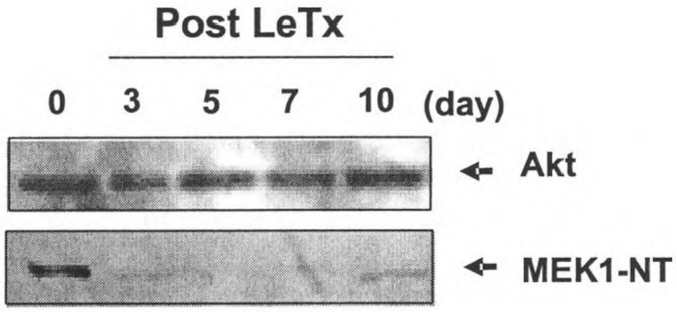


Figure 3.6. Persistent in vivo activity of LeTx in heart and lung tissues. C57BL6 mice were injected with a sub-lethal dose of LeTx (PA 100ug and LF 75ug). Mice were sacrificed 1, 3, 5, 7 and 10 days after LeTx challenge. Heart and lung tissues were harvested and prepared for western blot analysis using antibody against MEK-1 N-terminus and regular Akt as a loading control.

Heart


Post LeTx
0
3
5
7
10
(day)
Akt
MEK1-NT

Lung


Post LeTx
0
3
5
7
10
(day)
Akt
MEK1-NT

3.7). The most pronounced delay in cell proliferation was detected in THP-1 cells. In order to determine the stage of cell cycle arrest in LeTx treated cell, the total DNA content of the cells was stained with propidium iodide and analyzed by FACS. Cell cycle profiles were generated using Modfit LT 3.0 software. LeTx treated THP-1 and U937 cells showed an increase in the number of cells in G_0-G_1 phase, whereas the number of cells in S and G_2-M phase was greatly diminished. Therefore, our data suggests that LeTx inhibition on proliferation was mainly due to G_1 cell cycle arrest (Figure 3.8).

3.2.2. LeTx down-regulates major cell cycle associated proteins: Cyclin D1 & Checkpoint Protein Kinase 1 (ChK1).

To identify signaling molecules involved in LeTx-induced cell cycle arrest, levels of 25 different cell cycle-related proteins were examined using Kinetworks™ multi-immunoblotting analysis. Among them, expression levels of Cyclin D1 and ChK1 were greatly (~ 9 fold) diminished by LeTx at 48 hours after treatment (Figure 3.9). Further detailed kinetic analysis of the expression of cyclin D_1 and ChK1 showed that cyclin D1 protein was reduced by more than 50% by 3 hours and became almost undetectable by 6 hours post LeTx-treatments (Figure 3.10). Levels of ChK1 proteins were diminished slower than that of cyclin D_1 and became almost undetectable after 24 hours of LeTx treatments. Since these proteins are short-lived (t1/2 > 25min) [126], at least two mechanisms can be proposed for the rapid decrease of cyclin D1 and CHK-1 proteins; the degradation can be due to either direct cleavage by LeTx or indirect inhibition of protein expression at the levels of transcription or translation. The cleavage site of LF on MEKs

Figure 3.7. LeTx inhibit proliferation in immune cell lines. Jurkat (T cells), Lg2.1 (B cells) and THP-1 or U-937 (monocytes) were briefly exposed to LeTx (250 ng of LF and 500 ng of PA) for 5h and cultured in fresh medium after washing with 1X phosphate-buffered saline. LeTx transiently inhibited cell proliferation in Lg2.1, THP-1 and U-937 cells. Cells were stained with CFSE (0.5uM) and analyzed daily by flow cytometry for the 4 consecutive days.

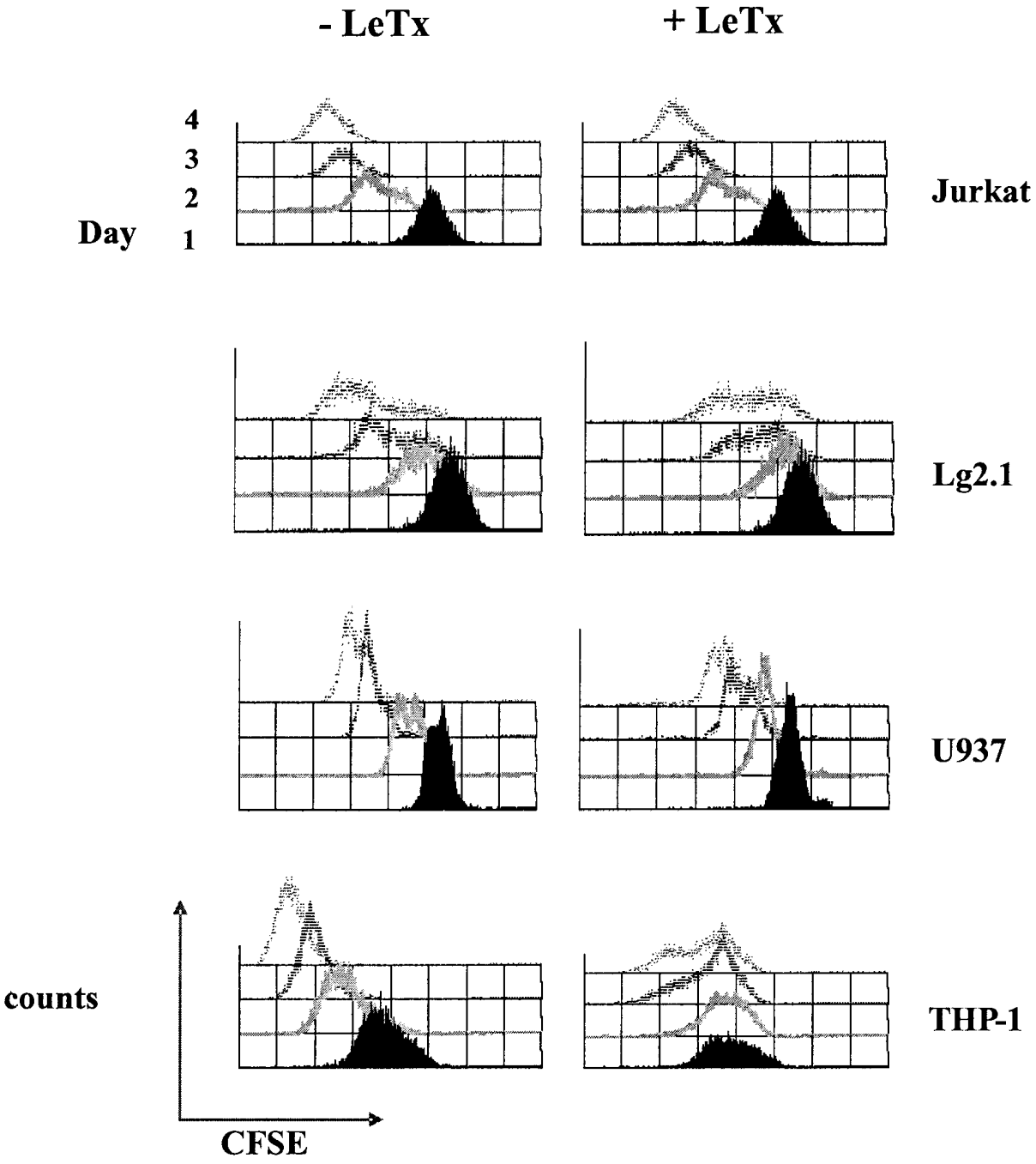
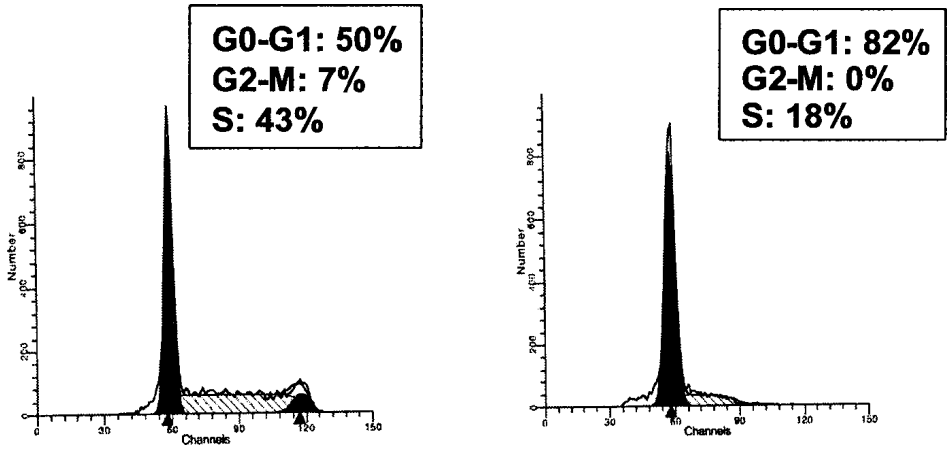
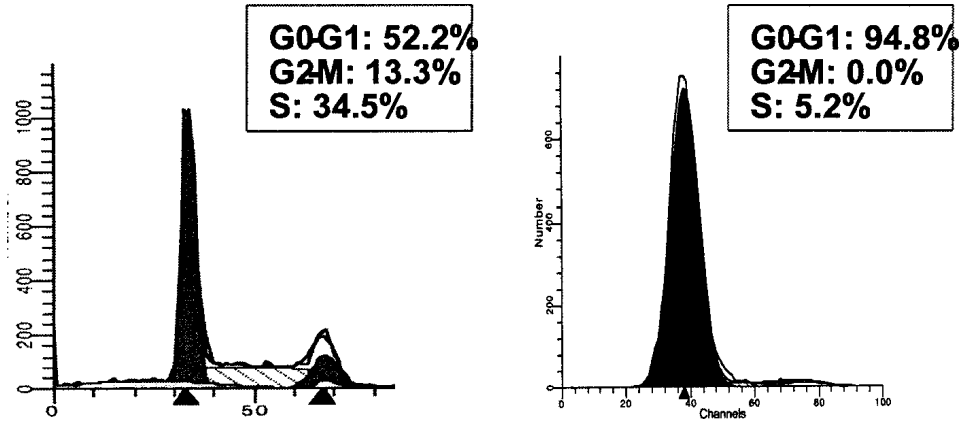

- LeTx
+ LeTx
Day
4
3
2
1
Jurkat
Lg2.1
U937
THP-1
counts
CFSE

Figure 3.8. LeTx induces G0-G1 phase cell cycle arrest in monocytic cell lines. THP-1 and U-937 cells were briefly exposed to LeTx (250 ng of LF and 500 ng of PA) for 5h and re-plated with flesh culture medium after washing off residual LeTx with 1X PBS. The percentage of cells at different cell cycle phases were analyzed using Modfit 3.0 software according to the PI content in cells 2 day after a LeTx exposure.

U937



THP-1



-LeTx +LeTx

Figure 3.9. Effect of LeTx on cell cycle related protein expression in THP-1 cells. 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 exposure using KinetworksTM multi-immunoblotting analysis (N.D presents samples that are not detected by the assay). 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.)

Post-LeTx day 2

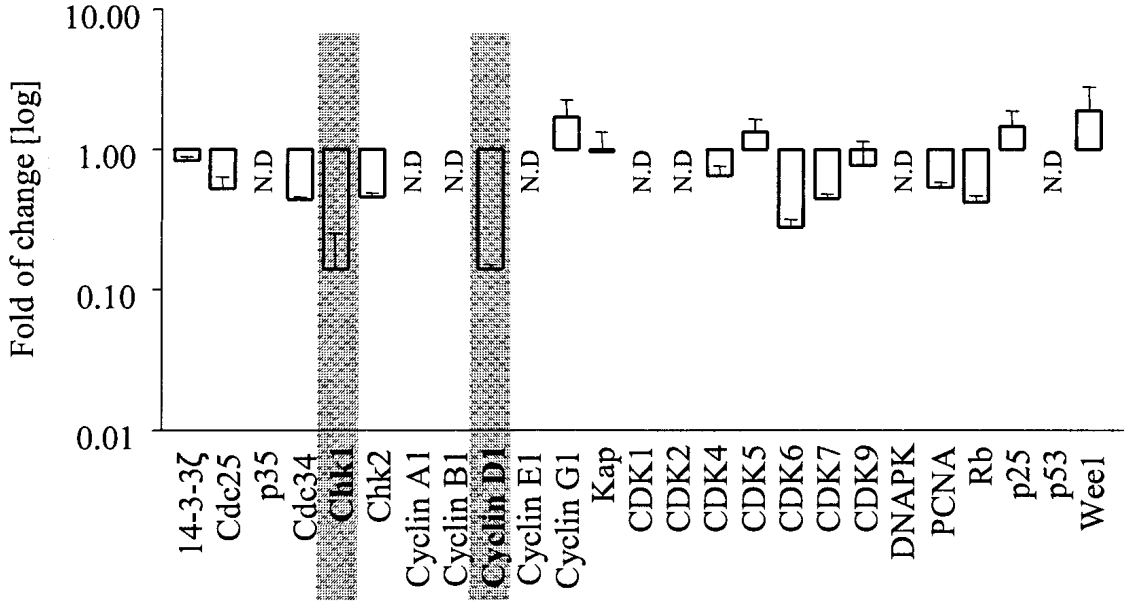
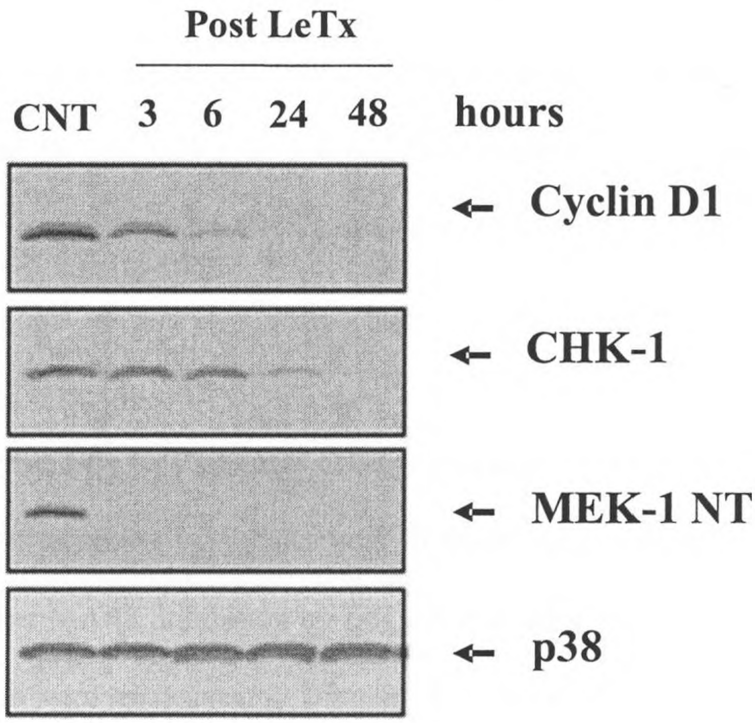


Figure 3.10. LeTx down-regulates CHK-1 and cycD1 protein level. THP-1 cells were similarly treated with LeTx and protein levels of cyclin D1, ChK1, MEK1, and p38 MAPK (as a loading control) were analyzed at 0, 3, 6, 24, and 48 h after the treatments.


Post LeTx
CNT 3 6 24 48 hours
Cyclin D1
CHK-1
MEK-1 NT
p38

is situated within the MAPK docking domain with a consensus sequence ++++XΦXΦ (basic and hydrophobic residues are indicated by + and Φ respectively, X represents any amino acid) [33]. I performed a BLAST search to compare the similarity between the MAPK docking sequence to cyclin D1 and CHK-1 to locate potential cleavage sites, but the results were negative. Furthermore, it has been suggested that the C-terminus of MEKs is essential for LF cleavage of the N-terminal domain [27, 33]; hence a direct LeTx cleavage on either protein is unlikely. When the mRNA expression levels of these proteins were examined using quantitative real-time PCR, LeTx caused rapid reduction of mRNA levels of both cyclin D_1 and ChK1 in 3 h and 6 h, respectively (Figure 3.11), suggesting that the loss of cyclin D1 and CHK1 was due to inhibition by LF at the transcriptional level.

3.2.3. Inhibition of ERK1 and 2 by LeTx causes cell cycle arrest

LeTx cleaves the N-terminus of MEKs and impairs downstream signaling cascades. To examine whether MAPK inhibition by LeTx is the cause of cell cycle arrest in macrophages, THP-1 cells were treated with different chemical inhibitors of MAPKs: U0126 (MEK1 inhibitor, 10 μM), SB202190 (SB; p38 MAPK inhibitor, 10 μM), and JNK inhibitor II (*JNKi*, 10 μM) for 2 days and cell cycle profiles were analyzed. MEK1 inhibition prevented cells from entering S phase, leading to an accumulation of cells in G_0-G_1 phase; whereas the p38 MAPK inhibitor affected the progression of S phase into G_2-M phase, and JNK inhibition showed no apparent effects on cell cycle (Figure

Figure 3.11. LeTx suppresses CHK-1 and CycD1 mRNA transcription. THP-1 cells were similarly treated with LeTx mRNA levels of cyclin D1 and ChK1 were analyzed after the treatments using real-time PCR analysis in LeTx-treated cells at various time periods as indicated in the panel. Samples were normalized by mRNA expression of GAPDH. Data are expressed as mean ± S.D. (n=3)

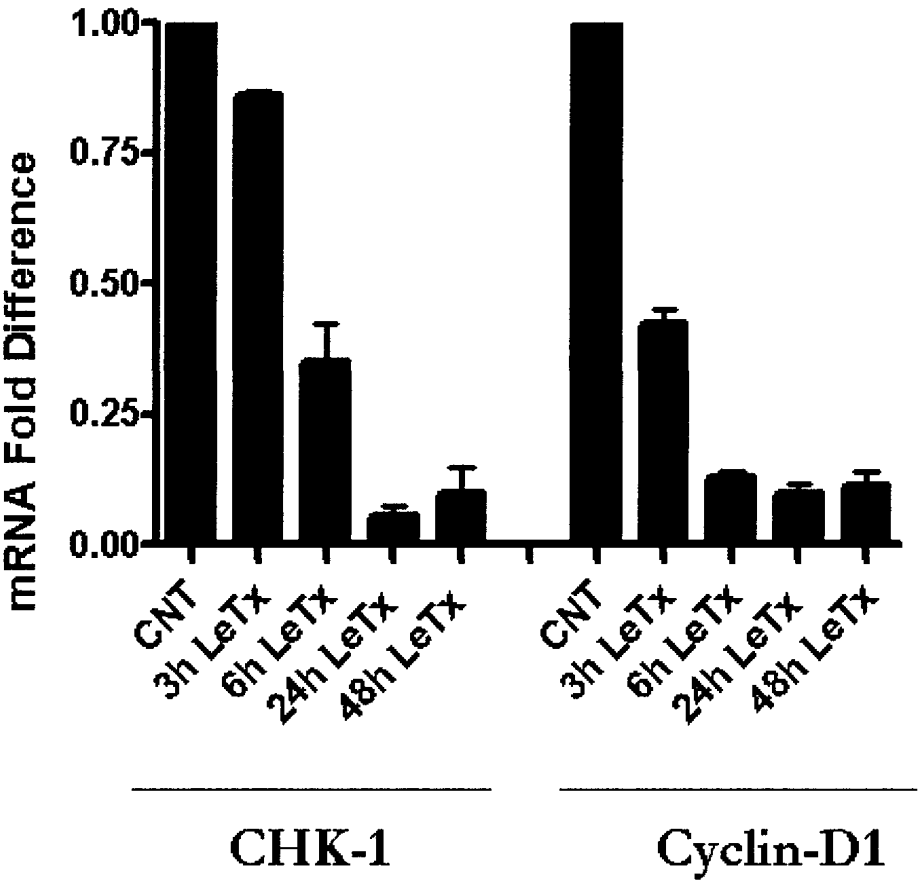
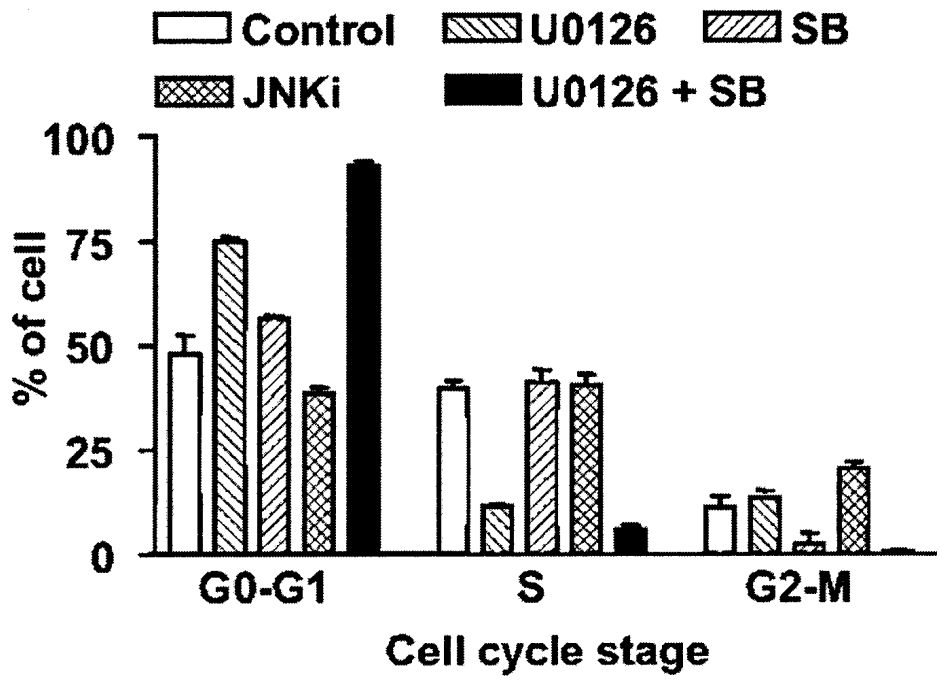

mRNA Fold Difference
1.00
0.75
0.50
0.25
0.00
CNT
3h LeTx
6h LeTx
24h LeTx
48h LeTx
CNT
3h LeTx
6h LeTx
24h LeTx
48h LeTx
CHK-1
Cyclin-D1

Figure 3.12. LeTx induced cell cycle arrest by inhibiting ERK 1 and 2 activity. 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 were stained with PI and analyzed by FACS. Cell cycle profiles were generated using Modfit 3.0. Data are expressed as mean ± S.D. (n = 3).


Control
U0126
SB
JNKi
U0126 + SB
100
75
50
25
0
% of cell
G0-G1
S
G2-M
Cell cycle stage

3.12). Expression levels of cyclin D1 and ChK1 were also downregulated by U0126 but not by SB or JNKi (Figure 3.13 A). Similarly, mRNA levels of cyclin D1, cyclin D2 and ChK-1 in the presence of LeTx or U0126 were down-regulated, but SB and JNKi had no such effect (Figure 3.13 B). Collectively, these results suggest that LeTx prevents cells from entering S phase and G_2 phase of the cell cycle through inhibiting ERK 1 and 2 and subsequently down-regulating cyclin D_1 and D_2.

3.3. PI3K/Akt/GSK3 axis drives cell cycle in LeTx-exposed THP-1 cells

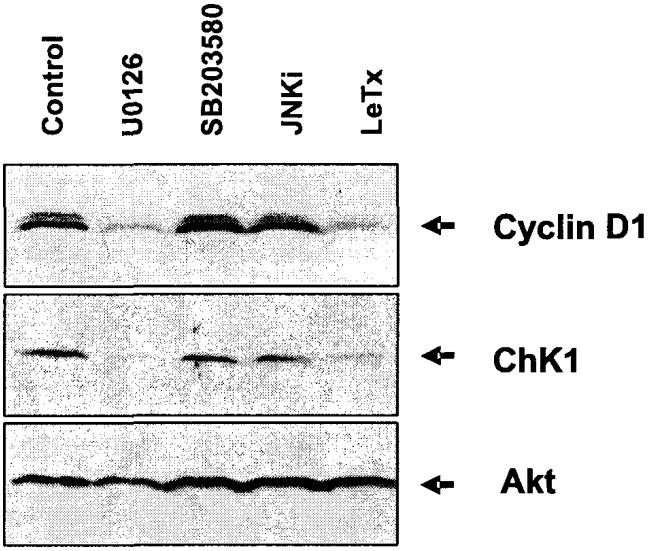
3.3.1. Spontaneous Akt activation in LeTx-exposed THP-1 cells.

Since LeTx causes permanent cytotoxic effects on non-proliferating macrophages (Figure 3.3), logically LeTx-induced cell cycle arrest should project similar permanent inhibitory effects on proliferating macrophages. However, the cell cycle profiles of THP-1 cells 3 days after LeTx treatments were normal and comparable to non-treated cells (Figure 3.14A), although MAPK signaling was impaired by LeTx (Figure 3.14B). Previous studies have shown that PI3K activation is sufficient to induce cyclin D1 expression in an ERK1 and 2-independent manner [127, 128] and overrides G_1 arrest in hematopoietic cells [129]. Therefore, I examined whether activation of PI3K/Akt/GSK3β signaling was involved in the cell cycle progression of LeTx-exposed, MEK1 cleaved THP-1 cells. I found that Akt became phosphorylated at Ser-473 two days after LeTx treatment (Figure 3.15). Maximum Akt activation was achieved on day 3 and lasted for more than 6 days.

3.3.2. Cell cycle re-initiation requires activation of Akt

Figure 3.13. ERK 1 and 2 inhibition down-regulates CHK-1, cyclin D1 and cyclin D2. *A* and B) 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 were analyzed 24 h after the treatment using Western blotting or real-time PCR, respectively. A, represents data of similar results observed in two independent experiments. *B*, data are expressed as mean ± S.D. (n = 3).

A)


Control
U0126
SB203580
JNKi
LeTx
Cyclin D1
ChK1
Akt

B)

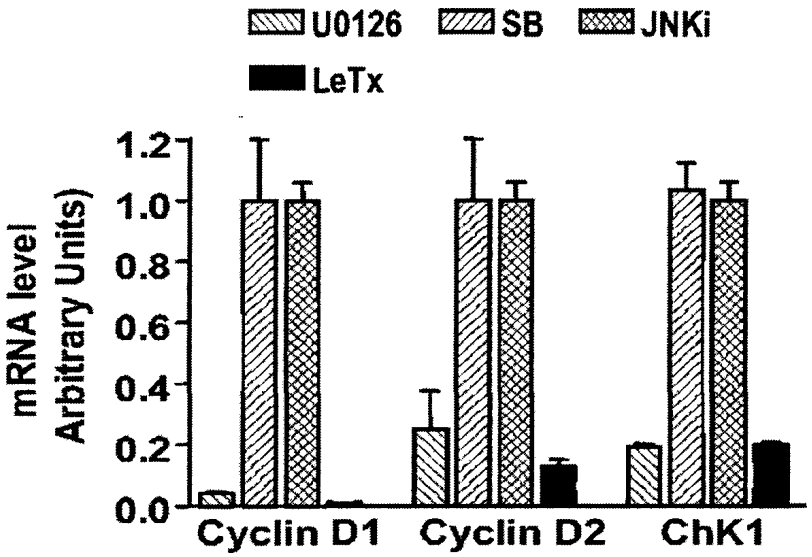
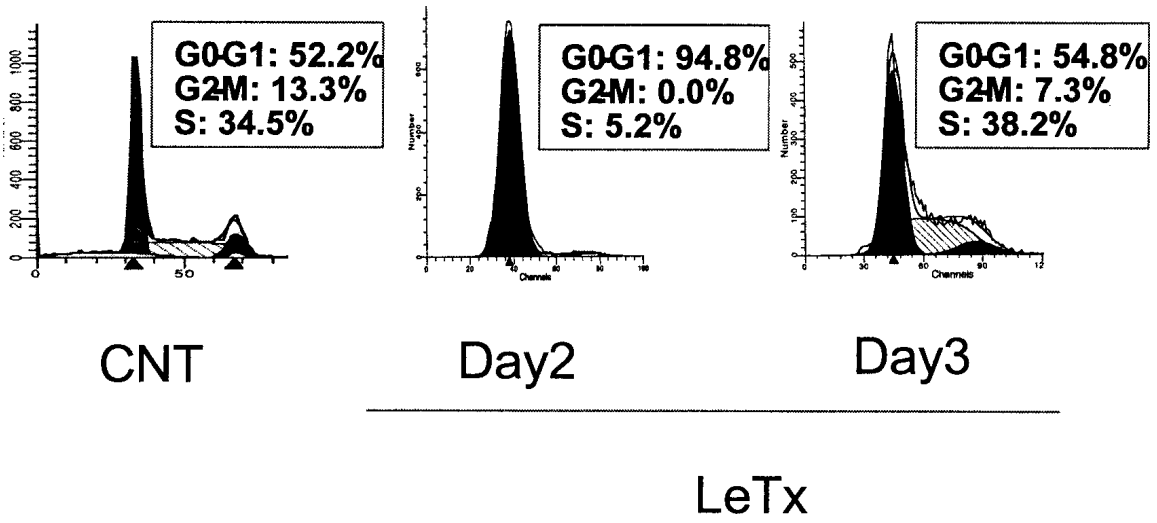

U0126
SB
JNKi
LeTx
mRNA level
Arbitrary Units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cyclin D1
Cyclin D2
ChK1

Figure 3.14. THP-1 cell cycle re-initiaition independent of MAPK signaling. A) THP-1 cells were briefly exposed to LeTx (250 ng of LF and 500 ng of PA for 5 h) and cultured for 3 days. The percentage of cells at different cell cycle phase was analyzed according to the PI-stained DNA content in cells 2 and 3 days after a LeTx exposure. B) Inhibition of MAPKs by LeTx was examined using Western blotting against N-terminal, C-terminal MEK1 and MEK3, and phospho-p38 MAPK (*p-p38 MAPK*) at different time point as indicated in the panel. Western blotting for total p38 MAPK was used for loading control. Results are a representative data obtained at least two separated experiments.

A)


G0-G1: 52.2%
G2-M: 13.3%
S: 34.5%
G0-G1: 94.8%
G2-M: 0.0%
S: 5.2%
G0-G1: 54.8%
G2-M: 7.3%
S: 38.2%
CNT
Day2
Day3
LeTx

B)

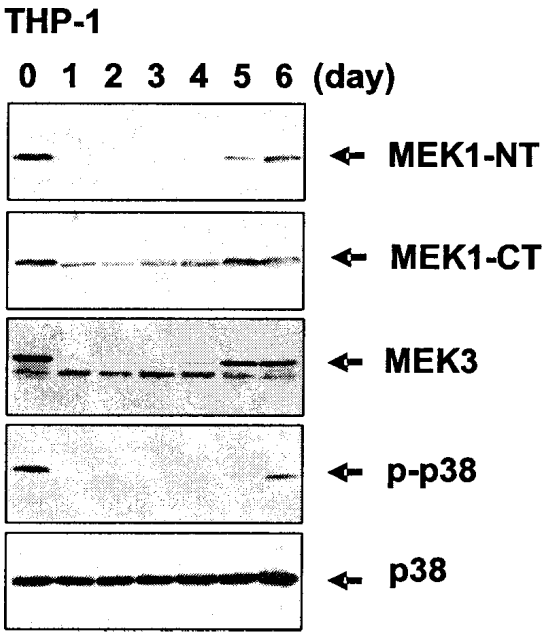
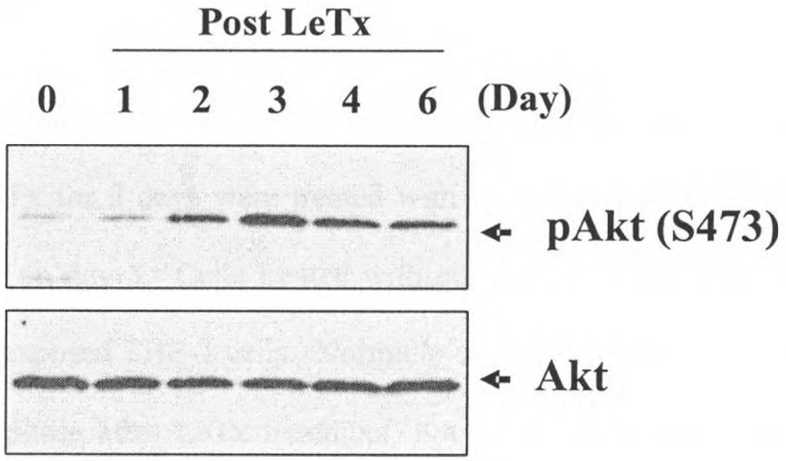

THP-1
0 1 2 3 4 5 6 (day)
MEK1-NT
MEK1-CT
MEK3
p-p38
p38

Figure 3.15. LeTx induces Akt Activation in THP-1 cells. THP-1 cells were briefly exposed to LeTx (250 ng of LF and 500 ng of PA for 5 h) and phosphorylation of Akt at Ser-473 (pAkt) at various time points was analyzed by western blot. Regular non-phosphorylated Akt used as control.


Post LeTx
0
1
2
3
4
6
(Day)
pAkt (S473)
Akt

Akt is a down-stream signaling molecule of PI3K and an up-stream kinase of GSK3 . To examine whether Akt is activated by PI3K and induces GSK3 phosphorylation, THP-1 cells pre-exposed to LeTx for 2 days were treated with PI3K inhibitor (Ly294002 (Ly), 10uM) or Akt inhibitor (Akt Inhibitor II (Akti), 5uM), and samples were western blotted on day 3 for detection of Akt activation. PI3K inhibition by Ly completely eliminated phosphorylation of S473 on Akt, whereas Akti was able to block LeTx induced activation while retaining a basal level of phospho-Akt (Figure 3.16).

To further examine the role of Akt activation in recovery from LeTx, THP-1 cells pre-exposed to LeTx for 2 days were treated with Ly (10uM) or Akti (5uM), and cell cycle was profiled on day 3. Cells treated with either Ly or Akti suppressed cell cycle recovery in LeTx-exposed THP-1 cells. Normally in 3 days, cells can gradually progress into S and G_2-M phase after LeTx treatment, but co-treatment with either Ly or Akti abrogated recovery (Figure 3.17). Furthermore, THP-1 cells were infected with retroviral vectors containing either inactive Akt mutant (Akt^{K179M}, containing an inhibitory mutation at the ATP-binding site) or membrane-targeted constitutively-active mutant Akt (Akt^{myr}, myristoylated Akt) [121], where both vector contained internal ribosome entry site sequences followed with green fluorescent protein, and allowed sorting of only retrovirus-infected cells expressing the mutant Akt. Similar to the results with Akt inhibitors, LeTx treatment in THP-1 cells containing the inactive Akt mutant demonstrated a slower recovery than vector control infected cells. Conversely, cells harboring the constitutively-active mutant Akt recovered more efficiently from cell cycle arrest (Figure 3.18).

Figure 3.16. PI3K mediates LeTx-induced Akt activation. THP-1 cells were un-treated (*CNT*), treated with LeTx (250 ng of LF and 500 ng of PA for 5 h), or treated with LeTx plus Ly294002 (*Ly*, 10uM) or Akt inhibitor (*Akti*, 5 uM). Two days later, phosphorylation of Akt at Ser-473 (pAkt), GSK3 on Ser-9 was and MEK-1 NT cleavage was analyzed using Western blotting.

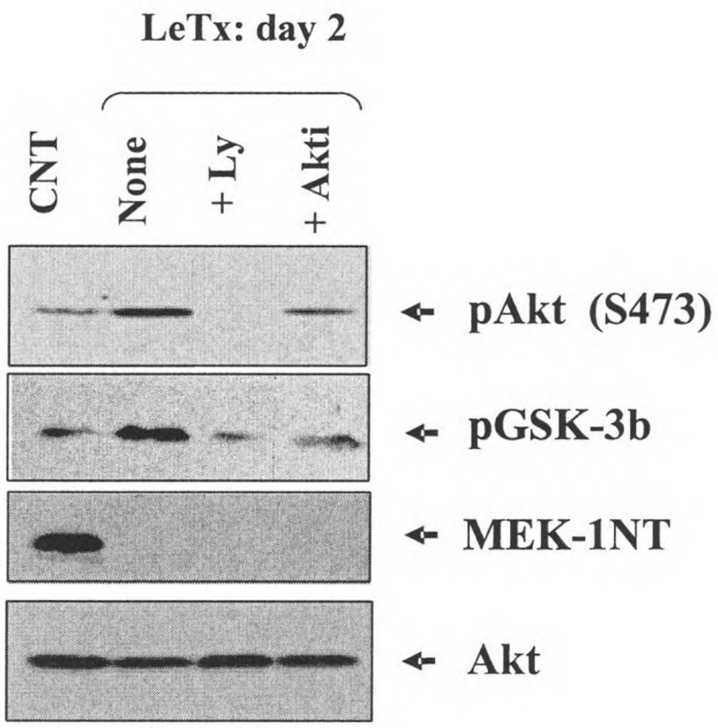
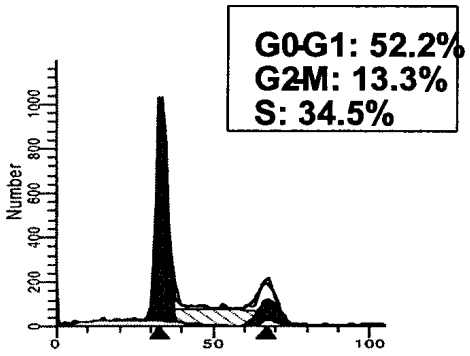
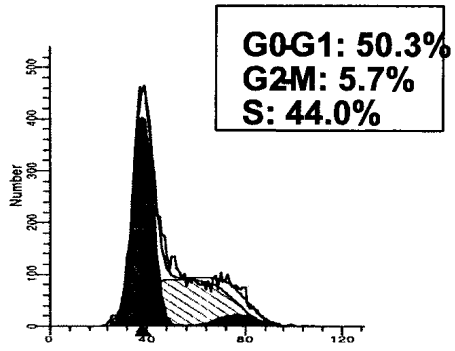

LeTx: day 2
CNT
None
+ Ly
+ Akti
pAkt (S473)
pGSK-3b
MEK-1NT
Akt

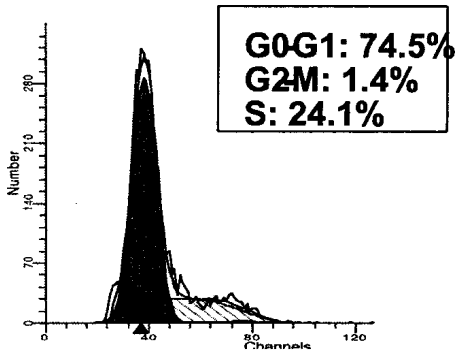
Figure 3.17. Akt inhibition attenuated THP-1 cells recovery from LeTx-induced cell cycle arrest. THP-1 cells were non-treated or pretreated with LeTx (250 ng of LF and 500 ng of PA for 5 h). Two days later, non-treated or pretreated cells were exposed to LeTx (250 ng of LF and 500 ng of PA), or LeTx plus Ly (10 μM) or LeTx + Akti (5 μM). The cell cycle distribution was analyzed 3 days later using Modfit 3.0 software according to the PI content in cells.



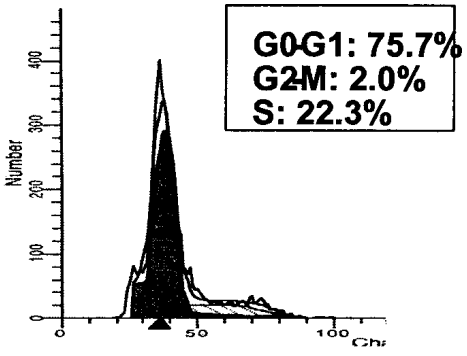
CNT



LeTx Day 3

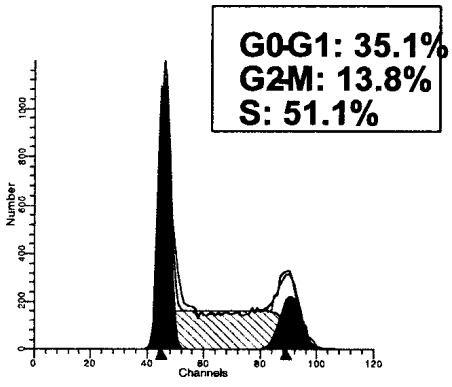


LeTx
Day 3 + Ly

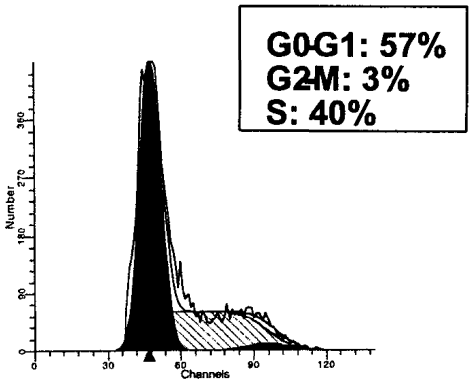


LeTx
Day 3 + Akti

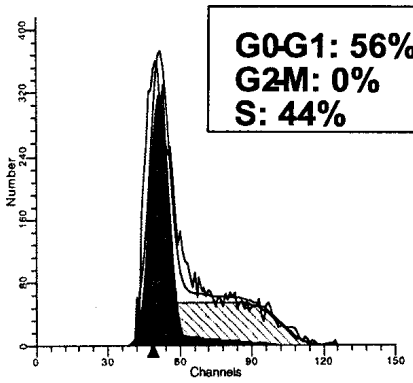
Figure 3.18. Akt activation rescues cells from LeTx-induced cell cycle arrest. THP-1 cells infected with control virus, dominant inactive Akt mutant (AktK179M) or dominant active Akt (Akt^{myr}) retroviruses were exposed to LeTx (250 ng of LF and 500 ng of PA for 5 h) The cell cycle distribution was analyzed 3 days after LeTx treatment using Modfit 3.0 software according to the PI content.



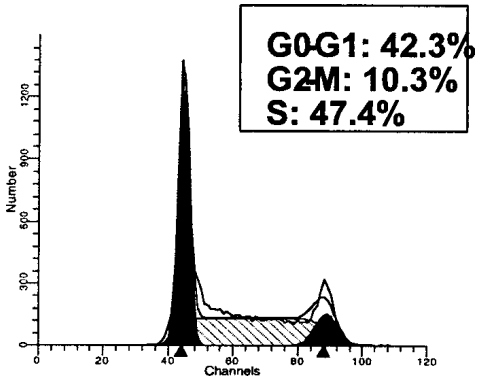
CNT RV



CNT RV
LeTx Day 3



AKT^{K179M}
LeTx Day 3



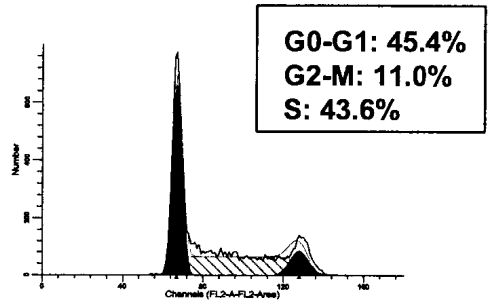
AKT^{myr}
LeTx Day 3

3.3.3. LeTx pretreatment protects cell cycle arrest of subsequent LeTx treatment.

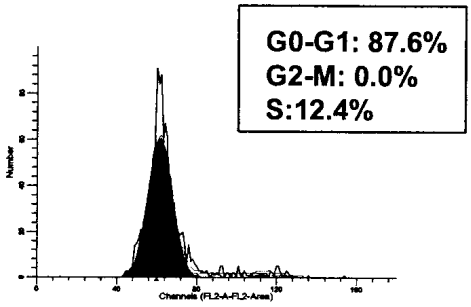
Our data suggest that LeTx prevents cell cycle progression through cleaving MEKs, and THP-1 cells adaptively activate Akt to re-initiate cell cycle activity in the absence of MAPK signaling. If adaptive cell adjustment confers resistance to LeTx-induced cell cycle arrest, it is possible that LeTx-pre-exposed cells could be resistant to further LeTx challenges. To examine whether LeTx exposure indeed induces resistance, THP-1 cells were first treated with LeTx for 5 hr and then re-treated 2 days later when cells begin to display Akt activation. As expected, the cell cycle profile of THP-1 cells showed that treatment with LeTx for 2 days inhibited proliferation, where a high percentage of cells remained in G0-G1 phase, and by day 4 cells were able to completely resume cell cycle activity. While LeTx re-challenged cells showed no defect in cell cycle progression (Figure 3.19). To determine whether this LeTx induced resistance is due to the inability of cells to further uptake LeTx or due to expression of intact MEKs, MEK-1 cleavage and ERK 1 and 2 phosphorylation were analyzed by western blotting using antibody specific against MEK-1 NT and phospho-ERK 1 and 2. While LeTx cleaved MEK-1 and inhibited activation of ERK 1 and 2 on day 2 as expected, full length MEK-1 and basal phospho-ERK could be detected on day 4. For cells that were further challenged with LeTx again, MEK-1 remained cleaved on day 4 suggesting that there was no defect in further incorporation of LF into the cells that were pretreated with LeTx (Figure 3.20).

3.3.4. Inhibition of GSK3β stabilizes cyclin D1 degradation

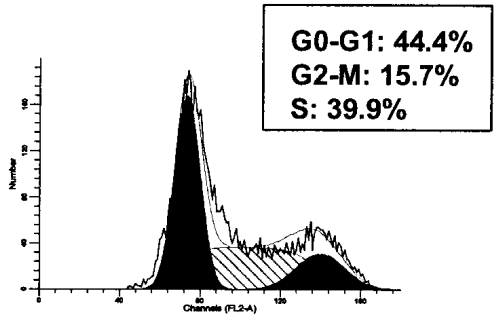
Figure 3.19. Pretreatment of LeTx protects LeTx-induced cell cycle arrest. THP-1 cells were non-treated or pretreated with LeTx (250 ng of LF and 500 ng of PA for 5 h). Two days later, non-treated or pretreated cells were exposed to LeTx (250 ng of LF and 500 ng of PA) again, and the percentage of cells at different cell cycle phase was analysis over the next 2 days using Modfit 3.0 software according to the PI content



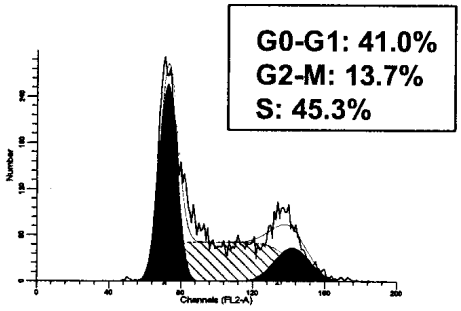
CNT



LeTx Day2

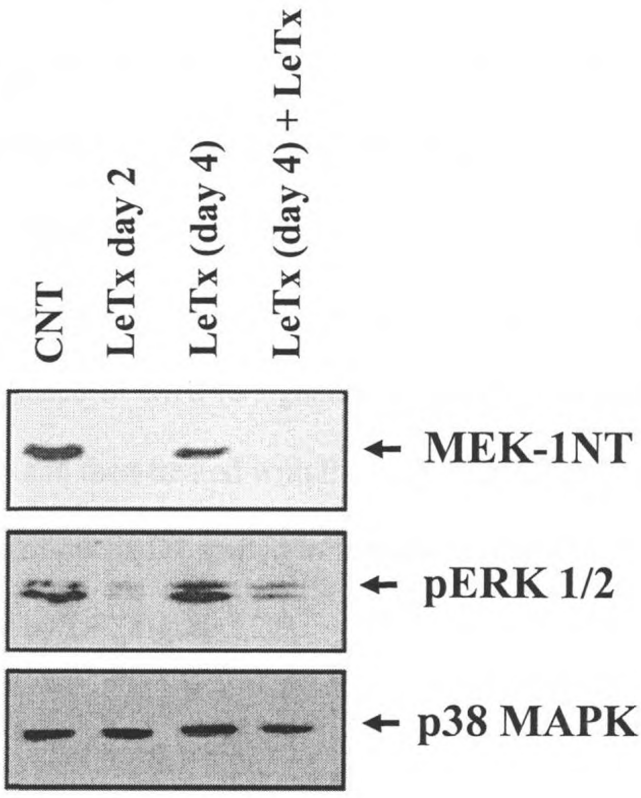


LeTx Day4



LeTx Day 4+ LeTx

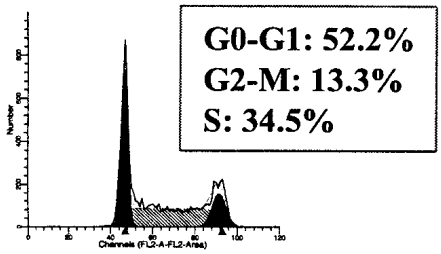
Figure 3.20. LeTx pretreatment does not protect MEK-1 cleavage from further LeTx challenge. THP-1 cells were non-treated or pretreated with LeTx (250 ng of LF and 500 ng of PA for 5 h). Two days later, non-treated or pretreated cells were exposed to LeTx (250 ng of LF and 500 ng of PA) again. MEK-1 N-terminal cleavage, phospho-ERK and p38 MAPK (as loading control) were examined by western blotting


CNT
LeTx day 2
LeTx (day 4)
LeTx (day 4) + LeTx
MEK-1NT
pERK 1/2
p38 MAPK

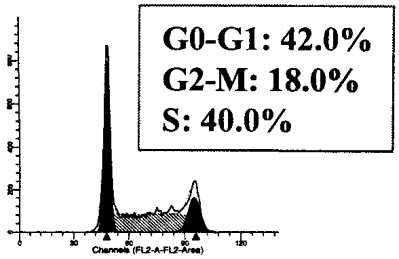
GSK3β is one of the major downstream components of the PI3K pathway in regulation of the cell cycle. GSK3β negatively regulates cyclin D1 levels by phosphorylating cyclin D1 on Thr-286 to promote proteasome mediated degradation [102]. Akt activation inhibited the activity of GSK3β and thus indirectly mediated cyclin-D1 stabilization [110, 130]. Akt activation in LeTx treated cells led to phosphorylation of GSK3β (Figure 3.13A). To further examine whether GSK3β inhibition protects from LeTx induced cell cycle arrest, the cell cycle profile of THP-1 co-treated with LeTx and a GSK3β specific inhibitor (SB216763, 10uM) were examined. Our data showed that GSK3β inhibition indeed protected LeTx induced cell cycle arrest found on day 2 (Figure 3.21). Furthermore, GSK3β inhibition alone is sufficient to promote stabilization of cyclin D in the absence of MAPK signaling. In THP-1 cells 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 past treatment, whereas MEK1 was cleaved by LF (Figure 3.22).

Figure 3.21. Inhibition of GSK protect LeTx-induced cell cycle arrest. THP-1 cells were exposed to LeTx (250 ng of LF and 500 ng of PA for 5 h) in the presence of absence of GSK3 inhibitor (*GSK3i*, 10uM), and 2 days later cell cycle distribution was examined by FACS and analyzed using Modfit 3.0 software according to the PI content in cells.

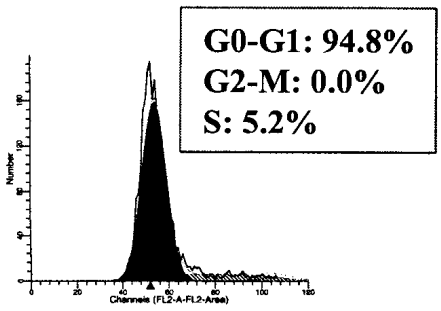
CNT



GSKi



LeTx day2



LeTx day2 + GSKi

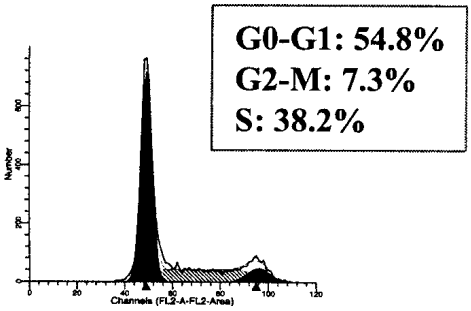
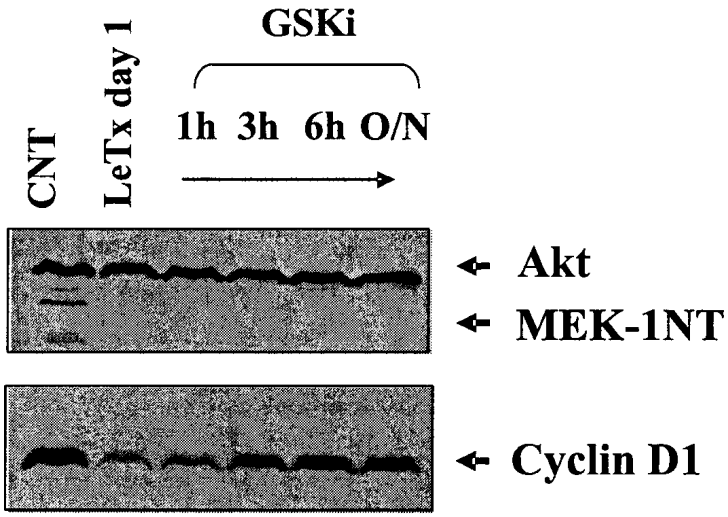


Figure 3.22. GSK inhibition stablizes cyclin D1 level in LeTx treated THP-1 cells. THP-1 cells were exposed to LeTx (250 ng of LF and 500 ng of PA for 5 h) and 1 day after, cells were treated with GSK3 inhibitor (*GSK3i*, 10uM) for 1, 3, 6 and 18 h. Mek-1 N-terminal cleavage, cyclin D1 and total AKT (as loading control) were examined by western blotting.


GSKi
CNT
LeTx day 1
1h 3h 6h O/N
Akt
MEK-1NT
Cyclin D1

CHAPTER 4 – DISCUSSION

Anthrax LeTx targets MAPK signaling pathways and inhibits various physiological functions in different immune cells. LeTx-induced immune impairments have been the focus of extensive studies [56, 57, 67, 68, 71, 73, 88, 131], but how LeTx causes cell cycle arrest and how cells recover from LeTx cytotoxicity have not yet been examined in detail. This study investigated the mechanisms of cell cycle arrest and recovery from cytotoxicity induced by LeTx in macrophages. Macrophages are key innate immune cells, and they are likely the first to encounter spores and germinating bacteria. Our *in vitro* observations on human PBMC briefly treated with LeTx showed that cleavage of MEKs and inhibition of TNF-α induction lasted over a course of 6 days (Figure 3.1 A&B), while primary peritoneal macrophages isolated from C57BL6/J mice resulted in similar persistent permanent inhibition, suggesting that macrophages exposed to LeTx in infected hosts are incapacitated for an extended period of time. The long term effect of LeTx in primary cells was likely due to persistent intracellular activity of LF. Our lab has previously demonstrated that LeTx can continuously cleave MEKs for up to 5 days in the murine macrophage cell line RAW246.7 after a single brief exposure [132]. In contrast to primary cells, proliferating monocytic cell line THP-1 and murine BMDM challenged with LeTx demonstrated that MEKs cleavage and inhibition of TNF-α expression can be recovered (Figure 3.4A & B). Thus cells with the ability to proliferate appeared to be able to overcome the extended intracellular MAPK inhibition promoted by LF.

C57BL/6J mice injected with a single sub-lethal does of LeTx led to MAPK impairment in various tissues. MEK-1 protein levels eventually returned in all tissues tested, but the timing of the recovery found in each cell type was quite different (Figure

3.5). Recovery of full length MEK1 was detected as early as 3 days of LeTx treatments in bone marrow cells, while it took 7 and 10 days before full length MEK1 was detected in the thymus and spleen, respectively. The rate of MEK-1 recovery in different organs correlates with the expected proliferation rates of cells presented in these organs. In the bone marrow, new leukocytes are constantly generated from hematopoietic stem cells [54]. In the thymus, premature thymocytes continuously undergo expansion to maintain a healthy TCR reservoir [46]. Both organs demonstrate vigorous proliferation in comparison to the spleen where many terminally differentiated and non-proliferating cells reside [54], which supports the idea of proliferation in promoting recovery from LeTx exposure. Although, further experiments are required to confirm our assumption, since other factors can also contribute to delayed recovery in various organs. For example, the distribution of LeTx through intraperitoneal injection may expose splenocytes with higher dose of the toxin when compared to the cells in the thymus and bone marrow. Furthermore, the level of expression for PA receptor may have been different among different cell types, and giving rise to different amount of toxin uptake. Finally, LeTx have been shown to impair migration of cells [56, 133], since B cells and T cells are originated from bone marrow, LeTx may have inhibited new cells with full length MEKs from reaching peripheral organs.

Given that proliferation is important for cells to recover from LeTx cytotoxicity, the ability of LeTx to cause cell cycle arrest will greatly impact on the course of the disease. This study demonstrates that LeTx specifically prevents the cell cycle entry of S phase and G_2-M phase, leading to an accumulation of cells in G_0-G_1 phase in THP-1, U-937 and Lg-2.1 (Figure 3.8). Kinetworks™ multi-immunoblotting analysis showed that LeTx-induced cell cycle arrest is likely mediated through down-regulation of cell cycle

associated proteins, particularly cyclin D1 and Chk1 protein levels that were reduced by ~ 9-fold (Figure 3.9). D-type cyclins as mentioned before are critical to drive cell cycle progression through G1 and into S-phase [134], and the importance of cyclin D1 in proliferation has been well described [43, 93, 96, 103, 110, 135-141]. Interaction of cyclin D1 interaction with CDK4 or CDK6 leads to inhibition of tumor suppressor pRb and initiates expression of various proliferative genes [142]. Hence, the loss of cyclin D1 is potentially a critical factor leading to G1 cell cycle arrest in LeTx treated cells. ChK-1 kinase is a major effector of S-phase checkpoint signaling which maintains proper cell cycle progression [143]. ChK-1 responds to DNA damage and prevents G2-M transition by inhibiting dual phosphatase Cdc25A and Cdc25C, resulting in inhibition of Cdc2 kinases [144]. ChK-1 was also shown to control the timing of early versus late replication origin firing during normal S phase [145], and maintains DNA replication through structurally unfavorable stretches of DNA [146, 147]. Thus, ChK-1 is required for cell cycle progress for S and G2-M phases in normal cell growth, and down-regulation of CHK-1 in LeTx treated cells may contribute to the inhibition of S to G2 phase transition.

Since LeTx treatment quickly down-regulated the mRNA level of Cyclin D1 and ChK-1 (Figure 3.11), the depletion of both proteins is may be mediated through disruption of MAPK signaling and prevented transcription of their genes. Many studies have shown that ERK 1 and 2 activity is critical for cells to pass the G1 phase restriction point and enter into S-phase [43, 148, 149]. ERK 1 and 2 activation phosphorylates various nuclear substrates such as cAMP response element-binding protein (CREB), Elk-1, STAT3 and activator proteins (AP-1) such as c-Jun or cFos leading to stimulation of early gene transcription to drive cell cycle progression [35]. And because the cyclin D1

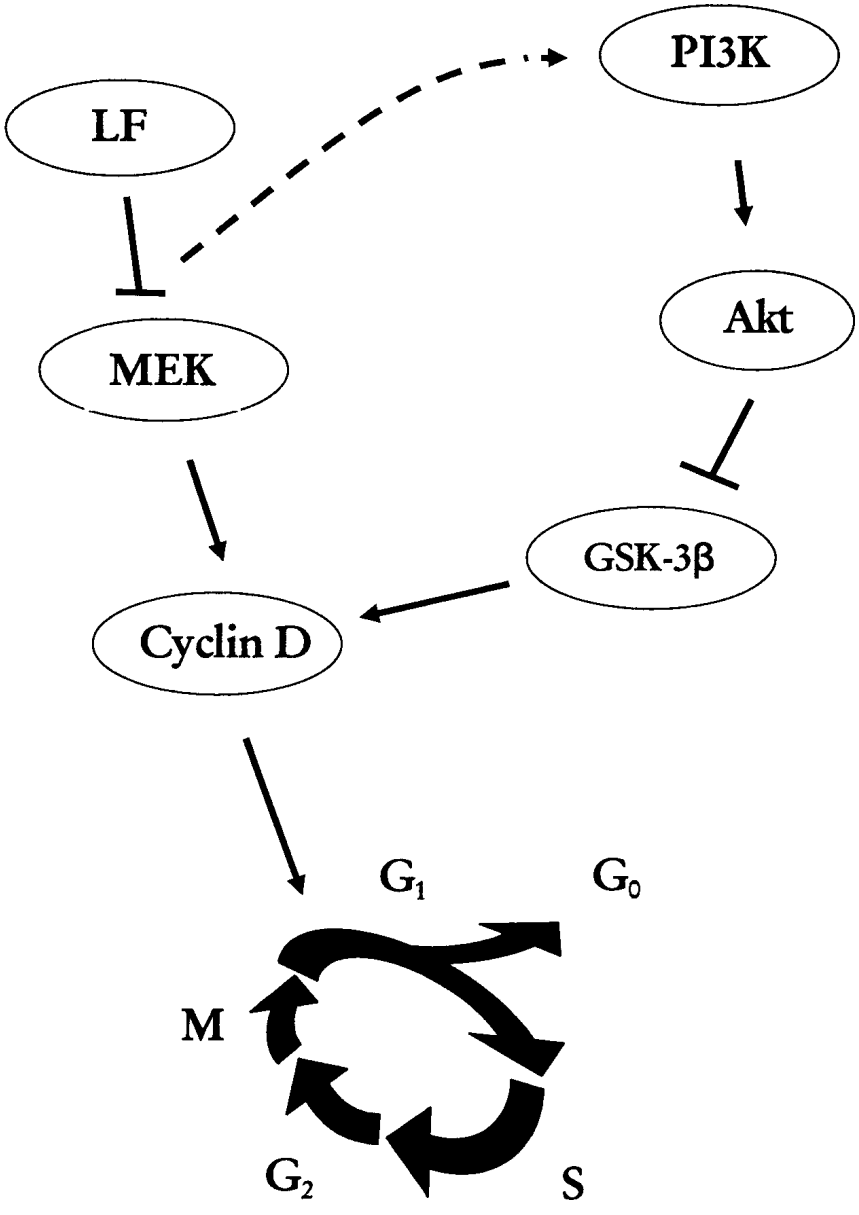
promoter contains a functional AP-1, CREB and E2F-1 binding site [93, 150], ERK 1 and 2 activities are essential for maintaining appropriate levels of cyclin D1 in THP-1 cells. Indeed chemical inhibition to suppress MAPK signaling reproduced the effects of LeTx on cell cycle arrest (Figure 3.12). Specifically, inhibition against ERK 1 and 2 activation with UO126 alone down-regulated cyclin D1, D2 and CHK-1 level which suggests a similar mechanism of action to LeTx (Figure 3.13 A&B).

Activation of the PI3K/Akt pathway was previously shown to regulate cell cycle through induction of cyclin D1 expression [102], cytoplasmic relocalization of the cyclin-dependent kinase inhibitor p27 [111, 112], and inhibiting transcriptional activation of a number of related forkhead transcriptional factors (FoxO1, FoxO3, and FoxO4) [115, 151]. Several studies have also demonstrated that PI3K activation is sufficient to induce cyclin D1 expression in an ERK 1 and 2-independent manner [127, 128] and override G_1 arrest in hematopoietic cells [129]. Hence, LeTx treatment-induced Akt activation in THP-1 cells suggests an adaptive response activated to compensate for inhibition of proliferation (Figure 3.14A). Chemical inhibition against Akt activation, or cells infected with retrovirus to express constitutively inactive Akt abrogated cell cycle re-initiation after LeTx challenge, whereas cells infected with virus expressing active Akt promoted cell cycle recovery (Figure 3.16 and 3.17). Given the importance of cyclin D1 in G_1 to S phase cell cycle transition, Akt mediated recovery is likely due to repletion of cyclin D1 protein. In proliferating cells, cyclin D1 has a rapid turnover rate ($t_{1/2}$>25min) promoted by ubiquitination and proteasome-mediated degradation of phosphorylated cyclin D1 [91]. Since GSK3 is the primary kinase that phosphorylates Cyclin D1 on Thr-286 and its activity is inhibited via Akt phosphorylation on Ser-9 [102], reagents activating PI3K/Akt or

inhibiting GSK3 should stabilize cyclin D1 protein level in LeTx treated cells. As expected, chemical inhibition of GSK3 protected the loss of cyclin D1 in LeTx exposed THP-1 cells (Figure 3.21), and at the same time protected cells against LeTx induced cell cycle arrest (Figure 3.20). Furthermore, cells that have been previously treated with LeTx and retained active Akt were protected from cell cycle arrest against subsequent LeTx challenge (Figure 3.18). Interestingly, this phenomenon is reminiscent of the adaptive response to LeTx-induced cytolysis in murine macrophages [132, 152]. As mentioned, certain murine macrophage cell lines or primary macrophages originated from inbred mice undergo rapid NALP1b-dependent cytolysis by LeTx. These macrophages adaptively respond to low non-cytolytic dose of LeTx and become resistant to subsequent cytolytic doses of LeTx, termed toxin-induced resistance [132, 152]. Our lab had previously shown that toxin-induced resistance in murine macrophages is mediated through down-regulation of two mitochondrial death proteins, Bcl-2/adenovirus E1B 19kDa-interacting protein 3 (Bnip3) and Bnip3-like [132]. However, human macrophages do not express NALP1b and are resistant to LeTx-induced cytolysis [62, 77, 86]. The adaptive response to LeTx-induced cell cycle arrest in THP-1 cells is mediated through activation of PI3K/Akt signaling (Figure 4.1).

Taken together, this study was the first to describe the mechanisms of cellular recovery from anthrax LeTx exposure. LeTx has been regarded as a key virulence factor in the pathogenesis of anthrax, causing immune impairments and cell death in host immune cells. However, the effects of LeTx against proliferation or on proliferating cells may also impact the recovery of immune system and allow *B. anthracis* to better survive within the host. In THP-1 cells, LeTx induces cell cycle arrest in G1 phase by rapid

Figure 4.1. LeTx-induced cell cycle arrest and recovery pathways. LeTx caused cell cycle arrest at least in part by inhibiting the MEK1 →ERK1/2 →cyclin D1 pathway, and possibly GSK3 activation, which is also catalyzed by MEK1/2. An adaptive response activating PI3K →Akt →GSK3β stabilizes cyclin D1, overrides cell cycle arrest, and facilitates recovery from LeTx intoxication.


PI3K
LF
Akt
MEK
GSK-3β
Cyclin D
G1
G0
M
G2
S

down-regulation of cyclin D1 protein through inhibition of MAPK signaling. Since LeTx receptors have been shown to be widely expressed, cell cycle arrest by LeTx is likely to occur in a number of cell types that require ERK 1 and 2 activity for proliferation as well. This study further showed that the persistent intracellular activity continuously cleaves MEKs almost indefinitely in non-dividing cells, indicating an extensive period of LF intoxication. This may explain how anthrax infected hosts succumb to the disease despite bacterial clearance by antibiotics. THP-1 cells recover from the effects of LeTx by activating PI3K/Akt/GSK3 signaling-mediated adaptive responses. Reagents activating the signaling pathways or inhibitors of GSK3 can therefore be novel tools for treating LeTx toxemia, whereas reagents that inhibit the adaptive signaling pathway can be detrimental to the host.

4.1. Future Research

Conventional treatments for anthrax involve a combination of antibiotics and serum proteases [153], but delayed administration often leads to toxin-induced fatality even though bacteria had been cleared [14]. This study examined a very different aspect of LeTx inhibition against proliferation in macrophages, and described a mechanism of cell recovery from LeTx-induced cytotoxicity. Thus, this work has laid a foundation for novel therapeutic development against anthrax, and our findings have opened up several avenues for future work in this area.

4.1.1. Persistent LF activity in non-proliferating cells

LeTx can impair MAPK signaling of non-proliferating cells for an extended period after a single brief exposure (Figure 3.1 & 3.3). Macrophages recruited to the site of infection are mostly non-dividing [54], hence it will be important to further investigate the ability of non-proliferating cells in recovering from LeTx. For example, do non-proliferating cells replenish MEKs when LF is suddenly inactivated? Can membrane permeable LF inhibitors rescue primary murine macrophages treated with LeTx? Furthermore, certain non-proliferating cells can be stimulated with growth factors to induce proliferation; thus, can growth factors such as IL-3 and insulin that signal through the PI3K pathway stimulate cell growth in LeTx-exposed hPBMC and promote recovery from LeTx inhibition?

4.1.2. Role of Akt in other immune cells

LeTx inhibits proliferation of THP-1, U937 and Lg2.1 (Figure 3.7), and is likely to inhibit other cells in a similar manner. Therefore, it is equally important to establish the role of PI3K/Akt/GSK3 pathway in other cell types against LeTx challenge. First, we need to determine if Akt is activated in response to LeTx treatment in other cell lines can then examine whether activation of Akt or GSK3 inhibition affect LeTx cytotoxic recovery. Furthermore, we need to confirm that PI3K activation is not restricted to cancer cell lines. Using isolated murine splenocytes, we can perform a mixed lymphocyte reaction and challenge them with LeTx to check for defects in proliferation and Akt activation. Similarly we can then apply reagents that inhibit or induce Akt activation in conjunction to LeTx challenge to further examine the role of Akt.

4.1.3. Therapeutic potential

One of the most important questions relating to this study is how LeTx induces Akt activation in macrophages and if it can be applied to therapeutic development. PI3K is the major activator of Akt in response to growth factors; however Akt has also been known to be activated by other pathways [99]. Interestingly, studies have suggested that the Raf/MEK/ERK pathway cross-regulates with PI3K/Akt/GSK3 [154], where Raf has been reported to activate Akt in hematopoietic cells [113]. Since LeTx effectively cleaved MEKs, cells exposed to LeTx are likely to accumulate activated Raf leading to Akt activation. Therefore, using a chemical inhibitor against MEK-1 such as UO126, we should be able to reproduce Akt activation similar to LeTx treatment. Similarly GSK3 inhibitor protected THP-1 cells from LeTx induced cell cycle arrest, hence GSK3 antagonists is another potential target for therapeutic development.

Finally, we need to assess whether Akt activation can benefit LeTx-induced toxemia in the host. LeTx has been shown to rapidly deplete circulating monocyte in sensitive mouse strains, but in resistant mouse strains, monocyte number replenish within 30 hours [25]. However, the cause of death to the animals in LeTx toxemia was not due to macrophage killing, but likely caused by endothelial cells dysfunction, leading to vascular collapse, hypoxia related tissue necrosis and systemic shock [25, 155]. LeTx has been shown to affect endothelial cells in cell density, morphology, barrier function, and can induce a low percentage of cell death [155]. Hence, if Akt activation can promote vascular endothelial layer regeneration, it will greatly benefit host recovery.

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