Novel Functions of Anthrax Lethal Toxin

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at George Mason University

By

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Spring Semester 2009
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DEDICATION

This is dedicated to my loving wife Karen and my wonderful son Gabriel.

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to the following individuals whose unwavering support and faith were critical to the completion of this research.

First, I would like to thank Dr. Charles Bailey for his valuable insights and intellectual support. Dr. Aigou Wu who provided the scientific initiatives that formed the basis of this study and is always so generous with his guidance, feedback and support. Dr. Serguei Popov who provided invaluable scientific comments and suggestions on my research. It is a honour to have them as members of my committee.

A special thank you to Dr. Ken Alibek for his mentorship and for allowing me the opportunity to tap into his expertise. I wish to acknowledge the past and present employees at AFG Biosolutions, Inc. namely, Svetlana, Darya, Hao, Caroline and Lena. As a result of their assistance, my research was able to progress smoothly.

Finally, I want to thank my family for standing by me every step of the way. To my parents who cheered me on despite the long periods of separation. To my wife, Karen, for being my best friend and critic. To my son, Gabriel, who brought immense joy and laughter to my life.

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LIST OF ABBREIVATIONS

3-MA	3-methyladenine
ALO	Anthrolysin O
AO	Acridine orange
ATCC	American Type Culture Collection
ATG	Autophagy related genes
AVA	Anthrax Vaccine Adsorbed
AVO	Acidic vacuoles
BHI	Brain Heart Infusion
CBA	Cytometric bead array
CDC	Centers for Disease Control
CGD	Chronic granulomatous disease
CMA	Chaperone-mediated autophagy
Cvt	Cytoplasm to vacuole targeting
CW	Cell wall
DCFDA	dichlorofluorescein *diacetate*
DCFDA	Dichlorofluorescein diacetate
DoD	Department of Defense
EBSS	Earles Balanced Salt Solution
EDTA	Ethylenediaminetetraacetic acid
EF	Edema factor
ET	Edema toxin
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
fMLP	formy-Methionyl-Leucyl-phenylalanine
FSC	forward scatter
GATE16	Golgi-associated ATPase enhancer of 16 kDa
GDI	GDP dissociation inhibitor
GFP	Green fluorescent protein
GI	Gastronintestinal
GSH	glutathione
GSH	Glutathione
HBSS	Hank's balanced salt solution
HIV	Human immunodeficiency virus
HSV	Human simplex virus

IACUC	Institutional Animal Care and Use Committees
IP	Intraperitoneal
JNK	c-Jun N-terminal kinase
LAMP	Lysosomal associated membrane protein
LC3	Light chain 3
LF	Lethal factor
LPS	Lipopolysaccharide
LT	Lethal toxin
MAPKK	Mitogen-activated protein kinase kinase
MHC	Major histocompatibility complex
MRSA	Methicillin-resistant *S. aureus*
NAC	N-acetylcysteine
NAC	N-acetyl-l-cysteine
NADPH	nicotinamide adenine dinucleotide phosphate
PA	Protective antigen
PAMP	Pathogen-associated molecular patterns
PAS	Phagophore assembly site
PBMC	peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
PI	propidium iodide
PI3K	Phosphatidylinositol 3-kinases
PMA	phorbol-12-myristate-13-acetate
PRR	Pattern recognition receptors
ROS	Reactive oxygen species
SSC	side scatter
STIMAL	Signal transduction methodology antioxidant liposomes
TLR	toll-like receptors
TOR	Target of rapamycin
VCC	*Vibrio cholerae* cytolysin
XMEA	X-linked Myopathy with Excessive Autophagy

ABSTRACT

NOVEL FUNCTIONS OF ANTHRAX LETHAL TOXIN

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George Mason University, 2009

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Bacillus anthracis is a gram positive spore-forming bacterium that can cause cutaneous, gastrointestinal or inhalational anthrax in many animals and humans. Vegetative *B. anthracis* generates two essential virulence factors: the anthrax lethal toxin and the poly-γ-D glutamic acid capsule. The primary virulence factor is a secreted zinc-dependent metalloprotease toxin known as lethal factor (LF), which is introduced into the cytosol by protective antigen (PA) through its receptors on the cells. LF exerts its toxic effect through the disruption of mitogen-activated protein kinase kinase (MAPKK) signaling pathway, which is essential in mounting an efficient and prompt immune response against the invading pathogen. LF also mediates the destruction of host cells through either necrotic cell death or apoptosis pathway depending upon the genetic background of the cell types.

Autophagy is an evolutionary conserved intracellular process whereby cells break down long-lived proteins and organelles. Accumulating evidences suggest increasing

physiological significance of autophagy in pathogenesis of infectious diseases. In addition to the myriad of effects that LT exerts on different cell types, we describe herein a novel effect of LT-induced autophagy on mammalian cells. Several autophagy biochemical markers including LC3-II conversion, increased punctuate distribution of GFP-LC3 and development of acidic vesicular organelles (AVO) were detected in cells treated with LT. Analysis of individual LT component revealed a moderate increase in LC3-II conversion for protective antigen-treated cells, whereas the LC3-II level in lethal factor-treated cells remained unchanged. Moreover, our preliminary findings suggest a protective role of autophagy in LT intoxication as indicated by accelerated cell death when autophagy was inhibited. Separately, LT was also shown to induce harmful levels of reactive oxygen species (ROS) in immune cells although antioxidant appeared to have some protective effects against its damaging effects. In addition, chemotaxis analysis revealed that LT not only fail to elicit chemokines production in immune cells but also suppressed the chemokines-inducing properties of lipopolysaccharides (LPS) and bacterial cell wall (CW). The wide array of effects that LT exerts on various immune cells reflects the intricacies of the intoxication mechanisms. These findings enhance our understanding of anthrax pathogenesis and may prove to be relevant to the development of a more effective countermeasure against anthrax.

Chapter 1: Introduction and Literature Review

1.1 Introductory overview

1.1.1 Discovery of anthrax

Anthrax is an acute zoonotic disease that affects primarily animals and to a lesser extent, humans. This ancient disease was described in Athens of Plague in 430 BC [1] and thereafter, episodes of outbreaks in human persisted throughout the centuries into the middle ages [2]. Notably, in the 18th century, approximately half of the sheep population in Europe was decimated by the disease [2] and in Iran, almost 1 million sheep were wiped out by the disease in 1945. Many reports subsequently described the occurrence of anthrax in numerous countries including South Africa, Namibia, Australia, Nepal, China and India between 1980s to 2000 [3].

The causative agent was first identified in 1876 by Robert Koch who was the first to demonstrate the life cycle of anthrax [4]. By inoculating healthy animals with anthrax culture and observing the progression of disease, he concluded that anthrax can be transmitted from one host to another and thereby developed the Koch's postulates germ theory based on those findings. To further demonstrate the theory of vaccination, Louis Pasteur subsequently inoculated 25 cattle with attenuated live bacteria followed by challenged with a virulent strain [5]. All vaccinated cattle survived while the cattle from control group succumbed to the disease [5]. It was these findings that Pasteur provided

more conclusive evidence on the germ theory. While the incidence of anthrax cases was high in the early 1900s, the number of cases decline dramatically during the late 20th century. Persistent decline of anthrax cases continued into the 1980s, which prompted Brachman of Centers for Disease Control (CDC) to prematurely conclude in 1980 that anthrax is "now primarily of historical interest" [6]. However, recent history witnessed several events that reignite the interest in anthrax not only in area of public health but also as a potential bioweapon.

1.1.2 Epidemiology

Although anthrax spores persists in many parts of the world (Figure 1.1), natural cases of human anthrax infection are usually confined to mill workers and those dealing with animal fibers and hence, the name woolsorter's disease [6]. Several other obsolete names that were used to describe the disease in the past included ragpickers' disease, charbon, milzbrand, black brain, "tanners" disease, and Siberian (splenic) fever.

Cases of human anthrax started to decline in the early 20th century. Several factors may account for the reduction in anthrax cases, such as the implementation of vaccination, improved animal husbandry as well as controls on imported animal products [7]. Despite the significant decline in the number of incidences, the first half of the 20th century saw 20,000 to 100,000 cases in Asia and Africa being recorded annually [7] and 2,000 cases yearly during the second half. The largest anthrax epidemic occurred in Zimbabwe with approximately 10,000 cases recorded from 1978 to 1985 [8].

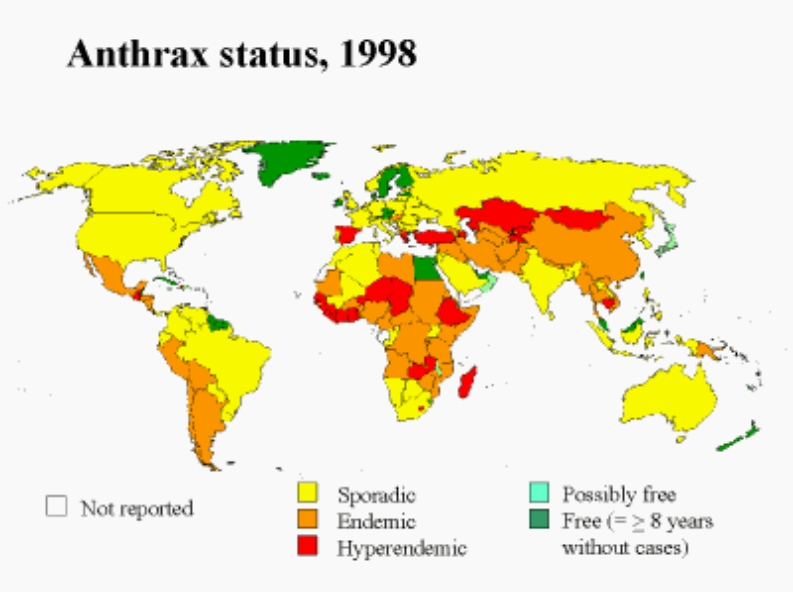


Figure 1.1: Geographical distribution of anthrax (1998)

Depending on the route of infection, anthrax infection can occur in 3 main forms; cutaneous, inhalational and gastro-intestinal [9]. Cutaneous anthrax represents the most prevalent form of anthrax with annual occurrence of 2000 cases reported worldwide [10]. In the USA, 224 cases were reported from 1944 to 1994 [11] and only 1 case was reported in 2000 [12]. Inhalational anthrax is the most severe form and often resulted in over 90% fatality if it is not treated early [13]. Inhalational anthrax, the much rarer form, had only 18 cases reported in the USA between 1900 and 1978 with the majority of the cases associated with occupational hazards for those who worked with livestock or their by-products. In contrast to cutaneous anthrax, the last case of natural occurring inhalational anthrax in USA occurred in 1976 [14]. With the rarity of inhalational anthrax and taking into account the recent anthrax letter cases in 2001, any new reported cases of

inhalational anthrax should warrant an investigation by the health authorities. Gastrointestinal anthrax is often associated with the ingestion of under-cooked contaminated meat and occur mostly in Africa and Asia [10, 15]. Although it is generally reported to be less common than cutaneous form [9], some researchers believe it is in part attributed to the lack of awareness in differential diagnosis in rural endemic area and therefore result in gross under-reporting [16].

1.1.3 Anthrax as a biological weapon

The use of biological agents as a possible weapon was explored by several countries in the last century. During World War I, Germany, England and France were allegedly having programs that involved the use of biological agents such as *B. anthracis* and *Burkholderia pseudomallei* in covert operations [17-19]. Several more countries including Russia and Japan joined the list of countries that began biological warfare research programs during World War II. Among the several biological agents, anthrax remained the most extensively researched and used [17]. During World War II, Japanese ran a covert biological weapon program, known as Unit 731, which conducted biological experiments on prisoners of war [20]. Agents tested by the Japanese include anthrax, botulism, brucellosis, cholera, dysentery, gas gangrene, meningococcal infection, and plague [20]. During the same period, British were researching on effective dissemination of anthrax and conducted a release on Gruinard Island near Scotland [21]. The aerosol release resulted in persistent contamination of viable spores on the island for almost 40 years till it was fully decontaminated with paraformaldehyde in 1986.

Recent history has also witnessed several incidents involving anthrax. In 1979, reports were emerging in Sverdlovsk, USSR, pertaining to a purported anthrax epidemic with the majority of the 96 cases involving gastrointestinal anthrax [22]. It was only after an American scientist conducted a joint investigation with the Russian clinician then did the actual cause of the outbreak was concluded to be caused by an release of anthrax aerosol from a military facility [22]. Although the aerosol release was accidental, it nonetheless demonstrated the feasibility and potency of employing anthrax as a weapon.

In Japan, a non-state sponsor terrorist group Aum Shinrikyo, attempted to disseminate anthrax spores in Tokyo in 1993 by spraying anthrax spores preparation from a rooftop [23]. Fortunately, no one was known to be infected from that dispersal. Subsequent analysis of the spores indicated that the group used a relatively non-virulent strain of anthrax that closely resembled Sterne 34F2 strain, which is commonly used for animal vaccination [24].

Most recently, letters laced with anthrax spores were sent to several prominent personnel in the USA, which resulted in a total of 22 cases of anthrax with 11 cases of cutaneous and 11 cases of inhalational anthrax [25]. 5 out of the 11 cases of inhalational anthrax died and all 11 cases of cutaneous anthrax survived. This anthrax episode which involved seemingly minute amount of spores was able to instill fear throughout United States and eventually millions of dollars were spent on decontamination [26]. It also sparked off a series of copycat anthrax hoaxes in countries around the world. When the first victim succumbed to the disease in Oct 2001, it marked the beginning of a series of long investigations by FBI [27]. Despite the enormous amount of resources committed to

the investigation, it was only until recently in late 2008 that the case came to a closure after FBI's lead suspect committed suicide [28].

Several risk assessment studies estimated the number of casualties as well as the extent of damage in the event of a biological release in populated area. World Health Organization issued an report in 1969 estimating that an aircraft dissemination of 50 kg of anthrax over a population of 500,000 would result in 95,000 killed and 125,000 incapacitated [17]. Another study conducted by the U.S Congressional Office of Technology Assessment revealed similar figures of up 3 million death would be expected if 100 kg of anthrax spores is released in Washington, D.C. [29]. Furthermore, CDC estimated the economic impact of such an attack in the suburban of a major city to be $26.2 billion per 100,000 persons exposed [30]. Most recently, Homeland Security released a report detailing terrorism threats to USA over the next 5 years [31]. Of note, prediction by intelligence official suggests that USA will encounter a destructive biological attack within the next 5 years [31]. According to the threat assessments, terrorists will focus on targets with "massive economic losses, casualties and political turmoil".

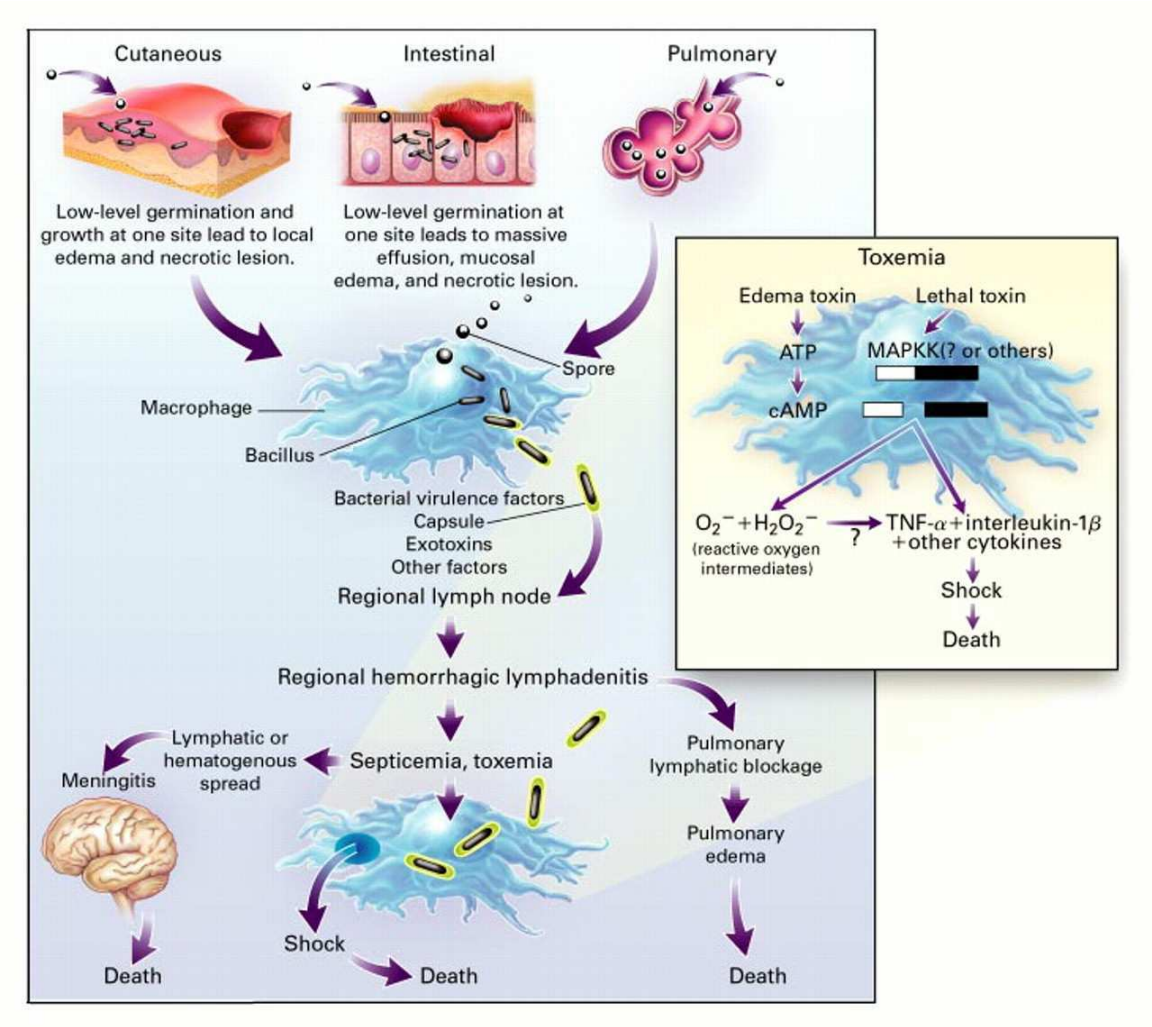


Figure 1.2: Pathophysiology of anthrax [9]

1.2 Bacteria pathogenesis

1.2.1 Microbiology

Bacillus anthracis is a rod-shaped, gram positive, spore forming and non motile bacteria that was first isolated in 1850 [5]. Together with *Bacillus cereus* and *Bacillus thuringiensis*, *B. anthracis* are classified under *B. cereus* group [32]. Despite the striking similarity among members of the *B. cereus* group, *B. anthracis* can be differentiated from other species by colony morphology, gamma phage susceptibility and capsule production.

It grows readily in a variety of laboratory media at 37^{o}C as white gray colonies. In infected blood or tissue, the vegetative bacteria multiply rapidly and are often present in short chains. When growth condition becomes unfavorable, vegetative cells form endospores which are resistant to drying, heat, ultraviolet light and many disinfectants [33]. The dormant spores can survive in soil for decades [34, 35] and upon exposure to nutrient-rich environment, it will germinate to form large numbers of rapidly growing bacteria.

1.2.2 Virulence factors of *Bacillus anthracis*

Vegetative *B. anthracis* generates two essential virulence factors: the anthrax toxin and the poly-γ-D glutamic acid capsule [36]. pXO1 encodes for Protective Antigen (PA), Lethal Factor (LF) and Edema Factor (EF) where it exert a wide array of effects on host cells [37, 38]. pXO2 encodes for capsule which helps to prevent the germinated bacteria from being engulfed by phagocytes [39]. Strains that are lacking in either of the plasmid, for example Sterne strain ($pXO1^{+}$, $pXO2^{-}$) and Pasteur ($pXO1^{-}$, $pXO2^{+}$) strains are greatly attenuated and do not normally cause disease in human [40].

Edema factor

ET is a calmodulin-dependent adenylate cyclase that convert cytosolic ATP to cAMP [41]. The dramatic increase in cAMP is responsible for the edema observed in tissues from anthrax-infected animals [42]. ET was also recently reported to cause an increase in anthrax toxin receptors (ANTRX) expression on cells which in turn provide

more sites for PA binding. The presence of more PA binding sites would further enhance of the rate of toxin internalization into the cell [43].

Lethal factor

LF exerts its toxic effect through the disruption of mitogen-activated protein kinase kinase (MAPKK) signaling pathway which is essential in mounting an efficient and prompt immune response against the invading pathogen [44-46]. LF is also a potent inhibitor on many functions of immune cells. It was shown to inhibit the secretion of pro-inflammatory cytokine in macrophages [47, 48] and dendritic cells [49]. It has also been found to slow down the proliferation of human moncytic cell lines such as U-937, HL-60 and THP-1 cells and inhibited phorbol myristate acetated-induced differentiation of HL-60 cells into macrophage-like cells [50]. It can also affect the neutrophil chemotaxis ability by blocking Hsp27 phosphorylation [51, 52]. Lethal toxin was reported to inhibit activation, proliferation and cytokine expression in T cells [53-55]. Similarly through MAPK inhibition, LT indirectly inhibits B-cell proliferation and antibody production [56]. The multiple effects of lethal toxin on various immune cells significantly thwart both the innate and adaptive immune response.

It is well-known that LF mediates the destruction of host cells through either necrotic cell death or apoptosis pathway depending upon the genetic background of the cell types [57]. Murine macrophage cell line RAW 264.7 was the first cell line found to be sensitive to lethal toxin [58]. Treatment of RAW 264.7 cells with cytolytic dose of lethal toxin cause rapid cell death via necrosis pathway. However, sub-lethal doses of

lethal toxin induce cell death through programmed cell death, apoptosis [59, 60]. LT also causes apoptosis in macrophage cell lines from several strain of murine and human macrophages [61]. Other strains such as Balb/C macrophages are more sensitive to LT and die rapidly by necrosis [57]. Certain differentiated human macrophage-like cells are also susceptible to LT-induced cell death[50]. Similarly, DC from human and mouse C57BL/6 were reported to undergo apoptosis after treatment with lethal toxin [57]. The differences in susceptibility of macrophages from different mouse strains to lethal toxin was initial reported to be dependent on *kif1C* gene that encodes a kinesin-like motor protein of the UNC104 subfamily [62, 63]. However, a subsequent study identified NACHT-leucine rich repeat and pyrin domain containing protein 1b (NALP1b) as the contributing host factor for LT cytotoxicity [64].

The importance of lethal toxin in anthrax pathogenesis is demonstrated by a marked reduction in virulence upon depletion of the toxin-bearing plasmid [65, 66]. In addition, the elimination of bacteria from body system with antibiotic may not prevent death [67]. This suggests that despite the absence of viable bacteria, remaining circulating LT and possibly other secreted compound may continue to have a detrimental effect on the body system. Together with the disabling of several important subsets of the immune system (Table 1.1) and the presence of high amount of LF (11-15µg/ml) in serum of infected animal [68], lethal toxin represents an integral part of the anthrax pathogenesis.

Germination operon

In addition to toxigenic genes, pXO1 also encodes a germination operon *ger*X which is critical for spore germination. Strains lacking *ger*X operon exhibited diminished germination rate and thus reduced virulence [69]. *GerX* operon encodes 3 proteins; GerXA, GerXB and GerXB, which together may form a receptor for detecting specific germinant in the host cells [69].

Capsule

The other virulence plasmid, pXO2, carry 3 genes that encodes for a poly-gamma-D-glutamic acid capsule [70, 71]. The capsule is responsible for inhibiting phagocytosis of the bacteria and consequently enables the bacteria to establish itself in the host cells. The simple homopolymeric structure of capsule also confers poor immunogenicity and therefore do not elicit a strong immune response [72]. Capsule appears to be an important virulence factor as capsulated nontoxigenic strains are virulent for certain mice strain [73]

Other putative virulence factors

While lethal toxin is generally accepted as the main virulence factor of anthrax, several studies suggest that *B. anthracis* harbour a number of other factors that may contribute to bacteria pathogenesis. Several secreted proteolytic enzymes from *B. anthracis* culture were reported to be toxic to mice and it was shown that treatment of mice with protease inhibitor and antibiotics protected mice from spores challenge [74]. Another two secreted proteases, Npr599 and InhA, were observed to significantly

enhance syndecan-1 shedding from cultured normal murine mammary gland cells resulting in the disruption of epithelial or endothelial integrity, hemorrhage, edema and abnormal cell signalling [75]. Npr599 and InhA were also found to contribute to hemorrhage and thrombosis mediated by the degradation of circulating von Willebrand factor [76] and may possess other factors such as degradation of host tissues, the increase of barrier permeability and host defenses modulation [77]. Additionally, a recent study demonstrated that secreted InhA1 initiates coagulation of human blood through the activation of prothrombin and factor X [78]. In addition to Npr599 and InhA, Sterne strain also produce another protein termed as anthrolysin O (ALO), which was shown to readily lyse human erythrocytes [79]. ALO was also hypothesized in part, to mediate the escape of *B. anthracis* from phagolysosome [79]. These potentially relevant but overlooked virulence or virulence enhancing factors may play a contributing role in disease pathogenesis.

1.2.3 Translocation of LT and ET into cytoplasm

PA is a 83kDa secreted protein that binds to at least 2 cell surface receptors; ANTXR1/TEM-8 and ANTXR2/CMG-2 [80, 81]. Another cell surface co-receptor, lipoprotein receptor-related protein 6 (LRP-6), is also determined to be essential to the endocytosis of anthrax toxin complex into the cell [82]. After binding to the cell surface receptors, PA_{83} is subsequently cleaved by a furin-like protease to release a 20kDa from the N-terminus [83, 84]. The truncated PA_{63} subunit then assemble itself into a heptameric configuration that allows the binding of LF or EF to form a toxin-receptor

complex [85]. The complex is subsequently transported into the cytoplasm via a raft-dependent and clarthrin-mediated endocytosis [86] where it is acidified. Acidification in endosome is an essential process as inhibitors that prevent endosomal acidification also prevent the translocation of LF and EF into the cells [87] . Subsequent processing in the endosomes culminates in the eventual release of free LF/EF into the cytoplasm (Figure 1.3).

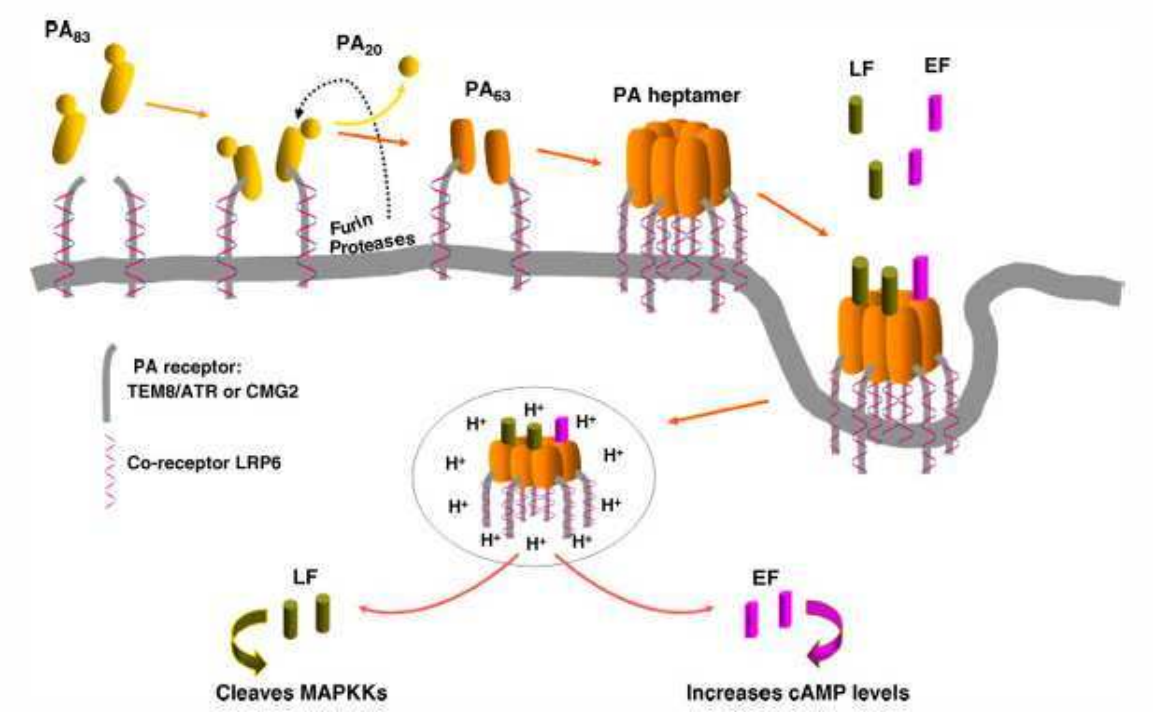


Figure 1.3: Internalization of anthrax toxin [38] PA83 binds one of two cellular receptors, TEM8/ATR or CMG2, which, in turn, associate with the LRP6 co-receptor. Upon binding, PA83 is cleaved by cellular furin proteases and the smaller fragment (PA20) is released. The larger fragment, PA63 forms a ring-shaped heptameric pre-pore, which can simultaneously bind up to three molecules of LF and/or EF. The toxin/receptor complex is then internalized in a LRP6-dependent manner. The endocytic vesicles are

subsequently acidified, initiating a conformational change of the PA heptamer which converts it from the pre-pore into a mature pore that can allow entry of EF and/or LF toxin enzymes into the cytoplasm of target cells.

Table 1.1 Cellular targets and effects of anthrax toxins [67]

Toxin	Target cell	Effect	Refs
Lethal toxin	Neutrophil	Inhibits mobility	42
	Monocyte	Inhibit proliferation and differentiation	73
	Activated macrophage	Causes cell death	73, 74, 75, 76
	Macrophage	Suppresses cytokine production	125
	Immature dendritic cell	Causes cell death	67
	Mature dendritic cell	Suppresses cytokine production, co-stimulatory molecule expression and T-cell stimulation	78, 79, 126
	T cell	Inhibits activation, proliferation, surface-molecule expression and cytokine expression	95, 96, 97
	Red blood cell	Causes cell death	69
	Platelet	Induces coagulopathy	112
	Endothelial cell	Causes cell death; promotes cytokine mRNA degradation; dysregulates barrier function	43, 45, 66, 68 82
Oedema toxin	Neutrophil	Inhibits phagocytosis	63
	Macrophage	Causes cell death	72
	Mature dendritic cell	Suppresses cytokine production	79, 126
	T cell	Inhibits activation, proliferation, surface-molecule expression and cytokine expression	96, 97
	Platelet	Induces coagulopathy	91

1.3 Clinical manifestation and diagnosis

Cutaneous anthrax represent the most common form that account for 95% of all anthrax infection in the United States [88]. While most cases of cutaneous anthrax arises from physical exposure of anthrax to a cut or a scrape on the skin [89], there were several cases that suggest mechanical transmission by insects, presumably after feeding on infected animal [90, 91]. The incubation period reported ranges from 12 hours to as long as 19 days [92] and followed by the appearance of local edema and the development of a ring of vesicles. It progresses to ulceration of the central papule and subsequently dries to form black eschar. Complete healing usually occurs in 1 – 2 weeks and the vesicle usually does not result in scarring. Although cutaneous anthrax is usually self-limiting and lesions can heal even without treatment [93], it can become systemic and fatal in about 20% of untreated cases. Treatment reduces the risk of death to less than 1%.

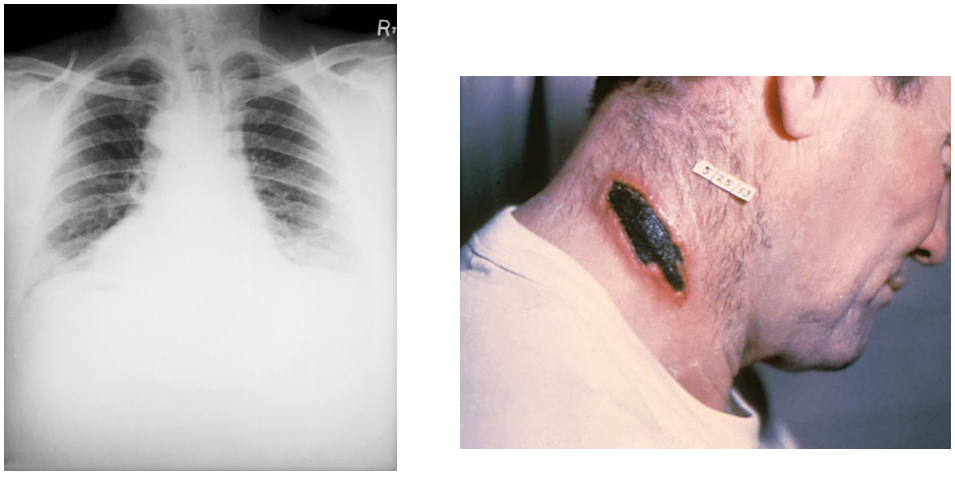


Fig 1.4 Left - Posteroanterior (PA) chest x-ray of a 46 yr. old male patient revealed revealed bilateral pulmonary effusion, and a widened mediastinum. Right - Cutaneous anthrax lesion on the neck. (CDC, Public Health Image Library, http://phil.cdc.gov)

Gastronintestinal (GI) anthrax are either very rare [94] or have not reported [9] in the United States but cases have occurred in other parts of the world [7]. Ingestion of anthrax spores does not appear to cause gastrointestinal anthrax as experiments involving direct gastrointestinal instillation of spores into monkeys did not cause disease [95]. Instead, it has been postulated that gastrointestinal anthrax requires the ingestion of large number of vegetative bacteria, as in the case of under-cooked contaminated meat [27]. Abdominal form of the disease is typically characterized by the formation of ulcers and eschars in the wall of the terminal ileum but may also affect other parts of the GI tract such as cecum, colon, stomach and the duodenum. Initial symptoms include nausea, vomiting, anorexia and fever and at later stages, severe abdominal pain, hematemesis and bloody diarrhea which may progress to septicemia and death [96]. Gastrointestinal anthrax is easily treated if diagnosed at an early stage, however, given the early nonspecific symptoms; diagnosis is difficult and may result in high mortality.

Inhalational anthrax describes the route of infection via the airway with the subsequent deposition in the lungs. Aerosol size of 1 – 5 μm represent the most effective lung retention size [97, 98], thus enabling the spores to reach to the bronchioles and alveoli. Although lungs represent the initial site of contact, there is no infection of the lungs in most inhalational cases, [99, 100]. Upon entry into the human lungs, alveolar macrophages and lung dendritic cells would engulf the spores and germination occurs in the macrophages en route to the regional lymph node [101]. Proliferating vegetative would escape from macrophages [69] and multiply quickly in the lymphatic system. Subsequently, it would spread to the blood system (Figure 1.5 & 1.6) where patients can

die from septicemia and septic shock [9]. Mortality rate for this form of anthrax is expected to be high with about 85% as in the case of Sverdlovsk incident [22]. However, the recent anthrax cases in United States depicted a lower mortality rate of 45% [25]. Rapid diagnosis accompanied by immediate antibiotic treatment and better health care support may account for the differences in survival.

Initial symptoms of inhalational anthrax 2 – 5 days after exposure includes "flu like symptoms" of mild fever, fatigue, malaise, myalgia and non-productive cough. The mild prodromal period which last about 2 days progresses to acute acute illness characterized by acute dyspnoea, stridor, fever, and cyanosis. Clinical findings includes tachypnoea, cyanosis, tachycardia, moist rales, and evidence of pleural effusion [96]. Chest X-ray and chest CT scans is diagnostically important with findings such as widened mediastinum, massive pleural effusion, air bronchograms and necrotizing pneumonic lesion [27]. At terminal stage, the patient becomes disoriented and may slip into a coma followed by death [102-104]. Although 50% of inhalational anthrax patients may develop meningitis, which is characterized by the presence of blood in the cerebrospinal fluid, some patients with late stage of other forms of anthrax may also developed meningitis [35]. Often, patients who developed meningitis from any form of anthrax infection have very poor prognosis.

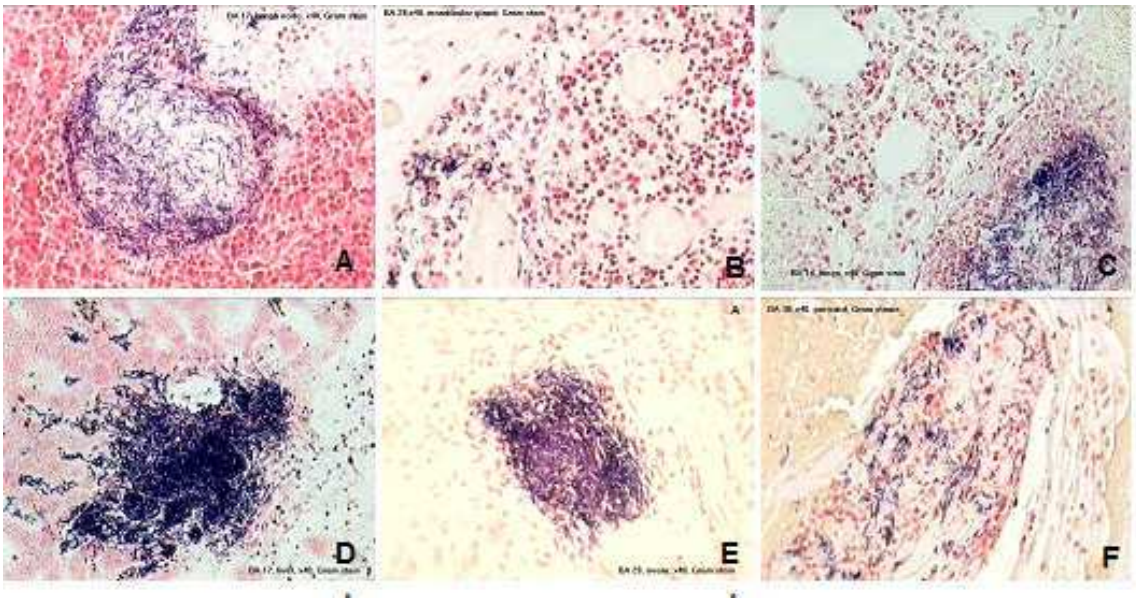


Figure 1.5: Dissemination of bacteria into different organs after challenge of *B anthracis* Sterne spores in rabbits. The animals were challenged with *B. anthracis* spores and organs were harvested immediately after expiration of the animal. Slides were made with a microtone and colored with Gram stain. Pictures were taken under microscope (40 x) using Motic Imagine Plus system. **A:** lymph node; **B:** mandibular gland; **C:** lung; **D:** liver; **E:** ovary; **F:** pericardium.

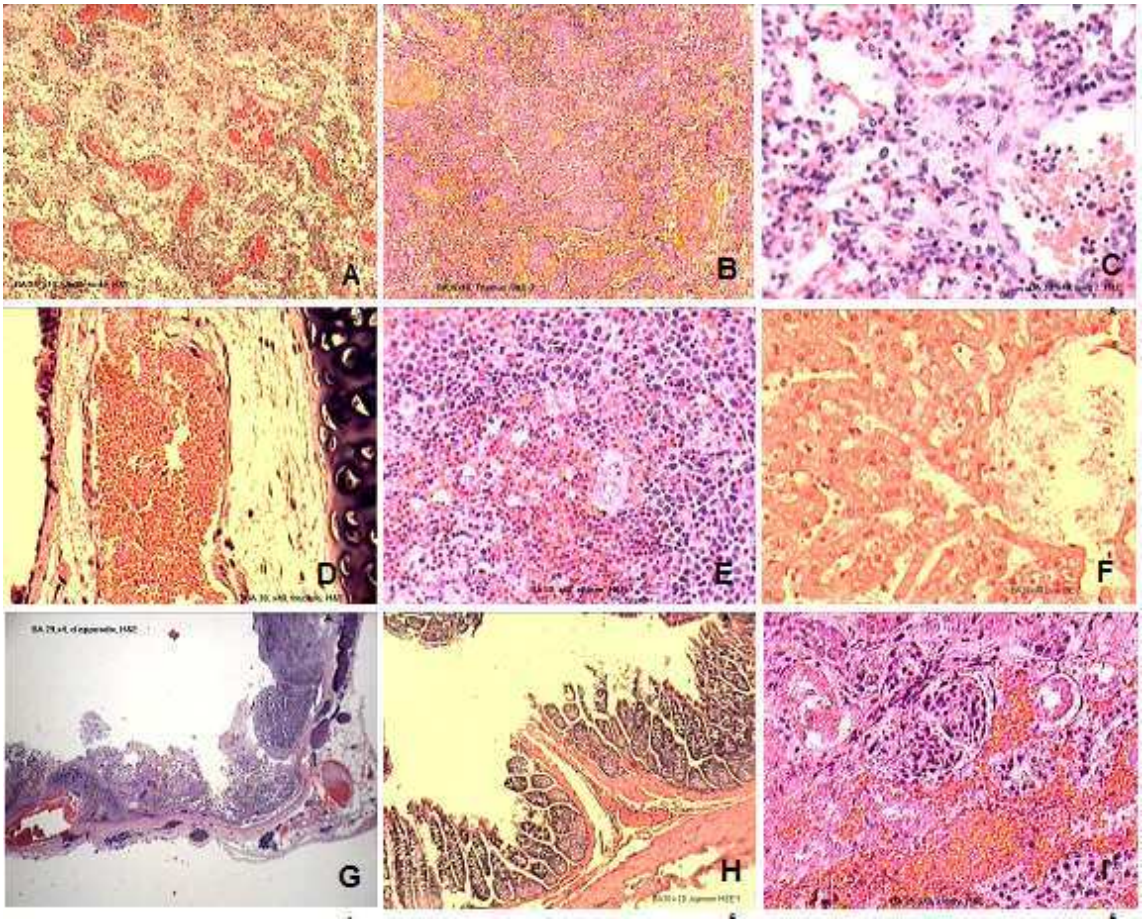


Figure 1.6: Pathological changes observed in the tested organs from expired rabbits after infection with *B. anthracis* Sterne spores. The organs were harvested immediately after death of the animals. Slides were made and stained with H&E. The common findings in different organs include congestion, degeneration, hemorrhage, and necrosis as described in the text. **A:** lymph node x10; **B:** thymus x10; **C:** lung x40; **D:** trachea x40; **E:** spleen x40; **F:** liver x40; **G:** appendix x4; **H:** ileum x10; **I:** kidney x40.

1.4 Prophylaxis and treatment

There is currently one anthrax vaccine approved by the Food and Drug Administration (FDA) in the United States. Manufactured by Bioport, the Anthrax Vaccine Adsorbed (AVA) is derived from aluminium hydroxide precipitated preparation of PA from *B. anthracis* Sterne strain [105]. Pre-exposure vaccination with AVA is shown to protect animals from subsequent challenge with live virulent strain of *B. anthracis* [106, 107]. Routinely provided to persons with higher risk of exposure to anthrax spores, AVA vaccination is administered subcutaneously as six initial doses at 0, 2, and 4 weeks and 6, 12 and 18 months followed by an annual booster [108]. In 1997, the United States Department of Defense (DoD) mandated that all military personnel be vaccinated with the AVA vaccines. Concerned about the safety of the vaccine and possible side effects, some personnel has rejected the compulsory vaccination in the United States Armed Forces and risked facing disciplinary actions [109, 110]. Due to the inherent shortcomings of the current vaccines preparation [27], there is a need for new vaccine that offers improved efficacy with simpler schedule.

Rapid treatment of anthrax infection is central to improving prognosis of patients, with the administration of antibiotics as one of the most critical aspect of therapy [111] and should be initiated pending confirmed diagnosis. Approved drugs for inhalational anthrax treatment includes penicillin, doxycycline and ciprofloxacin [111, 112]. Recommendations by CDC for inhalational anthrax treatment requires 60 days of ciprofloxacin treatment [27] taking into account the possibility of delayed spores germination as observed from experimental studies in animals [113] and the Sverdlovsk

incident [22]. In addition to antibiotics, post-exposure vaccination can also be administered to those who are suspected for exposure [9].

1.5 Study Objectives

Despite the advances made in our understanding on anthrax pathogenesis, the prognosis for certain forms of anthrax, in particular inhalational anthrax, is extremely poor. There is little doubt that LF is the primary virulence factor for anthrax [65, 66] and in view of this, contemporary research on new therapies has been focused on mitigating the harmful effect of this toxin. Indeed, several studies provide new insights into the possible use of drugs that inhibit the toxicity of LF *in vivo* and the corresponding protection in animal experiments [114, 115]. While it remains unclear if combination therapy will indeed improve survival, it seems to be a reasonable approach to augment the current treatment method of antibiotics regimens and supportive care with other novel approaches that may counter the action of LF.

In this study, we identified novel effects of LT on immune cells and discuss about its implication in anthrax pathogenesis. Our preliminary results suggest that LF may activate autophagy, an increasingly important process that plays a role in many cellular functions. More detailed experiments will be conducted to verify the induction of autophagy as well as to determine the role of autophagy in LF intoxication. Reactive oxygen species (ROS) is involved in many aspects of signaling transduction and its production is upregulated during exposure to LF. We seek to further investigate the effects of ROS overproduction on cell viability and apoptosis. In addition, the mitigation

of harmful effects of ROS production in both *in vitro* and *in vivo* model with a new formula of antioxidant liposome will also be evaluated. The last portion of this work will focus on the influence of LF on the functions of neutrophils by way of mediating chemokines production.

Chapter 2: Lethal Toxin and Autophagy

2.1 Introduction

Maintaining proper homeostasis involves interaction between biosynthesis and degradation of cellular constituents. Intracellular degradation of damaging or unnecessary constituents can be divided into two main mechanisms. In cells, short-lived proteins are degraded by non-lysosomal ubiquitin-proteasome system [116] . The proteins destined for proteolysis are first labeled with ubiquitin followed by 26S proteasome complex catalysed degradation [117]. On the other hand, the intracellular break down of long-lived proteins and organelles is mediated by autophagy and is morphologically characterized by the formation of many large autophagic vacuoles in cytoplasm [118]. This evolutionary process is conserved across all eukaryotic cells and is fundamentally important in normal and pathological cell physiology and development [119, 120]. Autophagy occurs constitutively at a basal level in quiescent cells but the process may be up-regulated during periods of starvation [121] and in response to other stress stimuli [122-124]. This catabolic pathway is highly regulated and its dysfunction is associated with many various disease states. Many recent studies suggest that autophagy occurrence is more common than previously thought. Indeed, it has been implicated in many physiological and pathological conditions such as neurodegeneration, death of cancer cells, tissue and organ formation, neonatal development, host cell response to bacterial

and viral infection as well as toxin intoxication [122-124]. New discoveries are continuously added to the growing list of autophagy functions [125].

Types of autophagy

Several various types of autophagy exist in mammalian cells which includes microautophagy, macroautophagy, chaperone-mediated autophagy, micro- and macropexophagy, piecemeal microautophagy of the nucleus, and the cytoplasm to vacuole targeting (Cvt) pathway [126, 127] (Figure 2.1). During microautophagy, vacuolar membrane sequesters cytosolic component directly into lysosomal lumen [128] without vesicular intermediates [129]. Various cellular components including vacuolar membrane can be degraded via this form of microautophagy. Chaperone-mediated autophagy (CMA) involves the transportation of selective proteins directly into lysosomes through a protein translocation pathway [130]. Several proteins including LAMP-2a transporter [131], HSC70 cytosolic chaperones [132] and HSC73 lysosomal lumen [133] are essential for CMA process. For macroautophagy, the process begins with the formation of isolation membrane or phagophore followed by sequestration of organelles or part of the cytoplasm to form autophagosome. The double-membrane autophagosome subsequently fuses with lysosome to form autolysosome where the inner autophagosomal membrane and its content are degraded and released into the cytoplasm (Figure 2.2) [134, 135]. This study will only focus on macroautophagy which will be referred to as autophagy hereafter.

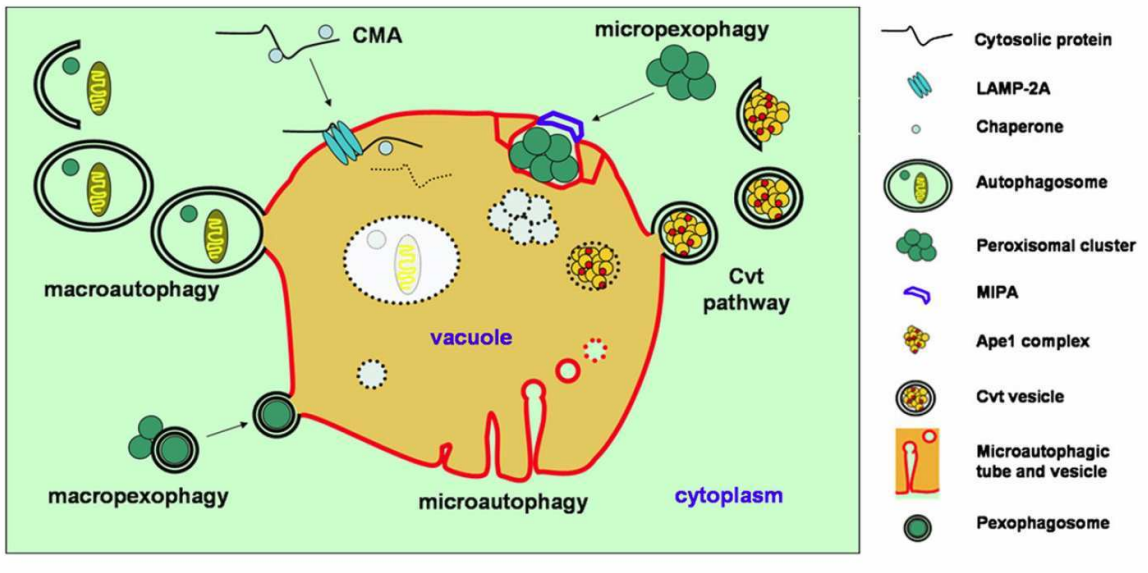


Figure 2.1: Schematic representation of autophagy process. Cytoplasmic components may be degraded via chaperone mediated autophagy, macroautophagy , microautophagy, macropexophagy, micropexophagy and the cytoplasm to vacuole (Cvt) pathway [136].

Autophagy related genes

A total of 31 autophagy related genes were identified and classified to date (Table 2.1) [135, 137]. Several important autophagy related genes (*ATG*) that are critical for autophagosome formation have been identified recently [137, 138]. Microtubule-associated protein 1 light chain 3 (LC3) is the mammalian orthologue of yeast Atg8 that is required for autophagosome formation [139]. During autophagy, cytosolic form of LC3 I is processed into a lipidated LC3 II which is tightly associated with autophagosome membranes [140-142]. In addition, Atg8 was also identified to be involved in the expansion of isolation membrane [143]. The other protein complex that is essential for elongation of the isolation membrane is Atg5-Atg12 complex [144, 145]. Many of these autophagy genes are also required for the Cvt pathway [146].

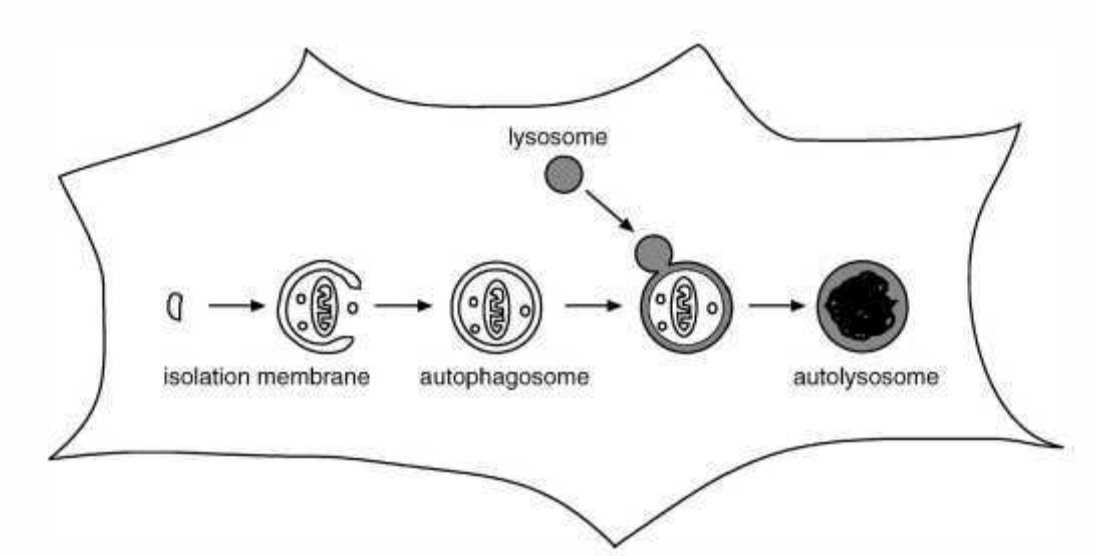


Figure 2.2: The simplified process of autophagy in mammalian cells. A small volume of cytoplasm is enclosed by the autophagic isolation membrane, which eventually results in the formation of an autophagosome. The outer membrane of the autophagosome then fuses with the lysosome where the cytoplasm-derived materials are degraded [147].

Table 2.1: Description of autophagy-related genes. [137]

Gene Designation							
Current	Former						
ATG	*APG*	*AUT*	*CVT*	*GSA*	*PAZ*	*PDD*	Protein Characteristics
1	1	3	10	10	1	7	Protein kinase
2	2	8	—	11	7	—	Peripheral membrane interacts with protein Atg9
3	3	1	—	20	—	—	E2-like enzyme conjugates Atg8 to phosphatidylethanolamine (PE)
4	4	2	—	—	8	—	Cysteine protease; cleaves C-terminal extension or PE from Atg8
5	5	—	—	—	—	—	Conjugated to Atg12 through internal lysine
(6)[a]	6	—	—	—	—	—	Component of PtdIns 3-kinase complexes I and II
7	7	—	2	7	12	—	E1-like enzyme activates Atg8 and Atg12
8	8	7	5	—	2	—	Ubiquitin-like protein conju gated to PE via C-terminal glycine
9	9	9	7	14	9	—	Integral membrane protein
10	10	—	—	—	—	—	E2-like enzyme; conjugates Atg12 to Atg5
11	—	—	9	9	6	18	Specific component involved in cargo recognition
12	12	—	—	—	—	—	Ubiquitin-like protein; conjugated to Atg5 via C-terminal glycine
13	13	—	—	—	—	—	Modifier of Atg1 activity; hyperphosphor-ylated in rich media
14	14	—	12	—	—	—	Component of PtdIns 3-kinase complex I
15	—	5	17	—	—	—	Putative lipase required for breakdown of intravacuolar vesicles
16	16	—	11	—	3	—	Component of Atg12-Atg5 complex
17	17	—	—	—	—	—	Modifier of Atg1 activity
18	—	10	18	12	—	—	Peripheral membrane protein; required for localization of Atg2
19	—	—	19	—	—	—	Cargo receptor for the Cvt pathway
20	—	—	20	—	—	—	PX domain protein needed for the Cvt pathway
21	—	—[b]	21	—	—	—	Specific to the Cvt pathway
22	—	4	—	—	—	—	Integral membrane protein; involved in autophagic body breakdown
23	—	—[c]	23	—	—	—	Needed for Cvt vesicle completion
(24)[d]	—	—	13	—	16	—	Sorting nexin; PX domain-containing protein involved in the Cvt pathway and pexophagy
25	—	—	—	—	—	4	Coiled-coil protein involved in macropexophagy
26[e]	—	—	—	—	4	—	UDP-glucose:sterol glucosyltransferase-containing GRAM domain
27[f]	—	—	24	—	—	—	PtdIns(3)P binding protein required for the Cvt pathway

[a] The standard name for this gene is *VPS30*.
[b] This gene was originally named *MAI1*.
[c] This gene was also named *MAI2*.
[d] The standard name for this gene is *SNX4*.
[e] This gene was originally named *UGT51*.
[f] This gene was originally named *ETF1*.

Autophagy process

For ease of illustration, the dynamic autophagy process can be divided into 7 static phases; (1) induction, (2) cargo selection and packaging, (3) vesicle nucleation, (4) vesicle expansion and completion, (5) retrieval, (6) targeting, docking and fusion of the vesicle with the lysosome/vacuole, and (7) breakdown of the vesicle and its contents [148].

As mentioned earlier, many conditions can induce autophagy and several of this stimulus activate autophagy via modulation of Tor [149]. Induction of autophagy upon Tor inactivation can occur either through direct binding or through other indirect inactivation mechanisms. Secondly, cargo selection and packing process, which is largely for selective autophagy, includes the Cvt pathway [150], pexophagy [151] and mitophagy [152] with each having their respective component for selective degradation.

The third step involves vesicle nucleation, which is proposed to initiate at phagophore assembly site (PAS) [153]. As autophagy progresses, many autophagy-related proteins are recruited to PAS where the intracellular membrane begins expansion to surround the sequestered cargo until the complete enclosure of the cargo. Essential to this elongation process are 2 ubiquitin-like conjugation process, the Atg8-phosphatidylethanolamine (PE) and Atg12-Atg5 [154]. Atg8-PE acts as a scaffold protein for the docking and expansion of membrane whereas Atg12-Atg5 conjugate promotes the lipidation of Atg8 to PE [154]. Similar to many protein targeting pathways, Atg proteins are also retrieved for multiple rounds of substrate delivery. As only Atg8 and Atg19

remained in completed autophagosomes, it is suggested that all other Atg proteins that were involved in previous vesicle forming process, including Atg2, Atg9 and Atg19 [155], were disassembled from the mature autophagosome before its completion [156, 157].

The next step in autophagy involves the targeting, docking and fusion of autophagosomes with lysosomes. Upon maturation of autophagosomes, fusion proteins facilitate the binding process in which the outer membrane of autophagosome fuses with the lysosomal membranes and releasing the inner vesicle into the lysosomal lumen. Some of the proteins required for fusion include SNARE proteins, NSF, soluble NSF attachment protein, GDP dissociation inhibitor (GDI) homologs, Rab proteins and the class C Vps/HOPS complex [138]. This fusion process is regulated by AAA ATPase SKD1, the small GTP binding protein Rab7, and possibly also the Alzheimer-linked presenilin 1 [158]. In the final step of autophagy, the inner membrane of the autophagy vesicle is degraded [159] along with the release of its cargo into the lysosome lumen for degradation by vacuolar hydrolases [160].

Autophagy regulation

Autophagy is regulated by several mechanisms (Figure 2.3) and amongst them, target of rapamycin (Tor) kinase represents the best characterized regulatory component to date [149]. Tor is a highly conserved Ser/Thr kinase that controls cell growth through regulating a series of anabolic and catabolic process [161]. It regulates autophagy through interaction with an autophagy mediator protein complex that consists of several Atg

proteins including Atg17, Atg11, Atg20, Atg24 and Vac8 [162, 163]. The protein complex composition is regulated by the phosphorylation state of Atg1 and Atg13 [162]. Under nutrient-rich conditions, Tor maintains Atg13 in a highly phosphorylated state which reduces its binding affinity for Atg1. Consequently, the lack of Atg13-Atg1 complex reduces Atg1 activity and thereby inhibits autophagy. Conversely, inactivation of Tor by rapamycin treatment or starvation results in dephosphorylation of Atg13 and thereby increases its affinity to Atg1. The corresponding increase in Atg1 kinase activity promotes autophagy via the control of the dynamic protein complex [162]. Although cells in nutrient rich condition do not promote autophagy, different amino acids have varying effect of inhibiting autophagy. Among the 20 types of amino acids, Leu, Phe, Tyr, Trp, His and Gln were determined to be strong inhibitor of autophagy [164] with Leu being the most potent inhibitor among all the amino acids [165, 166].

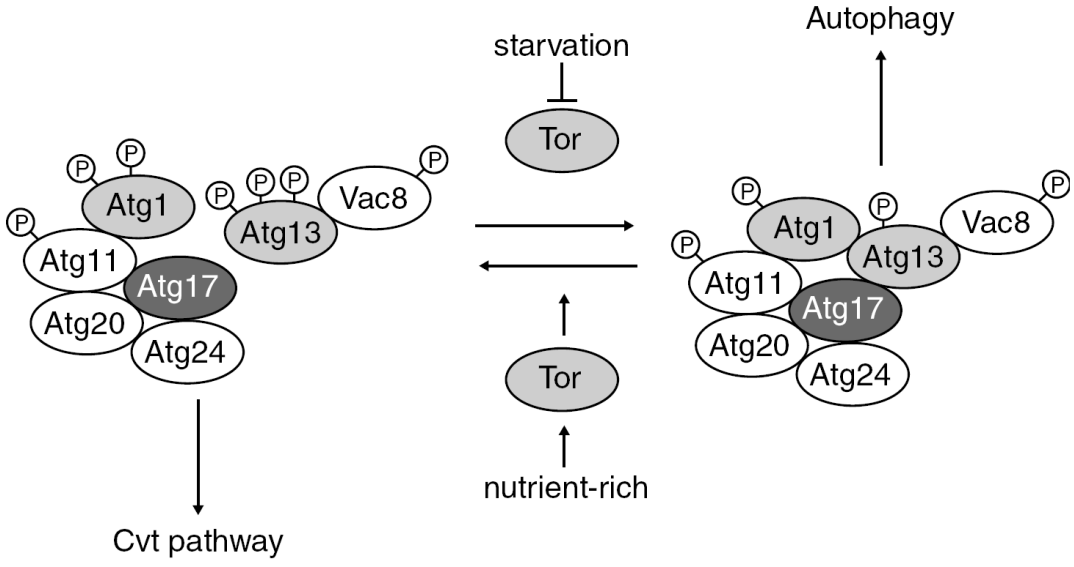


Figure 2.3 : Regulatory complex for autophagy induction. Tor kinase regulates the induction of autophagy upon sensing the conditions. Atg1 kinase, which is essential for

both autophagy and the Cvt pathway, forms a complex with several proteins that are characterized as being preferentially involved in the Cvt pathway (in white circles) or autophagy (in dark gray circle). Under nutrient-rich conditions, Tor kinase is active, and Atg13 is hyperphosphorylated. This highly phosphorylated Atg13 has a lower affinity for Atg1 and Atg17, and autophagy is downregulated. Dephosphorylated Atg13 interacts with Atg1 and Atg17 with a higher affinity. The enhancement of the formation of the Atrg1-Atg13-Atg17 complex mediates the induction of autophagy. The Atg20-Atg24 complex, Atg 11 and Vac8 might also belong to this complex [129].

In addition to amino acids, autophagy can also be modulated by certain hormones. It was demonstrated that glucagon rapidly promotes autophagy in liver cells whereas insulin has been shown to markedly inhibit autophagy [167, 168]. These observations are in agreement with the role of autophagic response towards metabolic requirements. Glucagon is typically upregulated during periods of starvation to convert stored glycogen into glucose. Autophagic breakdown of proteins synergistically provides additional nutrients for cellular metabolism and functions. Conversely, it is apparent that high glucose condition necessitates the conversion back to glycogen by way of insulin modulation, thus rendering recycling of nutrients via autophagy redundant.

Apart from Tor, autophagy is also reported to be regulated by other protein kinase including Ras/PKA signaling process [169], SNF1 kinase [170] and GCN2 kinase [171]. Recently, Osuna described another novel mTor-independent autophagy regulatory pathway as induced by trehalose [172]. Activation of autophagy by trehalose not only enhances the clearing of aggregate proteins that are responsible for Huntington's and Parkinson's disease, it also protects cells against apoptotic effects elicited via mitochondrial cell death pathway in Huntington's disease [172]. How these various complex pathways interact to mediate autophagy is currently not completely understood.

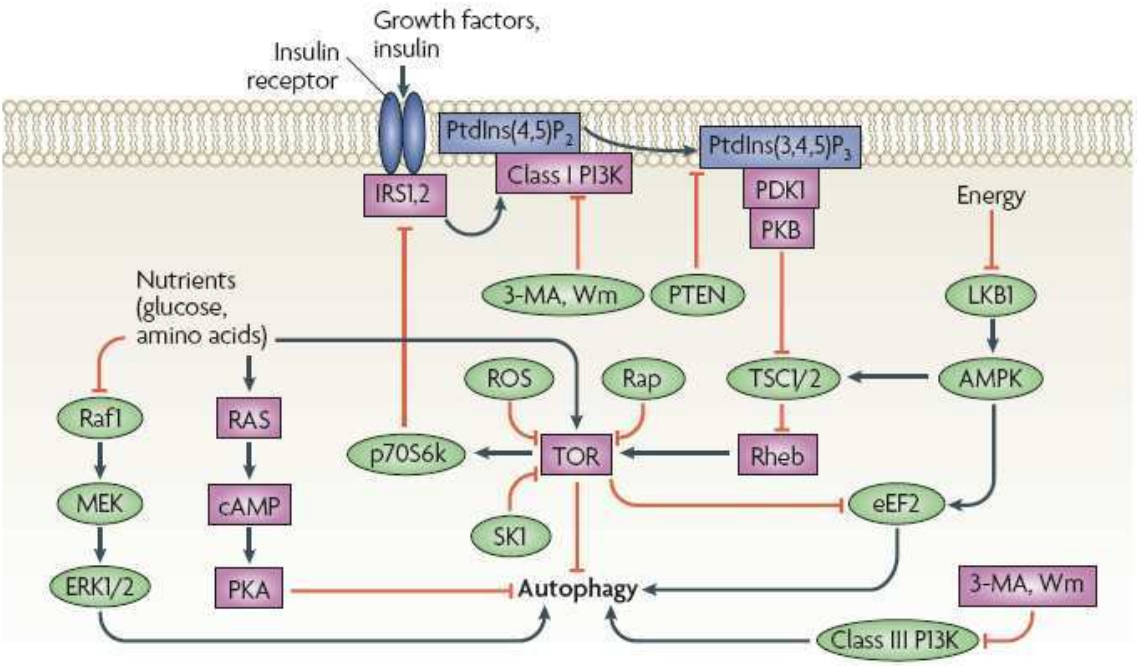


Figure 2.4: Regulation of autophagy in mammalian cells. In the figure, the green circles represent components that stimulate autophagy, whereas the purple boxes correspond to inhibitory factors. 3-methyladenine (3-MA) and wortmannin (Wm) also inhibit class I phosphatidylinositol 3-kinases (PI3K), but the overall effect of these compounds is a block in autophagy (because they inhibit the downstream class III enzyme that produces phosphatidylinositol-3-phosphate (PtdIns(3)P), which is needed for autophagy). The regulation of autophagy is complex and far from understood. Historically, TOR (target of rapamycin) has been considered to be the central regulator of autophagy, because TOR inhibition with rapamycin (Rap) induces autophagy. However, it is now clear that there are also TOR-independent types of regulation. For example, beclin-1 and Atg4 might be regulated by the c-Jun N-terminal kinase (JNK) and reactive oxygen species (ROS), respectively [135].

Autophagy functions

Autophagy and homeostasis

Initially discovered as a response to nutrients deprivation in the 1960s [173], it is now apparent that autophagy plays a role in a myriad of physiological conditions [123, 124] as well as other stress conditions.

In cancer, autophagy probably has dual role in promoting and preventing cellular growth. Autophagy functions as a suppressor of early cancer growth [174, 175] but promotes survival of cancer cells in unfavorable nutrient-limiting conditions during late stage of tumor growth [124]. Furthermore, autophagy may improve cells survival from radiation treatment, possibly by clearing of damaged mitochondria in an attempt to prevent apoptosis [176].

Several neurodegeneration disorders associated with proteins misfold and aggregates such as Parkinson's, Huntington's, Alzheimer's diseases, amyotrophic lateral sclerosis or transmissible spongiform encephalopathies display accumulation of autophagic vesicles [177, 178]. Accumulations of abnormal configuration of proteins prompt cells to response by activating chaperone system for protein refolding and cytosolic proteases for protein removal. With the increasing level of misfolded proteins, the accumulation enables the progression of abnormal proteins into aggregates. Being resistant to protease degradation, the aggregates can therefore only be removed by autophagy [179]. Autophagy thus plays a role by removing protein aggregates during the early stage of the disease and triggers cell death for irreversibly damaged cells during the late stage.

Muscular disorder is frequently linked to deregulation of autophagy [180]. Diagnosis of vacuolar myopathy, a form of muscular disorder, is typically established by the presence of elevated accumulation of autophagic vesicles [180] . Several other muscular disorders related disease, including Danon disease and Pompe disease, are caused by a defect in autophagy arise from a defect in LAMP-2 gene [181]. On the other hand, excessive prominent autophagy plays a role in X-linked Myopathy with Excessive Autophagy (XMEA) which leads to progressive muscle weakness [181].

Autophagy and immunity

Apart from maintaining homeostasis, autophagy also participates in host defense against pathogens. In recent years, rapidly growing number of micro-organisms were shown to induce autophagy (Table 2.1). Typically, autophagy attempts to clear intracellular pathogens by sequestration into autophagosome and subsequent delivery to the lysosome for degradation. However, certain microbes can subvert or exploit the autophagy process for their survival and replication. Tuberculosis is a highly infectious disease that infected many people around the world. The causative agent, *Mycobacterium tuberculosis*, evades the immune system by arresting the maturation of *M. tuberculosis*-containing phagosome with lysosome, thus establishing the vacuoles as a site for replication [182]. Gutierrez reported that physiological and pharmacological induction of autophagy abrogated the mycobacteria-induced blockage of phagosome and lysosome fusion and also suppressed intracellular survival of mycobacteria [183]. In a similar fashion, autophagy was found to effectively eliminate *Staphylococcus aureus* by

sequestration of bacteria into autophagosome [184]. However, the nosocomial variant, methicillin-resistant *S. aureus* (MRSA) strain, displayed a higher level of resistance to autophagy degradation employing an escape mechanism into the cytoplasm [184].

Autophagy also plays a role in viral infection. For HIV infection, autophagy was markedly down-regulated following infection of human peripheral blood CD4(+) T-cells with HIV [185, 186]. This antagonism of autophagy facilitates replication of viruses that do not utilize the autophagosomal membrane as replication sites, such as Human Simplex Virus (HSV-1) [187].

Paradoxically, autophagy mechanism can also be manipulated by certain pathogens for replication. Dengue virus, a vector borne disease, induces autophagy upon infecting mammalian cells [188]. The induction of autophagy enhances viral pathogenesis as evidenced by the increased in extracellular and intracellular viral titer. Autophagy is also critical for polio virus infection as RNA replication occurs on autophagosomal membrane [189] following the impediment of autophagosome and lysosome fusion.

Recently, autophagy was even implicated as playing a major role in transmissible spongiform encephalopathies, also known as prion disease [190]. Double membrane autophagic vacuoles of varying sizes were observed in a large area of cytoplasm in neuron cells and ultrastructural analysis suggest that autophagy may participate in the spongiform change in cells [190].

In adaptive immunity, autophagy unexpectedly also contributes in antigen presentation. Conventionally, major histocompatibility complex (MHC) class II presents

antigen largely from extracellular sources. However, it was shown that autophagosomes fuse with multivesicular MHC class II-loading compartments, leading to antigen presentation in MHC class II-positive cells [191]. Paludan et al demonstrated that Epstein-Barr virus antigen, EBNA1 is processed intracellularly via autophagy pathway for MHC class II presentation to $CD4^+$ T cells [192]. In addition, targeting of influenza MP1 to autophagosomes also greatly enhances MHC class II presentation to CD4+ T cells [191]. These studies underscore the functional significance of autophagy in adaptive immunity.

Table 2.2: Induction of autophagy by bacteria, viruses and toxins

	Autophagy beneficial to cell	**Autophagy detrimental to cell**
Bacteria	*Mycobacterium tuberculosis* [183]	*Brucella abortus* [193]
	Staphylococcus aureus & MRSA [184]	*Legionella pneumophila* [194]
	Group A Streptococcus [184]	*Coxiella burnetii* [195]
	Listeria moncytogenes [196]	*Porphyromonas gingivalis* [197]
	Burkholderia pseudomallei [198]	
Virus	Human immunodeficiency virus (HIV) [185]	Dengue virus [188]
		Poliovirus [189]
		Coxsackievirus [199]
		Mouse hepatitis coronavirus [200]
Toxin	*Vibrio cholerae* cytolysin [122]	Ricin [201]
		Diphtheria toxin [201, 202]
Prion		Prion [190]

Autophagy and Cell Death

While autophagy is generally recognized as a protective mechanism against stress stimuli, the association of its involvement in cell death is however still controversial. Many studies described the presence of autophagosomes in dying cells [118, 155] and it is debatable whether autophagic activity causes death or simply occurs in parallel with other mechanisms of cell death. Autophagic cell death is also known as type II programmed cell death and distinct from type I programmed cell death, apoptosis [118].

Several studies that presented evidence of autophagic cell death in fact only provided correlative evidence [155]. In an in vitro study of autophagic cell death, a mutation in Atg1 gene only inhibited vacuolization but not cell death in a slime mold model [203]. Another study that detailed the observation of autophagic cell death in *Drosophila* salivery glands showed that cell demise can be prevented with mutations in non-autophagy genes, thus casting doubts as to the causative role of autophagy in cell death [204].

On the other hand, other studies demonstrated the requirement of functional autophagy for the initiation of cell death. In mouse L929 cells treated with caspase inhibitor zVAD, knockdown of 2 essential autophagy gene, Atg7 and beclin 1 gene, prevented cell death [205]. In addition, the knockdown of Atg5 and beclin 1 inhibited cell death in murine embryonic fibroblasts (MEF) treated with staurosporine or etoposide [206]. However, as these studies do not have intact apoptotic machinery, it cannot conclusively ascertain that autophagy plays a major cell death pathway in cells with functional apoptosis.

Autophagy and toxin

Several toxins from microbial and botanical origins, such as, ricin, abrin, shiga toxin [201], diphtheria [201, 202] *Vibrio cholerae* cytolysin (VCC) [122] have been reported to induce autophagy in cells. Gram positive bacteria *Corynebacterium diphtheriae* produces diphtheria toxin which inhibits protein synthesis by catalysing ADP-ribosylation elongation factor 2. It has been shown that diphtheria toxin induces cell death through autophagy and inhibition of the autophagy process protects cell against diphtheria toxicity [201, 202]. Cholerae is a disease that usually affects mainly developing countries where water treatment system and sanitation system are not widely available and is characterized by profuse watery diarrhea [207]. In addition to producing cholerae toxin [207], certain *Vibrio cholerae* strains secrete a pore-forming *Vibrio cholerae* cytolysin toxin that causes vacuolization or cell lysis and necrosis in certain cells. Autophagy was rapidly induced in VCC-treated cells [122]. In contrast to diphtheria toxin, autophagy was found to have a protective effect against *V. cholerae* cytolysin toxin [122].

Specific Aims

Although the function of LF has been extensively researched and well characterized, its ability to induce autophagy in mammalian cells has not been documented. As autophagy is commonly activated to enhance clearing of undesirable intracellular compounds including intracellular pathogens and toxins, it is speculated that cells may possibly attempt to rid itself of LF via the same mechanism. In this chapter, I present evidence that LF induces autophagy in RAW 264.7 murine macrophage cells and discuss the possible role of autophagy in LF intoxication in mammalian cells.

2.2. Materials and Methods

2.2.1 Cells and Reagents

RAW 264.7 murine macrophage cells (ATCC) were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin (Cellgro, VA). Earles Balanced Salt Solution (EBSS) was purchased from Cellgro, (Herndon, VA). HL-60 human promyelocytic leukemia cells (ATCC) were cultured in IMDM containing 20% FBS. Lethal factor (LF) and Protective antigen (PA) were obtained from Lists Biological (Campbell, CA). Rabbit polyclonal anti-LC3 was purchased from MBL (Wood Hole, MA). HRP goat anti rabbit IgG (Fc) was from Serotec (Germany). Anti-actin antibodies, E64d, Pepstatin A, 3-methyladenine *(*3-MA), and RIPA lysis buffer were from Sigma (St Louis, MO), Rapamycin was from Calbiochem (Gibbstown, NJ), NuPage 12% Bis Tris gel, MOPS running buffer, nitrocellulose membrane, G418 Geneticin and acridine orange (AO) were from Invitrogen (Carlsbad, CA). Protease inhibitor was from Roche (Switzerland).

2.2.2 Bacteria culture supernatant preparation

B. anthracis Sterne and delta Ames strain were routinely cultured on Brain Heart Infusion (BHI) agar plate at 37^{o}C. For broth culture, a single colony from agar plate was inoculated into BHI broth for 12 hours incubation at 37^{o}C. Following which, the culture media was clarified by centrifugation at 4^{o}C. The pellet was discarded and the culture supernatant was sterile filtered with 0.2µm supor low protein binding membrane. Filtered supernatant were kept on ice until ready for use.

2.2.3 PCR detection of anthrax chromosomal and pXO2 gene

B. anthracis Sterne and delta Ames strain were used in this study. The absence of pXO1 gene in delta Ames strain was verified with PCR at the beginning of the study [208, 209] . Specific primers BA 813 amplify a chromosomal target on all *B. anthracis* strains and lef encodes for lethal factor that resides on pXO1. BA 813 R1: TTA ATT CAC TTG CAA CTG ATG GG; BA 813 R2: CGA TAG CTC CTA CAT TTG GAG; Lef 3: CTT TTG CAT ATT ATA TCG AGC; Lef 4: GAA TCA CGA ATA TCA ATT TGT AGC. Amplification cycle entails an initial denaturation at 94°C for 5 mins, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s.

2.2.4 Stable Transfection of RAW 264.7 cells

EGFP-LC3 plasmid (a gift from Dr Tamotsu Yoshimori, Osaka University, Japan) was recovered from paper substrate by soaking in 50µl of TE buffer at 4^{o}C for 5 hours. 30ng of plasmid was added into 50µl of single use aliquot GC5 *E. coli* competent cells (Genechoice, Frederick, MD) and incubated on ice for 30 mins. The tube was subsequently heated to 42^{o}C for 45 seconds followed by rapid cooling in ice. 200µl of SOC medium (Sigma, MO) was added into mixture and the tube was incubated in shaking incubator at 37^{o}C for 30 mins. Single colonies were selected on LB agar containing 20µg/ml kanamycin and inoculated in LB broth with 20µg/ml kanamycin. The

plasmid was purified using Midiprep (Carlsbad, CA) according to manufacturer's instructions, aliquoted into smaller quantities and stored at -20^{o}C.

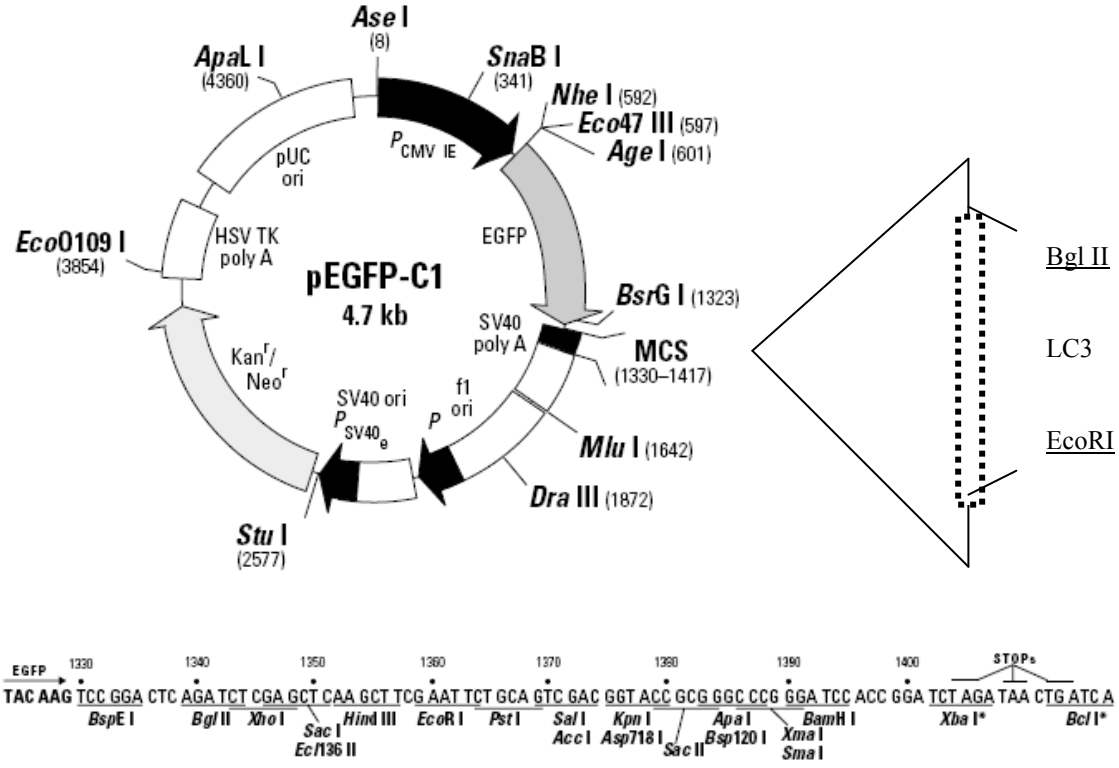


Figure 2.5: pEGFP-LC3 plasmid as provided by Dr. Tamotsu Yoshimori (Osaka University). The LC3 gene is inserted into the multiple clone site (MCS) between Bgl II and EcoRI. Cloning vector from Clontech, pEGFP-C1, GenBank Accession #: U55763

Transfection of adherent cells such as RAW 264.7 cells with lipid-based transfection agent often resulted in low transfection efficiency [210]. Furthermore, transient transfection of cells with pEGFP-LC3 exhibited autophagy-independent punctuate formation. Therefore, stable transfected cells were selected for subsequent experiments to overcome the above-mentioned limitations.

For transfection, RAW 264.7 cells were seeded in 12 well plate the day before transfection in complete medium. The following day, the medium was replaced with 2 ml of complete medium. 1.2µg of pEGFP-LC3 was diluted into 160µl of OPTI-MEM reduced serum medium followed by addition of 1.5µl of PLUS reagent. The mixture was incubated for 10 mins at room temperature. Next, 4µl of Lipofectamine LTX was added to the mixture and incubated at room temperature for 30 mins for DNA lipofectamine complex formation. After which, 160µl of the complex was added to each well and incubated at 37^{o}C. Following 2 days of incubation, the media was changed to selection medium containing 400µg/ml of Geneticin (Invitrogen). Single clones stably expressing EGFP-LC3 were selected and maintained in selection medium containing 400µg/ml of Geneticin.

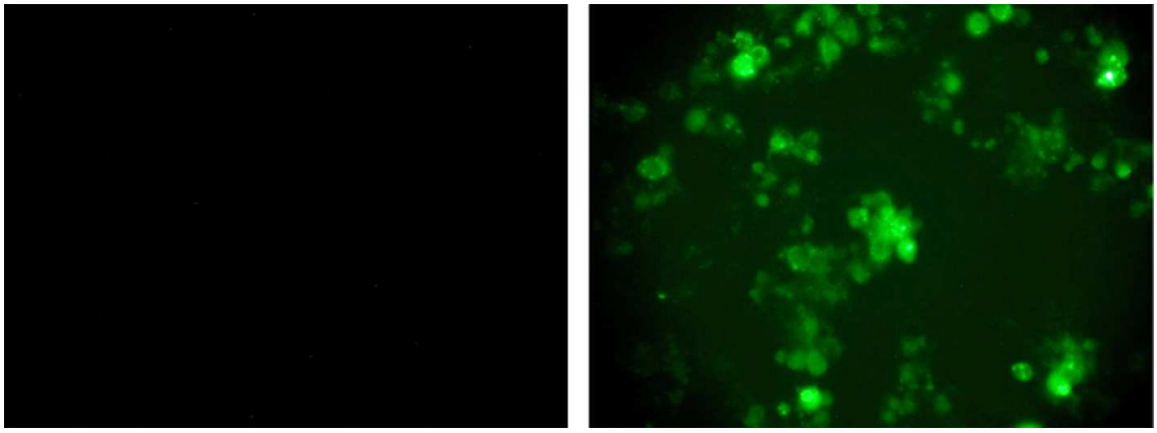


Figure 2.6: Transient transfection of RAW 264.7 cells with pEGFP-LC3. RAW 264.7 cells were transfected with pEGFP-LC3 using lipofectamine 2000 and observed under fluorescence microscope. (A) Non transfected control cells (B) cells transfected with pEGFP-LC3.

2.2.5 Immunoblotting

2 x 10^6 RAW 264.7 cells were seeded in 6 well plate in DMEM medium supplemented with 10% FBS. After overnight culture, the medium was refreshed and the cells were treated with lethal toxin (LT). The cells were washed twice with cold PBS and subsequently treated with 100µl of cold RIPA lysis buffer containing protease inhibitor in each well. Proteins were separated by using 12% NuPage Bis-Tris gel with MOPS buffer and blotted onto nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in PBS-T for 1 hour at room temperature and probed with 1:1000 dilutions of anti-LC3 and 1:5000 HRP conjugated anti-rabbit IgG Fc antibodies. Bands were visualized with HRP substrate and scanned for densitometry analysis with Image J according to instruction provided by the software developer (NIH).

2.2.6 Fluorescence microscopy and punctuate counting

Stably transfected RAW 264.7 cells expressing EGFP-LC3 were seeded on coverslips at a concentration of 1 x 10^6 cells/ml for overnight culture at 37^{o}C. Cells were treated with various combinations of protective antigen (PA) with or without lethal factor (LF). Rapamycin and EBSS were introduced into the culture for 2 hours as positive controls. The cells were washed with PBS and fluorescence was observed with Nikon Eclipse E600 microscope (Nikon, Japan) mounted with Nikon Y-FL-Epi-Fluorescence attachment and equipped with 100W mercury lamp, excitation filter 465-495nm, dichroic mirror 505nm and barrier filter 515-555nm. Images were obtained by Nikon E500

camera. The number of punctuate formed per cell was quantified under the fluorescence microscope.

2.2.7 Acidic vacuoles staining

RAW 264.7 cells were treated with various concentration of LT for 3 hours followed by removal from plate by trypsin-EDTA. Cells were stained with 1μg/ml AO in phenol red free media for 15 mins, washed and analysed by FACS (BD, San Jose, CA). Dot plots were analyzed by CellQuest and mean ratio of red:green fluorescent intensity was determined by FlowJo (Ashland, OR).

2.2.8 Cell viability

Cell viability was determined by CellTiter96 Aqueous Cell Proliferation Assay (MTS) according to manufacturer's instructions (Promega). It measures dehydrogenase enzyme activity in cells and is proportional to the number of living cells in the culture. Briefly, 4 x 10^4 cells were plated in 96 well in 100μl of complete medium for overnight culture at 37^{o}C. Cells were pre-treated with 3MA followed by introduction of PA and LF for 3 hours. 20μl of MTS solution was subsequently added to each well and incubated for another 2-3 hours at 37^{o}C. Absorbance reading at 490nm was measured with ELX808IU Biotek plate reader (Winooski, VT).

2.2.9 Statistical analysis

Statistical significance between groups was assessed by student t-test. P values < 0.05 were considered significant.

2.3 Results

2.3.1 Acridine orange staining showed increased acidic vacuoles (AVO)

Increased in AVO formation is a typical feature observed in cells undergoing autophagy [211, 212]. Hence, we examined the effect of LT on AVO formation in RAW 264.7 cells by using lysosomotropic agent acridine orange (AO). Acridine orange is an acidic probe that moves freely across membrane to stain acidic compartment red and cytoplasm green when observed under fluorescence light [213]. Therefore, the measurement of the mean ratio of red:green fluorescence intensity of AO stained cells would indicate the degree of AVO formation in cells. RAW 264.7 cells treated with LT displayed a dose-dependent increase in AVO formation as indicated by the higher mean ratio of red:green fluorescent intensity (Figure 2.7). Similarly, positive control starved cells also showed an increase in mean ratio of red:green fluorescence although the increase was less pronounced than the LT-treated cells.

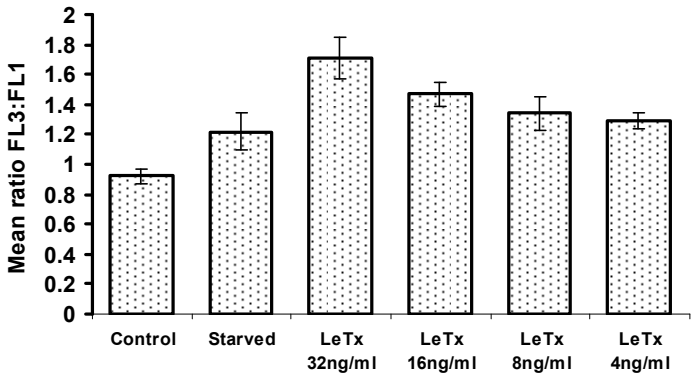


Figure 2.7: Supravital staining of acidic compartment with acridine orange. RAW 264.7 cells were treated with various concentrations of toxins for 3 hours and removed from plate by trypsin-EDTA. The cells were stained with 1µg/ml AO in phenol free medium for 15mins, washed and analysed by flow cytometer. Dot plots were analyzed by CellQuest and mean ratio of red:green fluorescent intensity was determined by FlowJo.

Correspondingly, the degree of AO staining of cells can also be visualized with fluorescence microscope [214]. Consistent with figure 2.7, treatment of RAW 264.7 cells with PA 100µg/ml and LF 30µg/ml for 4 hours displayed a more pronounced AO staining as compared to control, suggesting the increase in the acidic vacuoles of the cytoplasm (Figure 2.8).

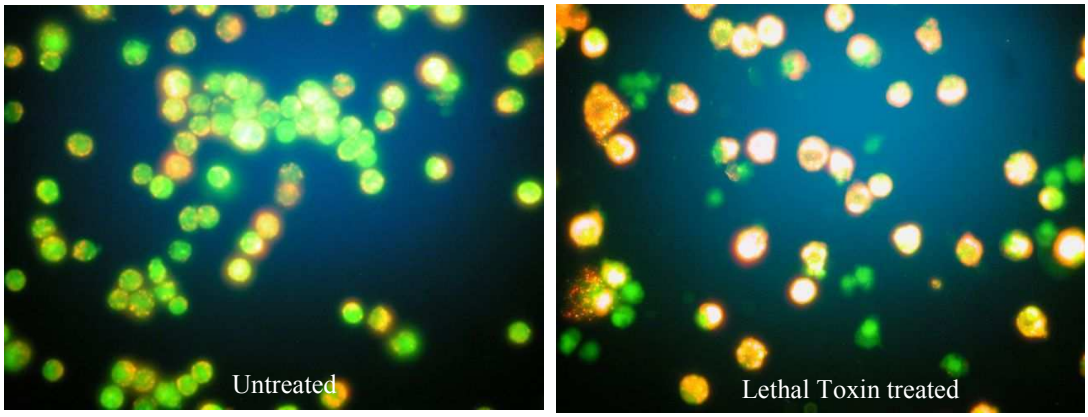


Figure 2.8: RAW 264.7 cells stained with acridine orange after LT treatment. Cells were cultured to 80% confluence and treated with PA concentration of 100 µg/ml and 30µg/ml of LF for 4 hours. Cells were stained with 1µg/ml of acridine orange for 15 mins at room temperature. Stained cells were observed with excitation 485 nm and emission 515nm long pass.

2.3.2 Increased GFP-LC3 punctuate when cells were treated with LT

Atg8 is an ubiquitin-like protein that undergoes conjugation process during autophagy and is determined to be an essential component for autophagy [215]. Atg8 related proteins exist in mammalian cells as microtubule-associated protein 1 light chain 3 (LC3), Golgi-associated ATPase enhancer of 16 kDa (GATE16), and g-aminobutyric-acid-type-A (GABAA)-receptor-associated protein (GABARAP) [142]. Among the 3 different families, LC3 is the most well studied and widely used protein marker for autophagic organelles. LC3, a 30kDa protein, is cleaved at C terminus of Gly120 to produce LC3-I immediately after synthesis [140]. During autophagy, cytosolic LC3-I is linked to phosphatidylethanolamine (PE) to form LC3-II [142] and remains tightly bound to the autophagosomal membranes [140]. This process can be indirectly monitored

through the use of reporter protein GFP conjugated to LC3 [147]. In order to determine if LT induces autophagy, we overexpressed GFP-LC3 in cells and observed for fluorescent punctuate distribution of GFP-LC3, which represents autophagosome formation.

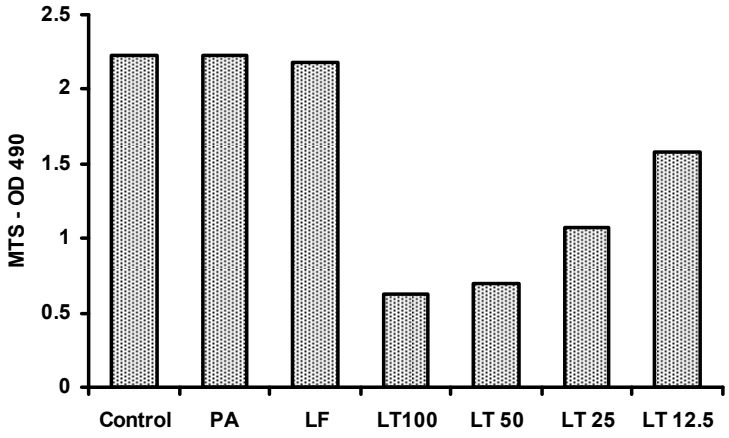


Figure 2.9: Stably transfected RAW 264.7 cells treated with LT. Cells were tested for LT susceptibility after selection with G418 in DMEM. PA was added to a final concentration of 500ng/ml in PA and all LT groups. Viability was determined with MTS as described in materials and methods.

Transfection of cells with GFP-LC3 for fluorescence microscopy analysis is commonly used to detect autophagosome. However, transient transfection may cause protein aggregates formation that is independent of autophagy and it is difficult to distinguish these protein aggregates from bona fide autophagosome under fluorescence microscope (Figure 2.10) [216]. Hence, stable cells expressing GFP-LC3 were used for all our studies.

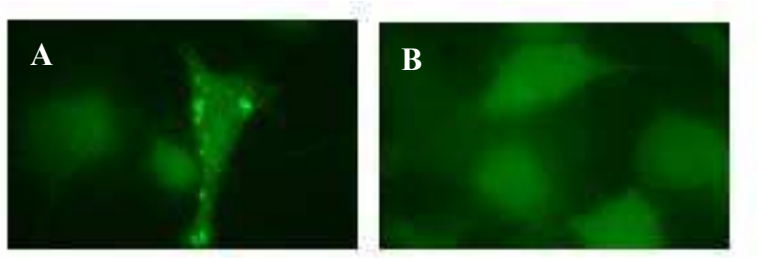


Figure 2.10: Transient vs stable transfection of RAW 264.7 cells with pEGFP-LC3. A) Transient transfection cells exhibited GFP-LC3 dot structure which may be independent of autophagy. B) Stable RAW 264.7 cells expressing GFP-LC3.

Over expression of GFP-LC3 also appears to have no effect on basal autophagy in cells [141]. Stable GFP-LC3 expressing RAW 264.7 cells were treated with anthrax LT for 2 hours and exhibited increased GFP-LC3 punctuates distribution, whereas untreated cells displayed a diffuse GFP-LC3 appearance (Figure 2.11). These punctuate fluorescent dots indicate autophagosomes formation. Most of these fluorescent dots were probably autophagosomes as autolysosomes had weaker or no fluorescence signals due to the presence of lower LC3-II proteins [140]. The reduced LC3-II level in autolysosome may possibly be due to degradation or recycling back to cytosolic LC3-I [140]. Further incubation of cells with lethal toxin for 3 hours caused massive cells lysis as observed under light microscope (data not shown), which indicates that the stably transfected RAW 264.7 cells were still susceptible to LT lysis. The punctuate distribution of GFP-LC3 in LT treated cells was similar to those treated with rapamycin positive control. Rapamycin binds to and inhibits mammalian target of rapamycin (mTOR), a negative regulator of autophagy [123]. Nutrients starvation can also trigger autophagy. Accordingly, cells incubated in nutrient-free salt solutions, EBSS, for 2 hours showed

punctuate distribution of GFP-LC3. Autophagy induced by nutrients starvation produced more pronounced fluorescence punctuates as compared to rapamycin or LT treated cells (Fig 2.11).

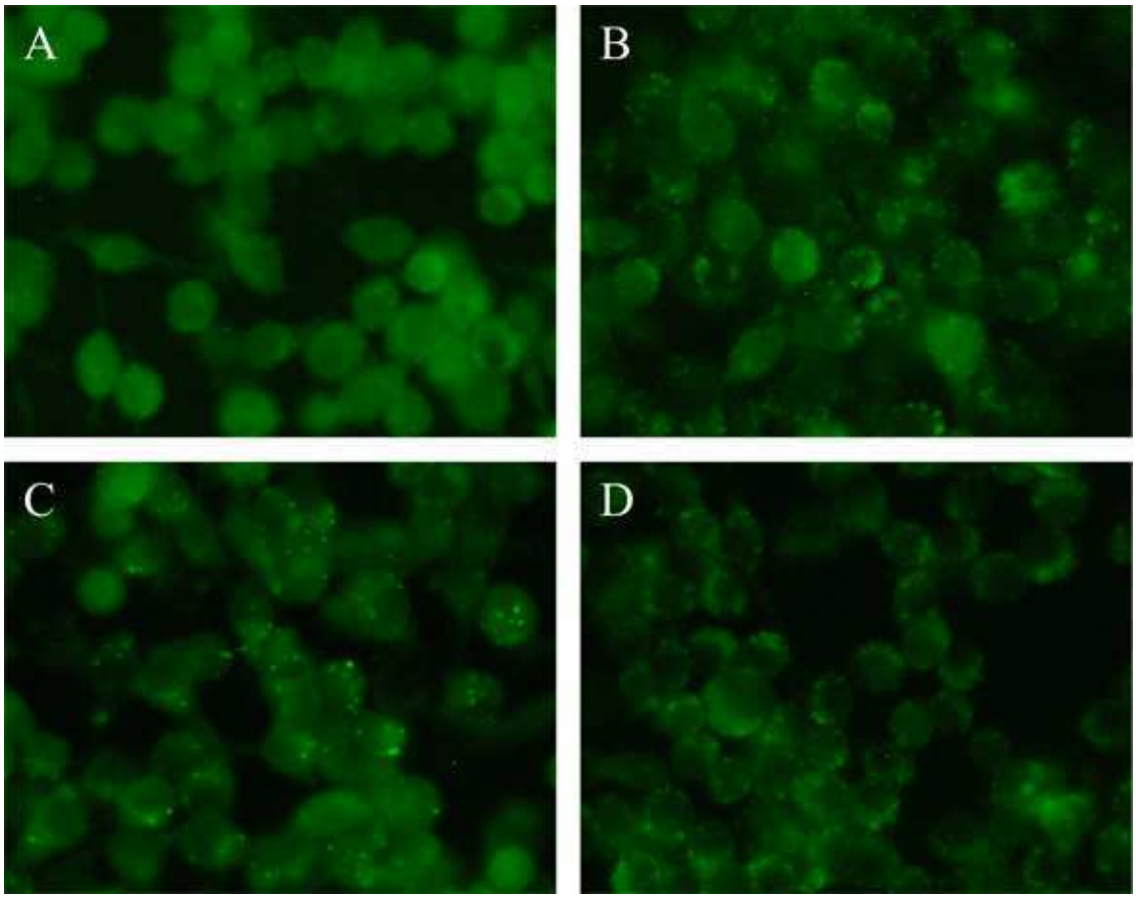


Figure 2.11: Lethal toxin induced punctuates EGFP-LC3 distribution in cells. Stably transfected RAW 264.7 cells expressing EGFP-LC3 were treated for 2 hours with (A) PBS, (B) 500ng/ml PA + 50ng/ml LF, (C) EBSS, (D) 4μM rapamycin. PBS, PA, LF and rapamycin were added directly into medium and EBSS treated cells were washed 3 times with PBS before incubation in EBSS. Images (40x) were taken from specimens under fluorescence microscope and are representative of 3 experiments.

The number of punctuates per cell was counted for quantitative assessment of autophagic activity in transfected RAW 264.7 cells. This method is reported to be more accurate than counting fraction of LC3 II positive cells [139]. Cells treated with LT showed up to 2.7 fold increase in the number of punctuates per cell versus untreated cell (Figure 2.12). Interestingly, cells treated with only PA also showed a significant increase in punctuate counts ($p < 0.05$), albeit at a level lower than the LT treated cells. Induction of autophagy by PA alone can occur because PA entry into cytosol is not dependent on LF [83]. Moderate induction of autophagy could be attributed to the rapid degradation of PA upon entry into the cytoplasm [86]. Treatment with LF alone produced similar punctuate count as control cells.

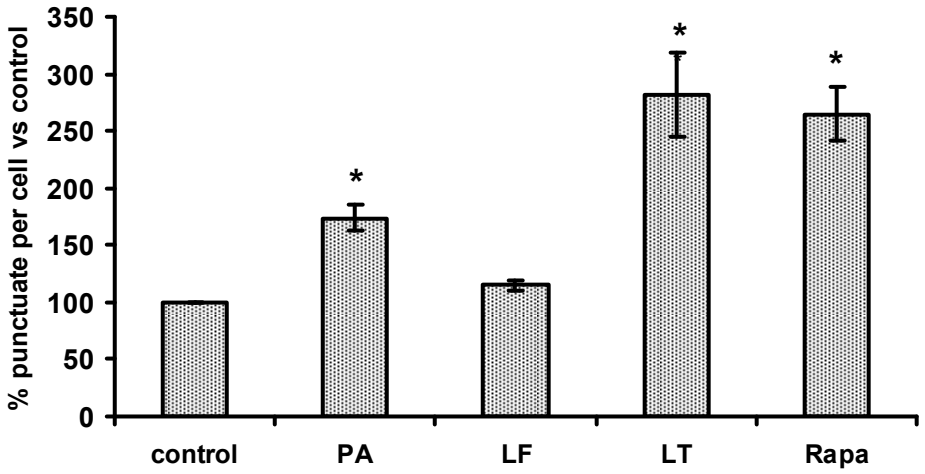


Figure 2.12: Lethal toxin and PA increased the number in punctuate EGFP-LC3 counts. RAW 264.7 cells stably expressing EGFP-LC3 were treated with 500ng/ml PA, 25ng/ml LF, 500ng/ml PA + 25ng/ml LF or 4μM rapamycin (Rapa) for 2 hours. Cells were observed immediately under fluorescence microscope as described in materials and methods. Data represent the mean of 3 independent experiments; error bars represent the standard deviation. * indicates that the values are significantly different from control with no treatment, $p< 0.05$.

2.3.3 Conversion of LC3 I to LC3 II

During autophagy, processing of cytosolic LC3-I to LC3-II permits autophagosomal membrane recruitment through an autophagic specific conjugation. As the amount of LC-II correlates with the extent of autophagosome formation [140], immunoblotting of LC3-II can be used to determine autophagy induction. To further corroborate that the GFP-LC3 punctuate observed was indeed autophagy induction by LT, we therefore examined the endogenous LC3-II levels in LT-treated cells.

The lipid conjugated LC3-II migrates faster than LC3-I and showed as a 16kDa band on western blot. In LC3 immunoblotting, antibodies often have higher affinity for LC3-II than LC3-I and therefore it is more meaningful to compare LC3-II amount with a loading control protein, such as actin, rather than LC3-I [142].

RAW 264.7 cells were pre-treated with 10μg/ml E64d and 10μg/ml pepstatin A for 1 hour to inhibit lysosomal proteases followed by incubation with LT for 1 and 3 hours. At both time points, increase of LC3-II level for LT treated cells was detected, although the ratio differs from 1 to 3 hours after incubation (Figure 2.13A). It is not unusual to observe fluctuation of LC3-II level across various time point during autophagy induction [217].

We further determined if LT components could also induce autophagy individually. Treatment of cells with PA alone showed moderate increase of LC3-II while LF alone produced similar ratio of LC3-II / actin as control cells (Figure 2.13B). RAW

264.7 cells treated with LT for 1 hour showed elevated amount of endogenous LC3 II (Figure 2.13B). These observations are generally consistent with the fluorescent punctuate count in GFP-LC3 transfected RAW 264.7 cells as indicated in figure 2.12. It may appear obvious that LF did not induce autophagy simply because it is not able to cross cell membrane in the absence of PA, although it was reported that a small fragment of LF can enter into the cell cytoplasm without the assistance from PA [218]. Apparently, this mechanism of PA-independent insertion of LF into cytosol did not have an observable effect on autophagy induction at the concentration tested.

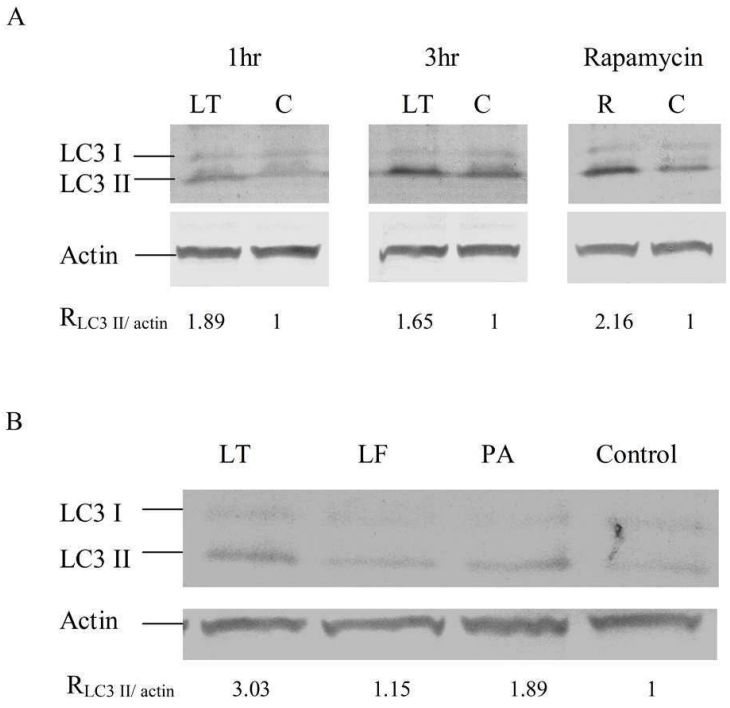


Figure 2.13: Immunoblot analysis of endogenous LC3 II conversion in RAW 264.7 cells. (A) Cells were pre-treated with 10µg/ml E64d and 10µg/ml Pepstatin A for 1 hour followed by incubation with LT (PA 500µg/ml + LF 100µg/ml) for 1 and 3 hours. Cells treated with 4µM rapamycin were used as positive control for autophagy induction. (B) RAW 264.7 cells were pre-treated with E64d 10µg/ml and Pepstatin A 10µg/ml for 1

hour followed by incubation with 500ng/ml PA, 50ng/ml LF and LT (PA 500ng/ml + LF 50μg/ml) for another 1 hour. Cells were lysed and total proteins were analysed by using anti-LC3 and anti-actin antibodies. Ratio of LC3 II/actin is shown under the blot.

2.3.4 Sterne and delta Ames strain contain other autophagy-inducing compound

In *V. cholerae*, cytolysin was determined to be the only secreted compound in culture supernatant that induces autophagy in mammalian culture [122]. Therefore, we examined further if *B. anthracis* secretes other compounds that may induce autophagy in addition to LT. *B. anthracis* delta Ames was used for this study as it lacks pXO1 which encodes for lethal factor, edema factor and protective antigen.

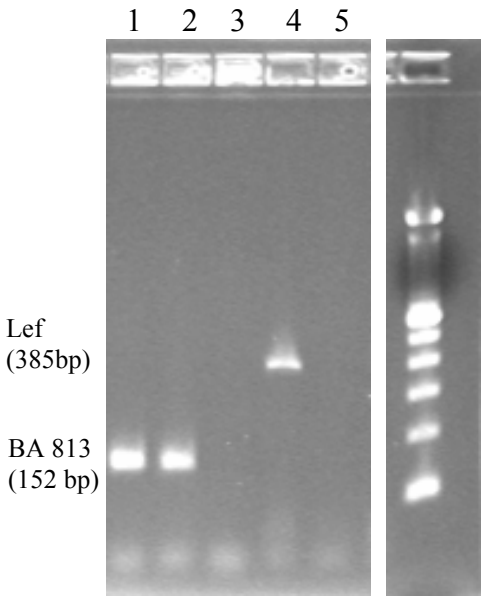
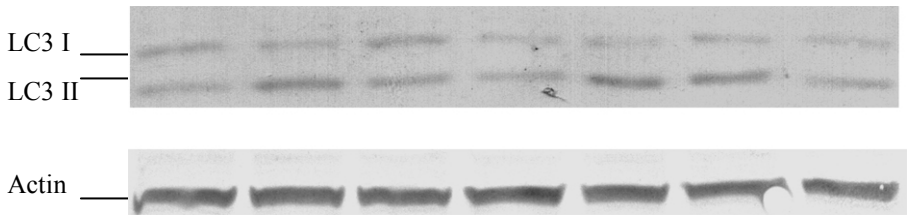


Figure 2.14: PCR detection of anthrax chromosomal and pXO1 gene in *B. anthracis* Sterne and delta Ames strain. Lane 1 – Sterne strain chromosomal gene; lane 2 – delta Ames chromosomal gene, lane 3 – blank, lane 4 – Sterne Lef, lane 5 – delta Ames Lef.

Filtered culture supernatant from both Sterne and delta Ames were shown to induce LC3 II conversion in RAW 264.7 cells after 2 and 3 hours treatment relative to control BHI broth (Figure 2.15). Supernatant from non-toxigenic delta Ames appears to provoke a higher conversion of LC3 II than toxin producing Sterne strain supernatant. This observation suggests that in addition to LT, *B. anthracis* indeed produces other autophagy-inducing compounds. However, the effect of autophagy induction by the unknown compounds in the supernatant on bacteria pathogenesis is not known.



$R_{LC3\ II/\ actin}$ (NT)	1	1.77	1.24	0.87	1.78	1.34	0.78
$R_{LC3\ II/\ actin}$ (BHI)		2.04	1.43	1	2.27	1.71	1

Figure 2.15: *B. anthracis* culture supernatant induced conversion of LC3 II. Bacteria were cultured in BHI broth for 12 hours and the culture were sterile filtered. Sterile filtered culture supernatants were diluted to a final concentration of 10% in medium and added to RAW 264.7 cells. Cells were lysed and subject to western blot as described in materials and methods. $R_{LC3\ II/\ actin}$ (NT) and $R_{LC3\ II/\ actin}$ (BHI) refer to normalization of LC3 II/actin ratio to non treated cells and control BHI broth as baseline respectively.

2.3.5 Autophagy inhibitor may increase cell death

Inhibition of autophagy process can be used to investigate the role of autophagy in cellular response to toxins, bacteria or virus. Depending on the interaction between

autophagy mechanism and stimulus, the induction of autophagy may sometimes be beneficial or detrimental to the cells. Autophagy protects cells against *Vibrio cholerae* cytolysin intoxication [122] but has opposite effect when autophagy is activated in response to diphtheria toxin treatment [201, 202]. Hence, we attempt to study the effect of autophagy on LT intoxication.

RAW 264.7 cells were treated with 10mM 3MA for 1 hour to inhibit autophagy followed by 2 and 3 hours of incubation with LT. Cells viability as determined by MTS assay showed no differences in 3MA treated and untreated cells (data not shown). This could be attributed to rapid lysis of RAW 264.7 cells when subject to LT treatment. Hence, we decided to use another cell line that is also susceptible to LT induced cell death but at a slower lysis rate than RAW 264.7. LT does not appear to cause cell death in monocytic cell line HL-60 but is cytotoxic when HL-60 cells are differentiated into macrophage-like cells with PMA [50]. Differentiated HL-60 cells were pre-treated with 3MA for 1 hour followed by LT treatment. Cells pre-treated with 3MA showed accelerated cell death compared to control cells at all the time points tested (Figure 2.16). This suggests that autophagy may function as a defense mechanism against LT intoxication. Although 3MA is often used as a specific inhibitor of autophagy [122, 219, 220], it also has effects on various aspects of metabolism that is unrelated to autophagy [221]. More studies have to be conducted to further understand the role of autophagy in LT intoxication.

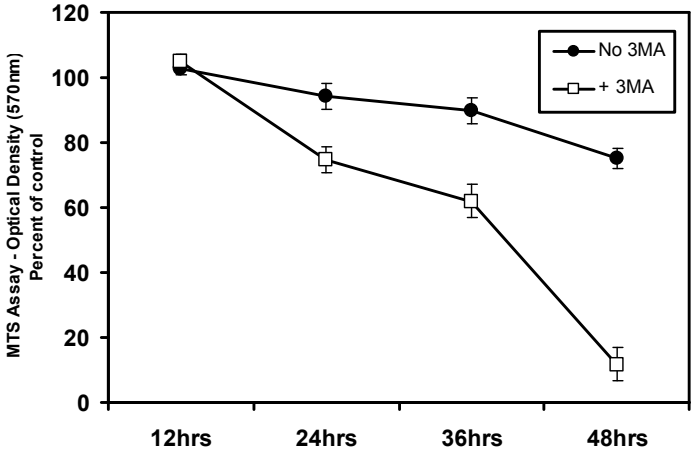


Figure 2.16: Autophagy inhibitor (3MA) accelerated cell death in LT-treated cells. HL-60 cells were differentiated by PMA in 96 well plate for 24 hours. Cells were pre-treated with 10mM 3MA for 1 hour followed by the addition of LT (500ng/ml PA + 30ng/ml LF) for the time indicated. Viability was determined by MTS according to manufacturer's instruction.

2.4 Discussion

LT is recognized as a critical virulence factor in *B. anthracis* pathogenesis. Having been extensively researched for numerous years, LT pleiotropic actions on many cellular mechanisms have been described. Autophagy is activated during periods of physiological stress such as starvation as a means to sustain cell viability in a nutrient-limiting environment [121]. In addition, autophagy is also implicated as a protective cellular response for the elimination of infectious agents [122, 222, 223]. However, certain pathogens are able to manipulate autophagy by altering certain processes for its survival and proliferation [188, 224]. Recently, autophagy became a rapidly growing biomedical marker as more studies unravel the role of autophagy in many physiological and pathological processes [135]. In this study, we demonstrate the induction of autophagy as a novel effect of LT intoxication in mammalian cells.

During autophagy, isolation membranes or phagophores elongate to sequester cytoplasmic components and become enclosed to form a double membrane autophagosome. Herein, we report LT induced autophagosome formation in cells as demonstrated by the punctuate GFP-LC3 distribution in the cytoplasm and the corresponding increase in the punctuate counts (Figure 2.11 & 2.12). Another frequently used method as an indicator of autophagy is the monitoring of LC3-II conversion. LC3-II protein associates tightly to autophagosome and was determined to be correlated with autophagosome in cells [140]. Indeed, LT-treated cells displayed enhanced LC3-II conversion, which is a typical representative of autophagosome formation. As expected, PA was determined to be a critical component for autophagy induction. By itself, PA

caused a moderate increase in LC3-II levels compared with non-treated controls (Figure 2.12B). This could be due to a self-protection response of the host cells upon PA exposure. However, cells treated with LT (PA + LF) caused a dramatic increase in LC3-II levels (Figure 2.12B). This could be mainly the result of cellular stress and its defence mechanism against the rapid toxic effect of LT. Effect of LT is believed to persist longer in the cells than PA alone, as indicated by its continuous enzymatic cleavage of substrate in the cells for 4-5 days [86, 225]. The prolonged presence of active LF in the cytoplasm may possibly play a contributing role for the dramatic increase of autophagy.

V. cholerae cytolysin induced autophagy in cells and the toxin was found to be localized in autophagic vacuoles [122]. If LT-mediated autophagy also resulted in the presence of LF in autophagosome, it may occur through two possible mechanisms. During the translocation of LT into cell cytoplasm, PA binds to cell surface receptors; ANTXR1/TEM-8 and ANTXR2/CMG-2 [80, 81] and is subsequently cleaved to release a 20kDa from the N-terminus [83, 84]. The remaining larger PA_{63} subunit then assembles itself into a heptameric configuration. Following the binding of LF or EF to form a toxin-receptor complex [85], the complex is subsequently transported into the cytoplasm via a raft-dependent and clarthrin-mediated endocytosis [86] where it is further processed in the endosomes. The first plausible mechanism of LF trafficking into autophagosome may occur from the direct binding of LF-containing endosomes with autophagosome (Figure 2.17) [135]. The alternate mechanism could be the direct sequestration of free LF in the cytoplasm by isolation membrane following its release from the endosomes. The former mechanism appears to be more physiologically favourable to cells as LF will be

eliminated before it can even be released into cytoplasm to exert its effect. Consequently, physiological intervention that can modulate the fusion of endosome that contains LT with autophagosome followed by lysosome may potentially contribute in reducing cellular exposure to LT intoxication.

Figure 2.17: Autophagy visualized by freeze-fracture electron microscopy. The fusion of an autophagosome, with its typical smooth limiting membrane that is devoid of transmembrane proteins, and an endosome with a particle-studded limiting membrane in a rat hepatocyte. The resulting structure is an amphisome [135].

Autophagy may function as a defensive mechanism against invading pathogens but at other times, it may be exploited by microbes for survival/replication or even leading to host cell death. In our preliminary experiment, autophagy was determined to be beneficial to differentiated human promyelocytic leukemia HL-60 cells exposed to LT as cells blocked from autophagy expressed accelerated cell death (Figure 2.16). Probably similar to the cellular response to *V. cholerae* cytolysin intoxication [122], autophagy was presumably activated to enhance LT clearance from cytoplasm by diverting them to

autophagosome and eventually eliminated by lysosomal degradation. As this study involved the use of cell lines, it is integral that the defensive role of autophagy be further determined on human primary macrophages or other immune cells. Other more specific autophagy gene knockdown or knockout studies can be carried out to confirm the results obtained from the commonly used autophagy inhibitor 3MA.

Meanwhile, circumstantial evidence from other non-autophagy related LT studies also suggests a possible link between lethal toxin and autophagy [226, 227]. As described earlier, autophagy proceeds from nascent vacuoles to become degradative autophagosomes by acquiring lysosomal proteins, including lysosome associated membrane protein (LAMP)-1 [228]. The maturation culminates with the subsequent fusion of the autophagosome with lysosome to form autolysosome where it then degrades and releases its contents into the cytoplasm. LAMP-1 protein is also a major component of lysosomes [229]. Kuhn et al analysed the proteomic profile of macrophages treated with LT and reported that LAMP1 protein was one of the highly upregulated protein [226], conceivably to increase lysosome capacity for fusing with autophagosomes and binding to late autophagosomes. In a separate study, several compounds were tested for its ability to modulate LT-induced cell death in macrophages [227]. Interestingly, the presence of rapamycin, an autophagy inducer, protected macrophages from LT-induced cell death. In contrast, macrophages co-treated with autophagy inhibitors, wortmannin or LY294002, exhibited accelerated cell death upon treatment with LT. Although autophagy was not part of their experimental design [227], it is worthy to note that the only compound tested in that study that protected macrophage from LT death in that

experiment is a well known autophagy inducer, rapamycin. The results from these studies are in agreement with our current findings that LT activate autophagy and it may also function as a cellular defense mechanism against LT intoxication.

Taken together, this study provides new insights into a hitherto undescribed effect of LT on cells, the induction of autophagic response in cells by PA and LT, and the plausible role of autophagy in *B. anthracis* infection. Looking beyond, modulation of autophagy may potentially counter the detrimental effects of LT exposure in cells and remains a subject for further investigation.

Chapter 3: Lethal toxin and ROS production

3.1. Introduction

Macrophages produce reactive oxygen species (ROS) as part of a coordinated effort to effectively combat against invading pathogens [230]. Reactive oxygen species are generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [231] and its production can be initiated by stimulus such as microbial products, IFN-g, IL-8 or by IgG-binding to Fc-receptor [232]. In addition to its direct antimicrobial properties, there are other evidences that suggest ROS is central to several innate and adaptive immunity functions, such as regulation of cytokine response, regulation of apoptosis and several immunological relevant signaling pathways [232]. For instance, mutation of genes that encode NADPH oxidase subunits in phagocytes is known to negate the ability of the phagocytes to produce ROS and is clinically linked to chronic granulomatous disease (CGD) [233]. CGD is characterized by phagocyte's inability to neutralize certain pathogens due to defective ROS production and may result in recurrent infections with microorganisms [231, 234]. While the production of ROS is essential for protecting cells against pathogens, its regulation must also be tightly controlled as overproduction may result in its own death. Essentially, when the highly reactive ROS accumulates beyond a certain threshold, it can inflict a wide range of molecular damage including lipids,

proteins and DNA. Subsequently, the molecular damage can lead to mitochondrial dysfunction, ion balance deregulation, loss of membrane integrity, and cell death.

Induction of cell death by excessive ROS production can occur via several mechanism including necrosis, apoptosis and autophagy [235, 236]. Progression of cell demise via necrosis is usually triggered by toxic insults and severe trauma conditions which can result in rapid cell lysis and releasing of cytoplasmic contents into extracellular spaces. Consequently, the released lysosomal enzymes may further inflict damage to the surrounding tissues and initiate adjacent cells to die.

In contrast to necrosis which elicited an inflammatory response, apoptosis describes a controlled manner of cell termination known as programmed cell death. It is characterized by cell shrinkage and blebbing, nucleus fragments, chromatin condensed, DNA degradation, exteriorization of phosphatidylserine and activation of caspases [237]. Additionally, the exteriorization of phosphatidylserine also functions as signaling mechanism to phagocytes for disposal of dying cells in an organized manner without eliciting any inflammatory response.

Lethal toxin was reported to induce the production of ROS in macrophages which ultimately resulted in cell death [238, 239]. Macrophages which were incapable of producing ROS due to mutation were not susceptible to lethal toxin-induced cell death further supported the notion that ROS is implicated either directly or indirectly in the cell demise. This chapter seeks to further investigate the effects of ROS overproduction on cell viability and apoptosis. In addition, the mitigation of harmful effects of ROS

production in both *in vitro* and *in vivo* model with a new formula of antioxidant liposome will also be discussed.

3.2 Materials and Methods

3.2.1 Spores preparation

B. anthracis Sterne strain 34F2 (pXO1+, pXO2-) was obtained from Colorado Serum (Denver, CO). Spores were inoculated in brain heart infusion (BHI) broth and incubated overnight in a shaker at 37^{o}C. Subsequently, the overnight culture was plated on BHI agar and incubated at 37^{o}C till sporulation reached to more than 99%. The extent of sporulation was confirmed by phase contrast microscopy or malachite green staining. The spores were harvested and heat shocked at 70^{o}C for 30 minutes followed by three rounds of washing. The final spore preparation was stored in distilled water at 4°C until the time of infection. The concentration of the stock was determined from the average of triplicate CFU results. Appropriate dilutions of the stock were subsequently made prior to challenge.

3.2.2 Cell culture

RAW 264.7 murine macrophage cells (ATCC) were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin (Cellgro, VA), and routinely sub-cultured with cell scrapper.

3.2.3 Blood isolation

Human blood purchased from Seracare Diagnostics was shipped and stored at 4^{o}C and blood components were isolated within 2 days after collection. Human peripheral

blood mononuclear cells (PBMCs), monocytes, and neutrophils were isolated before use in assay.

PBMCs were isolated by mixing each 10 ml of blood with 20 mls of DPBS, followed by the addition of 10 ml of Fico/Lite-LymphoH (Atlanta Biologicals, GA) to the bottom of the tube. The suspensions were centrifuged at 900 g for 30 minutes and the buffy coat containing PMBC were collected and resuspended in DMEM + 10% FBS for subsequent experiments. Monocytes were purified from PBMC using adherence method which involved incubating in serum-free media for 1 hr and two washes with DPBS to remove any unattached cells. Neutrophils were purified from the pellet obtained after ficoll density gradient separation. The pellet was resuspended with equal amount of PBS and 3% dextran, followed by 30 min incubation at room temperature. The neutrophil-rich plasma suspension was removed and centrifuged at 300g for 5 minutes at room temperature. Residual red blood cells were removed by hypotonic lysis with 0.2% cold NaCl for 30 sec followed by addition of 1.6% NaCl to resume osmotic pressure balance. Cells suspension was washed twice with DPBS and resuspend in media + 10% FBS.

3.2.4 ROS production

The amount of LT-induced reactive oxidant intermediates (ROIs) produced by human monocytes and neutrophils were measured by cell permeable DCFH-diacetate reagent. Briefly, monocytes or neutrophils were resuspended to final concentration of 10^6cells/ml in Hank's balanced salt solution (HBSS) containing 10mM HEPES. Cells were then incubated with LT (PA500ng/ml + LF 100ng/ml) for 30min, 60min, or

120min. After centrifugation at 125g for 5min, the cells were resuspended in Krebs-Hensleit buffer supplemented with 12.5 mM HEPES and 5uM DCFH-diacetate. The cells were incubated at 37^{o}C for 15 min, and then analyzed by flow cytometer.

For detection of ROS production in RAW 264.7, cells were seeded in 6 well plates at a concentration of 1 x 10^6 cells/well and incubated for 2-3 hours to allow attachment to the plate. The cells were washed twice with PBS and treated with 500 ng/ml PA + 100 ng/ml LF or 2 μM PMA as a positive control for 60, 75, 90, 105 and 120 minutes. Before harvesting, the cells were loaded with 5 μM of carboxy-DCFDA for 30 minutes. Cells were dislodged from the plate and immediately analyzed by flow cytometry. The cells were gated by forward scatter (FSC) and side scatter (SSC) and fluorescence intensity monitored by FL1 channel. A minimum of 10,000 events were collected in order to achieve statistical significance.

3.2.5 Apoptosis assay

Whole blood was collected in sodium citrate and subsequently used for the isolation of PBMCs using the Ficoll gradient method. Briefly, 10 mls of whole blood were mixed with 20 mls of DPBS and followed by addition of 10 mls of Ficoll to the bottom of the tube. The tubes were centrifuged at 2000 rpm for 30 minutes and the buffy coat interphase was transferred to new tube. The cells were further washed twice with media and seeded in 6 well plates at a concentration of 1 X 10^6 cells per well. Cells were activated with 100 U/ml of IFN γ for 24-48 hours prior to treatment. Lethal toxin was added at a final concentration of 500 μg/ml PA and 100μg/ml LF for 24 hours. After

which, cells were detached from the plate, washed and resuspended to 1 x 10^6 cells/ml. 100 µl of the suspension were transferred to a FACS tube and 5 µl of each Annexin V-FITC and 5 µl of propidium iodide (PI) were added to the tube and incubated for another 20 minutes in the dark. 400 µl of FACS binding buffer were added to the FACS tube and analysed by FACS within 30 minutes.

3.2.6 Murine anthrax model

Eight to nine weeks old female DBA/2 mice weighing 20-24 grams were purchased from Harlan (Bar Harbor, NE). The animals were quarantined for one week before commencement of experiment. The animals were challenged with 1 x 10^7 spores per mouse in PBS via intraperitoneal (I.P.) injection and treatment regime was initiated 24 hours after the challenge with 50mg/kg ciprofloxacin (cipro) alone or in combination with different dosages of N-acetyl-l-cysteine (NAC)-liposome (40mg/kg, 70mg/kg, 100mg/kg) in 200µl injection volume. Ciproflocaxin was prepared in sterile water and stored at -20^{o}C and fresh tube was thawed each time before use.

Freshly synthesized NAC-liposome were received from University of Tennessee the day before experiment begins and stored at 4^{o}C. The liposome was diluted in PBS and prepared each time before use. NAC-liposome was administered in the morning, and ciprofloxacin was administered in the evening starting from day 2 to day 10. NAC-liposome was administered simultaneously with ciprofloxacin on day 1 (24 hour post spore challenge).

All mice were monitored twice daily for 24 days and remaining surviving animals were euthanized using carbon dioxide inhalation. Sick animals that appeared moribund (exhibiting a severely reduced or absent activity or locomotion level, an unresponsiveness to external stimuli, an inability to obtain readily available food or water; along with any of the following accompanying signs: a ruffled haircoat, a hunched posture, an inability to maintain normal body temperature or signs of hypothermia, respiratory distress, or any other severely debilitating condition) were euthanized on the same day. All animal experimental procedures were reviewed and approved by Institutional Animal Care and Use Committees (IACUC).

3.2.7 Statistical analysis

Statistical analyses of survival data using Kaplan-Meier method were performed with Prism 4.0 software (GraphPad, San Diego, California)

3.3 Results

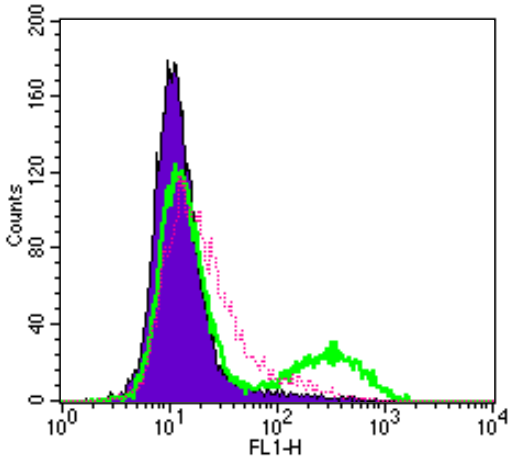
3.3.1 Induction of ROS production by lethal toxin

Dichlorofluorescein diacetate (DCFDA) is a commonly used reagent to probe for cellular ROS. Cells are usually loaded with DCFDA which diffuses into the cytoplasm and deacetylated by esterase to DCFH. The non fluorescent DCFH is converted to highly fluorescent DCH by ROS. An improved version of DCFDA, carboxy-DCFDA, has enhanced retention in cells because of additional negative charges on the compound and provided better signal when used as a probe for ROS measurement.

To examine the effect of LT on ROS production, RAW 264.7 cells were treated with LT followed by ROS measurement using fluorescent probes and FACS. Figure 3.1 A shows an increase in ROS production when RAW 264.7 cells were treated with commercial recombinant LT, as indicated by the increase in the fluorescence intensity when probed with carboxy-DCFDA. Among all the various time point tested, only cells treated with lethal toxin for 120 minutes (Figure 3.1 A) showed an increase in the FL1 intensity. This time point coincides with toxin-induced cell death as observed by microscope before cell harvesting.

In an attempt to determine if the production of ROS in cells is detrimental to its survival, RAW 264.7 cells were treated with lethal toxin in the presence and absence of various amount of antioxidant liposome. Glutathione (GSH) is an antioxidant that is commonly found in the cells and N-acetyl-l-cysteine (NAC) is a precursor amino acid for GSH production. Incubation of cells with exogenous antioxidant can help to increase the intracellular antioxidant by several folds. When cells were co-treated with either

A



B

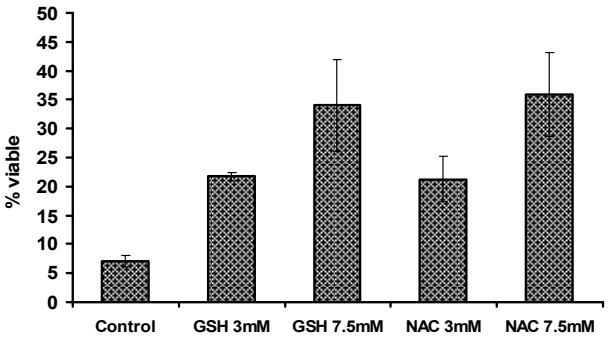


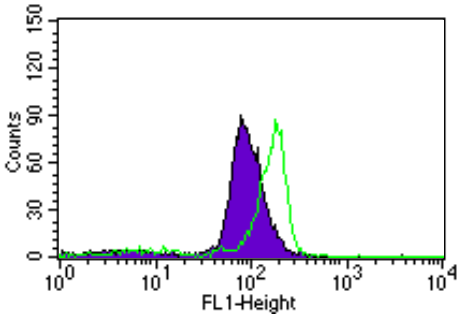
Figure 3.1: Production of ROS in RAW 264.7 cells A) Reactive oxidative species (ROS) produced by RAW264.7 cells after challenged with LT for 120 minutes (control-shaded purple; LT -green line; PMA-dotted red line). PMA treatment serves as positive control. The highly fluorescent DCH was measured by flow cytometer. B) Protection of lethal toxin-treated RAW264.7 cells with antioxidant. RAW 264.7 cells were seeded in 96 well plate and incubated for 16 hours. Lethal toxin (PA 500µg/ml + LF 100µg/ml) and various concentration of antioxidant were added simultaneously to the wells and cells viability was determined after 3 hours with MTS (Promega). Results are presented as percentage over untreated cells. Control–blank liposome, GSH–glutathione lipsome, NAC-N-acetyl-l-cysteine liposome.

exogenous GSH or NAC and lethal toxin, it provided up to 35% protection relative to lethal toxin-treated cells in a dose-dependent manner (Figure 3.1 B). This suggests that the induction of ROS may be one of the mechanisms for lethal toxin-induced cytotoxicity in macrophages cell line.

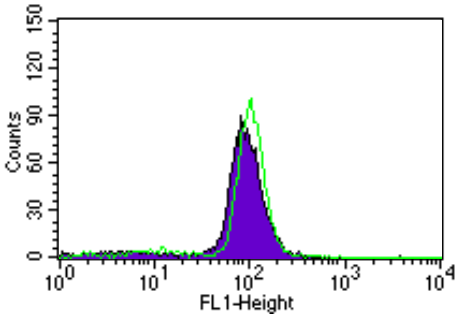
The induction of ROS by lethal toxin and the subsequent protection of antioxidant liposome were further assessed in other immune cells. In neutrophils, ROS production was up-regulated in LT-treated cells at 30 minutes post-challenged but not at 60 or 120 minutes after challenge (Figure 3.2). Treatment with NAC-liposome or GSH-liposome at 7.5 mM reduced the amount of ROS produced by neutrophils at all the time points. Oddly, blank liposome appeared to enhance the production of ROIs by neutrophils at 30 minutes post treatment. However, ROS enhancing effects were not observed in cells treated for 60 and 120 minutes. Although the optimal induction of ROS is different from RAW 264.7 and neutrophils, these results suggest that LT indeed induces ROS production in both cells lines and human neutrophils, and possibly through similar activation mechanism.

A.

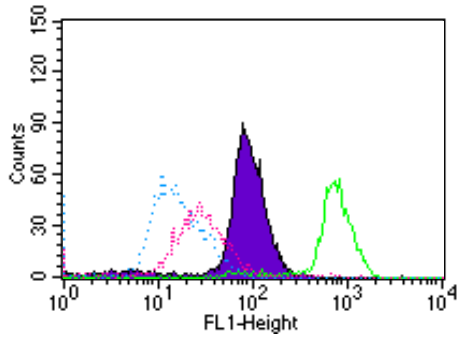
Human neutrophils treated with LeTx - 30mins



Key	Name	Parameter	Gate
	30 mins -ve control .001	FL1-H	G1
	30 mins LPS.002	FL1-H	G1



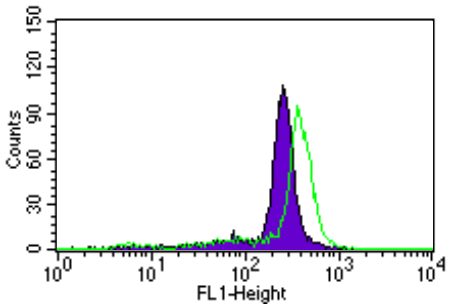
Key	Name	Parameter	Gate
	30 mins -ve control .001	FL1-H	G1
	30 mins LeTx.003	FL1-H	G1



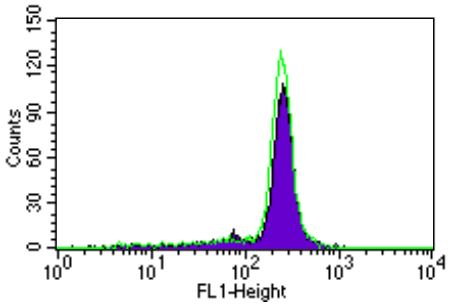
Key	Name	Parameter	Gate
	30 mins -ve control .001	FL1-H	G1
	30 mins LeTx + blank.004	FL1-H	G1
	30 mins LeTx + GSH.005	FL1-H	G1
	30 mins LeTx + NAC.006	FL1-H	G1

B.

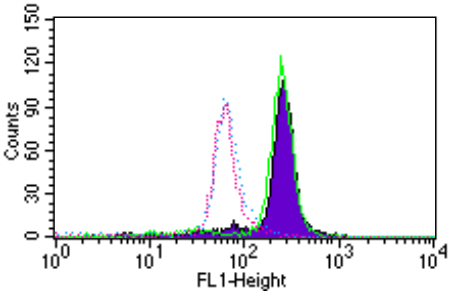
Human neutrophils treated with LeTx - 60mins



Key	Name	Parameter	Gate
	60 mins -ve control.007	FL1-H	G1
	60 mins LPS.008	FL1-H	G1



Key	Name	Parameter	Gate
	60 mins -ve control.007	FL1-H	G1
	60 mins LeTx .009	FL1-H	G1



Key	Name	Parameter	Gate
	60 mins -ve control.007	FL1-H	G1
	60 mins LeTx + Blank.010	FL1-H	G1
	60 mins LeTx + GSH.011	FL1-H	G1
	60 mins LeTx + NAC.012	FL1-H	G1

C.

Human neutrophils treated with LeTx - 120mins

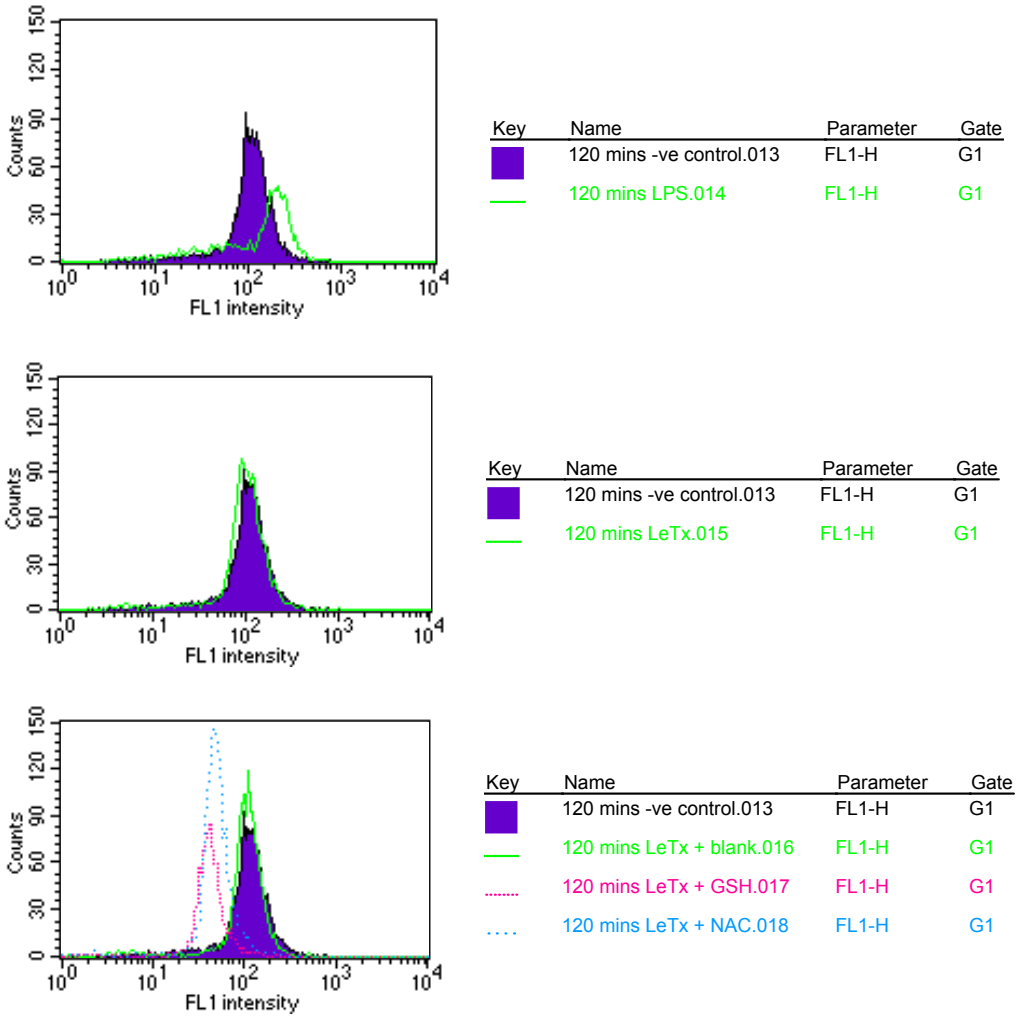


Figure 3.2: Lethal toxin induces ROS production in neutrophils. A) Lethal toxin treated human neutrophils displayed a higher amount of ROS production than untreated cells. 1 x 10^6 neutrophils were incubated in the presence and absence of lethal toxin for 2 hours. DCFDA were added 30 minutes before cell collection. Cells were washed twice with PBS and immediately analyzed with flow cytometry. Control cells – shaded blue, Lethal toxin treated cells – green line. Reactive oxidant intermediates (ROIS) produced by neutrophils after challenged with LT and treated with antioxidant liposomes (7.5mM NAC-liposome or 7.5mM GSH-liposome). LPS treatment serves as positive control. The highly fluorescent 2',7'-dichlorofluorescein was measured post challenge at 30 min (A), 60 min (B), and 120 min (C) by flow cytometer.

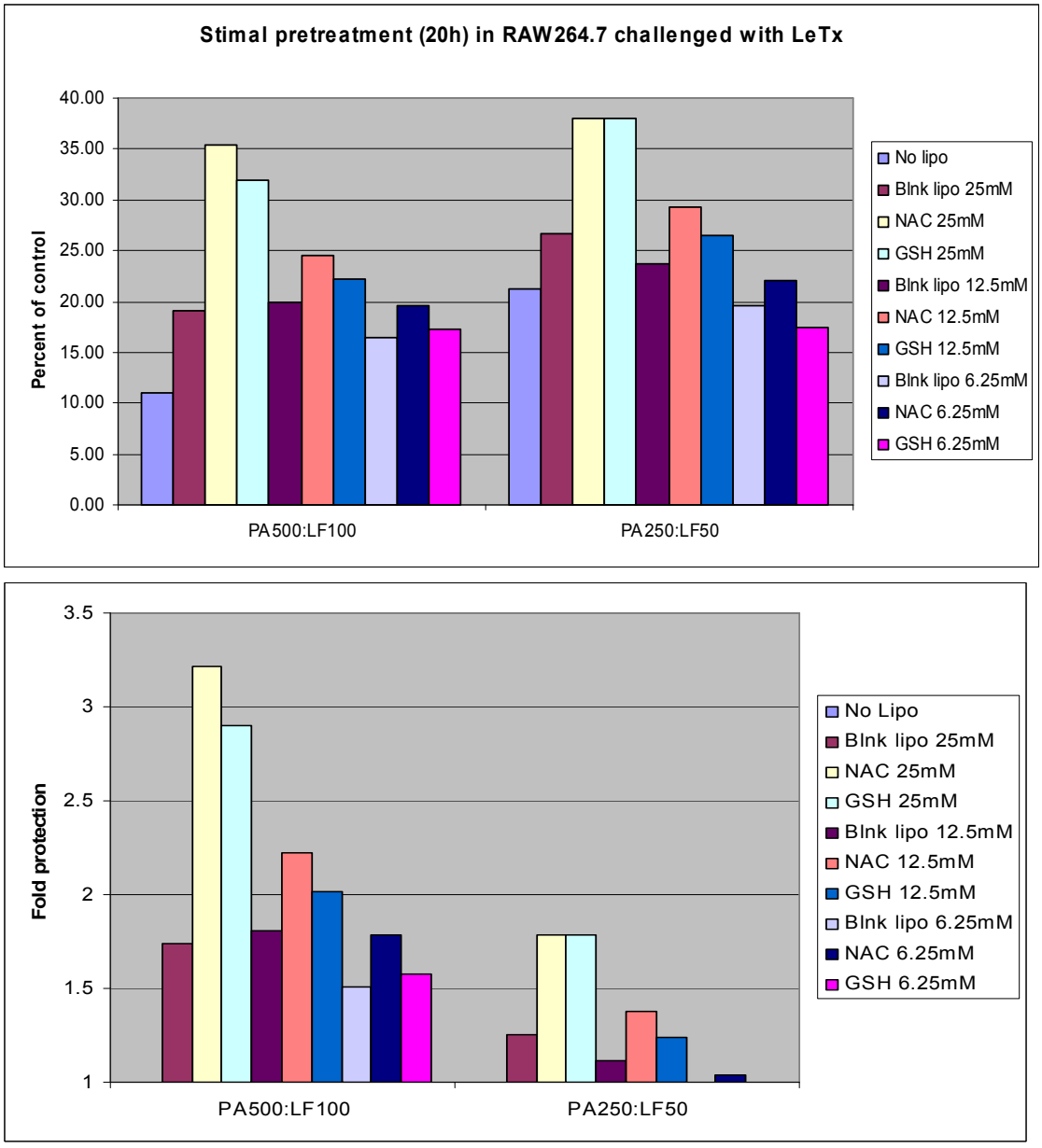


Figure 3.3: STIMAL protects RAW 264.7 from cytolysis after treatment with LT. Antioxidant liposomes at 25 mM, 12.5 mM, or 6.25mM were preincubated with cells for 20 hours before LT challenge. Before LT treatment, antioxidant liposomes were removed and washed to remove any residual liposomes. LT at PA 500 ng/ml : LF 100 ng/ml or PA 250 ng/ml : LF 50 ng/ml were then added to the well. After three hours, cell cytotoxicity was determined by CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega).

3.3.2 STIMAL protection of PBMCs against Apoptosis

To investigate the effect of Signal Transduction Methodology Antioxidant *Liposomes* (STIMAL) treatment on lethal toxin-induced apoptosis, an apoptosis detection kit containing Annexin V-FITC was employed. Annexin V-FITC assay is based on the specific affinity of Annexin V to the exposed phosphatidylserine that is externalized to the outside of the cell surface when apoptosis is activated [240]. PBMC that were treated with lethal toxin showed an 8-10% increase of apoptotic cells after 24 hours as observed by the higher number of cells with Annexin V +, PI – (Figure 3.4). This effect could be attributed to the mixed population of PBMC and the susceptibility of the various cell types to LT.

Pre-treatment of PBMC with STIMAL for 24 hours followed by LT treatment were found to reduce the ratio of apoptotic cells by up to 13% as compared to just LT (Figure 3.5). NAC liposome appears to provide a slightly better protection against apoptosis as compared to GSH liposome at 12.5mM. Both GSH and NAC liposomes confer protection in a dose-dependent fashion. However, blank liposome controls containing the same concentration of liposome was found to be highly toxic to the cells with more than 90% death (Figure 3.6). This observation is in contrast with those obtained with RAW 264.7 cells as no toxicity was observed for cells treated with blank liposome for 24 hours. This toxic effect of the blank liposome may also be relevant in interpreting the subsequent *in vivo* experiments.

(A) (B)

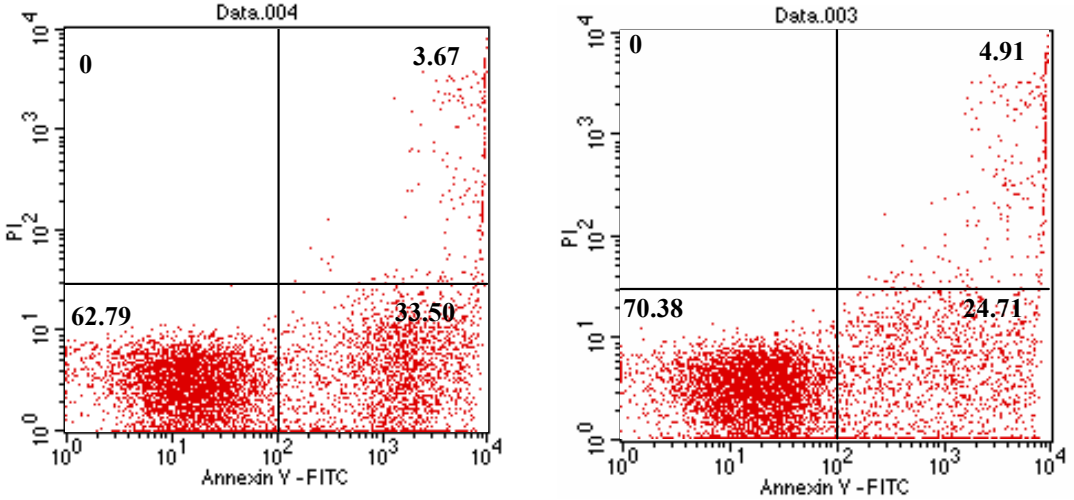


Figure 3.4: Scatter plot of PBMCs treated with (A) lethal toxin - 500µg/ml PA + 100µg/ml LF for 24hours and (B) PBS control. Live cells (PI -, Annexin -), apoptotic cells (PI -, Annexin +), dead cells (PI +, Annexin +)

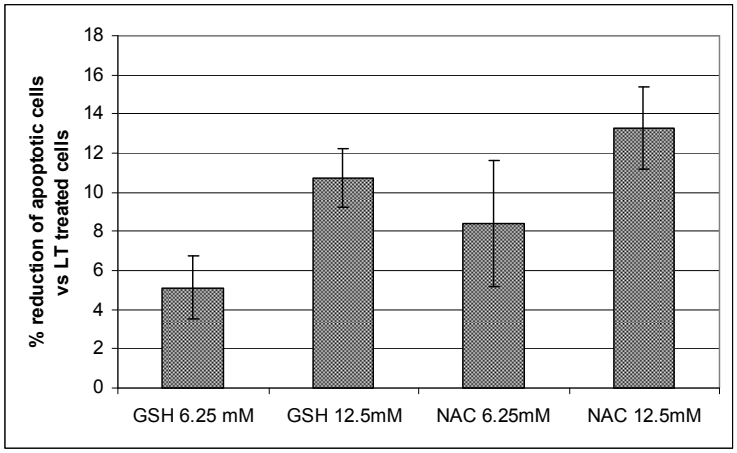


Figure 3.5: Protective effect of STIMAL against LT-induced apoptosis in PBMC. Error bar represent standard error.

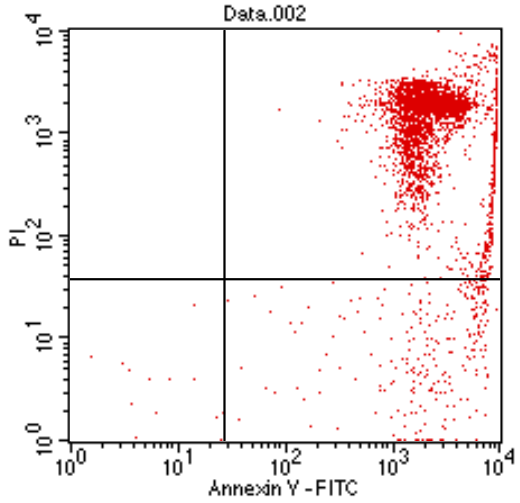


Figure 3.6: Toxic effects of blank liposome on PBMC. PBMC were treated with 12.5mM and 6.25mM blank liposome for 24 hours and subject to Annexin V staining assay. Graph shown is representative of 12.5mM. PBMC treated with 6.25mM showed similar profiles (data not shown). Live cells (PI -, Annexin -), apoptotic cells (PI -, Annexin +), dead cells (PI +, Annexin +)

3.3.3 Murine anthrax model

Titration study was initially conducted to determine the LD_{50} of a new batch of *B. anthracis* Sterne strain in murine anthrax model. Death curve were generated and found to be comparable to previous studies (Table 3.1). Subsequently, ciprofloxacin titration study was performed and determined $1x10^7$ cfu/mouse as the appropriate challenge dose for the animal model.

The protective effect of NAC liposome in combination with ciprofloxacin was assessed. Following challenge with *B. anthracis* 34F2 spores per mouse by intraperitoneal injection, the mice were treated after 24 hours with 50 mg/kg ciprofloxacin alone or in combination with different dosages of NAC-liposome (40, 70 and 100 mg/kg) through intraperitoneal injection.

All mice including NAC-liposome treated started to show signs of hemorrhages and accumulation of fluid within the peritoneal cavity (ascites) on day 2 after challenge. All animals had ruffled fur by day 3. Mice survival count was recorded (Table 3.2) and survival curve was graphed (Figure 3.7). We observed a delayed death in NAC-liposome treated group especially in 100mg/kg group (Figure 3.7). Hazard ratio, which is the slope of the survival curve or a measure of how rapidly subjects are dying, was calculated. The hazard ratio was 2.5, which means that the ciprofloxacin only group dies 2.5x faster than 100mg/kg NAC-liposome group. There were 60% survivors in 100mg/kg NAC-liposome treated group as compared to 30% survivors in ciprofloxacin only group. All mice in the untreated group expired by day 7.

Similar to previous results pertaining to the effect of blank liposome-induced toxicity on neutrophils (Figure 3.6), the animal model also showed a lower survival count for animals treated with blank liposome than NAC liposome. Both concentration of 40mg/ml and 100mg/ml of blank liposome only provided 30% survival as compared to 60% survival for 100mg/ml NAC liposome. Difference in genetic makeup may account for the disparity in the apparent toxic effect of blank liposome between human immune cells and RAW 264.7

However, based on analysis using GraphPad Prism 4, p value was 0.1202 (>0.05) and the survival curves between ciprofloxacin alone and NAC-liposome 100 mg/kg + ciprofloxacin were not significantly different. This could be attributed to the small number of sample size in each group. On the other hand, if the animal number had been increased to 20 mice per group, and assuming similar death curve, p value will improve to 0.02.

Table 3.1: *Bacillus anthracis* sterne spores (34F2) were administered to DBA2 mice intraperitoneally. The animals were monitored every day for 10 days for mortality. Ten mice were used in each dose group

	Mice Surviving (n=10)									
Group (cfu/mouse)	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**
4.0x10^7	10	10	7	3	0	0	0	0	0	0
2.0x10^7	10	10	8	5	0	0	0	0	0	0
1.0x10^7	10	10	8	7	2	0	0	0	0	0
5.0x10^6	10	10	9	8	5	3	1	1	0	0
2.5x10^6	10	10	10	10	5	2	1	1	1	1
1.25x10^6	10	10	10	10	9	8	8	8	6	5
6.25x10^5	10	10	10	10	10	7	4	1	1	1
3.13x10^5	10	10	10	10	10	7	6	6	6	6
1.56x10^5	10	10	10	10	10	10	8	8	8	7

Table 3.2: Protective effect of NAC liposome in combination with ciprofloxacin in DBA2 mice infected with *B. anthracis* sterne spores. The treatments were administered for 10 days and observed for mortality up to 24 days. NAC-liposome was administered in the morning, and ciprofloxacin was administered in the evening starting from day 2 to day 10. NAC-liposome was administered simulatenously with ciprofloxacin on day 1 (24 hour post spore challenge).

	Mice surviving (n=10)										
Group*	Day 0	Day 1	Day 2**	Day 3***	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Control-Non treated	10	10	7	2	1	1	1	0	0	0	0
Cipro alone	10	10	10	10	9	8	7	6	6	4	4
Blank liposome 40mg/kg + cipro	10	9	9	9	9	7	5	4	4	3	3
Blank liposome 100mg/kg + cipro	10	10	10	10	10	10	9	7	6	5	4
NAC-liposome 40mg/kg + cipro	10	10	10	10	10	9	8	7	7	7	7
NAC-liposome 70mg/kg + cipro	10	10	10	10	10	8	6	5	5	5	5
NAC-liposome 100mg/kg + cipro	10	10	10	10	10	10	10	10	9	7	6

	Mice surviving (n=10)									
Group*	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 18	Day 20	Day 22	Day 24
Control-Non treated	0	0	0	0	0	0	0	0	0	0
Cipro alone	4	4	4	4	3	3	3	3	3	3
Blank liposome 40mg/kg + cipro	3	3	3	3	3	3	3	3	3	3
Blank liposome 100mg/kg + cipro	4	4	4	4	3	3	3	3	3	3
NAC-liposome 40mg/kg + cipro	7	7	7	7	6	6	6	6	6	6
NAC-liposome 70mg/kg + cipro	5	5	5	5	5	5	5	5	5	5
NAC-liposome 100mg/kg + cipro	6	6	6	6	6	6	6	6	6	6

*All mice were challenged with 1x10^7 cfu/mouse *B. anthracis* 34F2 spores
** Signs of hemorrhages and ascites in almost all animals
***Ruffled fur

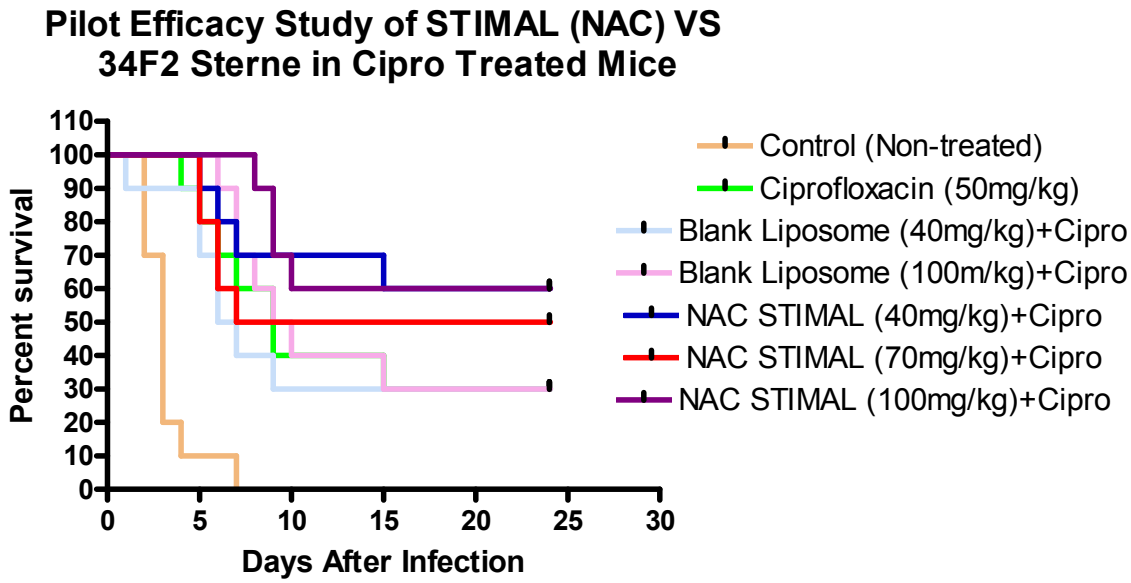


Figure 3.7: Survival curve for DBA2 mice infected with *B. anthracis* sterne spores and treated with ciprofloxacin alone or in combination with various dosages of NAC-liposome or blank liposome.

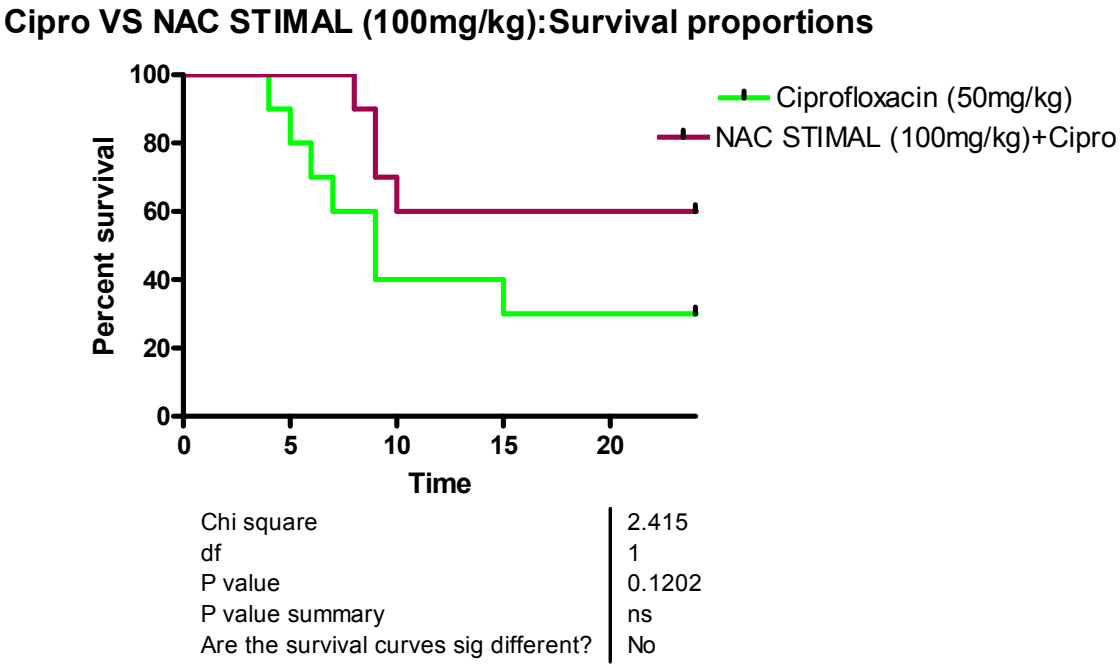


Figure 3.8: Survival curve for DBA2 mice infected with *B. anthracis* sterne spores and treated with ciprofloxacin alone or in combination with 100mg/kg NAC-liposome

3.4 Discussion

ROS is implicated in many cellular functions and play important roles in signal transduction. Although moderate amount of ROS is key to maintaining homeostasis, excessive amount can also cause deleterious effects to cells including programmed cell death [241-243]. In innate and adaptive immunity, ROS contributes to the control of pathogen by mediating several anti-microbial pathways [244]. For instance, ROS confers cytoprotection to cells by elimination of intracellular pathogens or acts as signal transducers that are essential for immune functions [232]. Depending on the various types and intensity of stimilus, ROS can be produced through several sources in the cells. Endogenous ROS are derived in moderate amount from membrane localized NADPH oxidase (Nox) enzymes [245], peroxisomes [246], cytochrome p450 system [247] while the major source is usually produced via mitochondrial electron transport chain (ETC) [248].

In this study, it was demonstrated that ROS production was upregulated in RAW 264.7 cells and human neutrophils upon exposure to LT (Figure 3.1 & 3.2). Apparently, LT-induced ROS production was shown to have harmful effects on cells as antioxidant liposome-treated cells resulted in diminished cell death. This observation is consistent with earlier study on LT effects on ROS production in several cell types [238]. Since LT has been shown to induce apoptosis in cells [59], the protective effects of antioxidant liposome on PBMC were also investigated. In agreement with previous studies [249], PBMC treated with LT displayed characteristic of apoptosis [237] as indicated by the binding of annexin V to external cell surface phosphatidylserine (Figure 3.4 and 3.5). The

induction of apoptosis by LT was partially abolished when cells were treated with NAC and GSH antioxidant liposome, suggesting that ROS may be responsible for triggering apoptosis in LT treated cells. Indeed, high level of ROS can mediate the induction of apoptosis by initiating the opening of the mitochondrial permeability transition (PT) pore [236], a pre-requisite process for apoptosis progression via the intrinsic pathway. Consequently, it results in the rupture of mitochondrial membrane and the release of apoptotic proteins [237]. The targeting of immune cells for elimination prevents the secretion of chemokines and cytokines that can attract other immune cells to the presence of pathogen in the body system. This represents an effective strategy undertaken by the bacteria to circumvent the immune system and evidently plays a major role in pathogenicity.

It has been previously reported that administration of antioxidant increased the resistance of mice to anthrax LT intoxication [238]. Notably, our *in vivo* murine model also demonstrated similar protective roles of antioxidant liposome in mice that were challenged with anthrax spores (Figure 3.7). The protective mode of action of liposome is presumably brought about by scavenging the excessive RO before it can accumulate to inflict damage on various molecular components. These findings are in agreement with results obtained from *in vitro* experiment with cell lines (Figure 3.3) and isolated immune cells (Figure 3.5). This suggests that LT-induced ROS production, conceivably in cytotoxic concentration, represent an effective method employed by the bacteria to evade the immune system.

Several other studies also provided compelling evidence suggesting the induction of ROS as a cellular response to LT intoxication. Proteomics study of RAW 264.7 and J774.1A cells treated with cytotoxic concentration of LT revealed considerable upregulation of ROS related proteins [250]. As upregulation of these proteins were more profound in J774.1A than RAW 264.7, it may partially explain the higher susceptibility of J774.1A to LT as compared with RAW 264.7 cells. In addition, RAW 264.7 cells also upregulated the production of thioredoxin, a protein which can function as antioxidant. This results in RAW 264.7 cells having a higher degree of protection from harmful effects of ROS as compared to J774.1A. Another proteomic study also observed the downregulation of a mitochondrial superoxide dismutase, a key antioxidant in protecting mitochondria. This reduces cellular ability to neutralize the onslaught of LT-mediated ROS production [226] which may lead to mitochondria dysfunction and subsequently apoptosis induction. From these observations, it is reasonable to speculate that ROS upregulation mediates mitochondria-dependent apoptosis [236].

In addition to apoptosis, ROS induced by LT may be associated with autophagy induction. As described in Chapter 2, autophagy in RAW 264.7 was upregulated as a response to LT intoxication. While the autophagy activation mechanism by LT is currently not yet identified, ROS produced from LT intoxication could possibly play a contributing role in autophagy activation through a pathway that is similar to starvation-induced cells [251]. During starvation, mitochondria upregulates ROS production which in turn inhibits cysteine protease Atg4 catalytic function. Atg4 has dual function of conjugating and deconjugating Atg8, another essential protein for autophagosome

formation. Almost immediately after synthesis, Atg8 is cleaved by Atg4 into a form permitting the conjugation with phosphatidylethanolamine (PE) [140]. Subsequent processes necessitate the inactivation of Atg4 to allow the binding of Atg8-PE onto autophagosomal membrane. It was postulated that it is at this stage which ROS regulate autophagy by targeting a conserved cysteine residue near the Atg4 active site for oxidation. The resultant inactivation of Atg4 thereby leading to enhancement of Atg8-PE binding and autophagosome development. However, as autophagy progresses with the fusion of autophagosome with lysosome, catalytic activity of Atg4 is subsequently required to delipidate and recycle Atg8. Given the existent of the various autophagy activation pathways, the possibility of ROS involvement in mediating autophagy through Atg4 redox cannot be ruled out at this point.

In addition to the work done by Scherz-Shouval *et al* [251], several other studies also point to ROS as possible signaling molecules to autophagy induction [252]. For instance, it was demonstrated that mitochondrial oxidation which involves ROS production is critical to the induction of autophagy [253]. In another study, ROS-dependent autophagy was showed to be essential in inducing caspase-independent cell death [211]. Separately, superoxides were reported to mediate autophagic cell death in selenite-treated glioma cells [254]. Taken together, these studies suggest that ROS may indeed play a contributing role in autophagy induction.

Although excess ROS may be harmful to cells, complete abrogation of cellular ROS is physiologically not desirable as it suppresses the proper functioning of immune cells, whose presence is essential to combat infections. Thus, it is crucial that proper

balancing of ROS concentration in cells must be taken into consideration if antioxidant administration is subsequently deemed to be a suitable method of treatment without suppressing other ROS-dependent protective functions against pathogens. Given the pleotropic effects of LT, ROS production is most likely a part of the coordinated effort of anthrax pathogencity in thwarting the immune system.

Chapter 4: Neutrophils chemotaxis

4.1 Introduction

Neutrophil is an important subset of innate immunity and is involved in phagocytosis and other modes of bacterial killing. During infection, neutrophils are mobilized from the bone marrow, where they are produced, to the blood stream from where they can promptly migrate to the infection sites [255, 256]. In the case of cutaneous anthrax infection, neutrophils appear to play an essential role in combating the bacterial spreading. Accordingly, neutrophils were shown to engulf anthrax spores and efficiently kill the vegetative cells through a ROS-independent mechanism following germination [257]. In addition, dermal neutrophils were found in large numbers at the infection site in resistant mice that were epicutaneously inoculated with anthrax spores. In contrast, mice that were susceptible to cutaneous anthrax infection did not exhibit any accumulation of neutrophil around infection site [258].

It is widely recognized that LT has a deleterious effect on many immune cells by suppressing essential functions or by inducing cell death [259]. Several studies suggest that the certain aspects of neutrophils immune functions were impaired during anthrax infection. For instance, pathological studies indicated a lack of neutrophils infiltration in the infected tissues harvested from anthrax victims [257]. In addition, the 10 inhalational anthrax patients who were admitted to hospital during the anthrax attack in 2001

displayed normal or only slightly elevated polymorphonuclear neutrophil (PMN) count [25, 260].

Chemotaxis describes the mobilization of cells to the infection site and is a key element for neutrophils to effectively mount an immune response during an infection. It is launched by several mechanisms such as the assembly of actin filaments for cell motility [261] and the production of chemotactic stimulus [262]. Neutrophil movement can either be positive or negative, which means that cells may move towards an increasing concentration gradient of chemotactic factors or in the opposite direction. Chemotactic factors can be categorized into exogenous and endogenous factors. Exogenous chemotaxins include bacterial oligopeptides of the formy-Methionyl-Leucyl-phenylalanine (FMLP) type, lectins, denutured proteins, certain lipids and lipopolysaccharides whereas endogenous chemotaxins are produced by the host organism and can be further subdivided into humoral or cellular type.

Neutrophil migration is powered by actin cytoskeleton reorganization. LT directly paralyzes neutrophils chemotaxis by impairing actin assembly in the cells [52]. However, the effect of LT on the production of chemokines has not been investigated. Due to the absence of neutrophils at the site of infection, we postulate that anthrax LT might inhibit the production of endogenous chemotactic compounds such as chemokines and complement factors. This could result in the absence of a gradient elevation of chemotactic factors that would prevent neutrophil infiltration to the sites of injury and therefore enabling the bacteria to multiply rapidly.

The objective of this work is to study the effects of LT and its components on the production of chemokines by human peripheral blood cells (PBMCs) and to compare their effects with cell wall (CW) components of *B. anthracis*. Here, we show that protective antigen (PA) and bacterial CW components induce chemokine production and this effect is negated when lethal factor (LF) was introduced. The chemotactic function of neutrophils was severely damaged when LT is formed.

4.2 Materials and methods

4.2.1 Human cells preparation and activation

PBMCs were isolated by mixing each 10 ml of blood with 20 ml of PBS, followed by addition of 10 ml of Fico/Lite-LymphoH (Atlanta Biologicals, GA) to the bottom of the tube. The suspensions were centrifuged at 900 g for 30 minutes and the buffy coat containing PMBC were collected and resuspended in DMEM + 10% FBS for subsequent experiments. Cells were treated with 500ng/ml PA, 100ng/ml LF and 1μg/ml CW for 24 hours at 37^{o}C. Supernatants were harvested for determination of chemokines.

4.2.2 RNA isolation

PBMC were treated with different stimuli for 2, 4 and 24 hours. Total RNA was isolated with Trizol (Invitrogen, CA). Briefly, PBMC cells were pelleted by centrifugation and lysed in Trizol reagent by repetitive pipetting. The homogenized suspension was incubated for 5 mins at room temperature followed by addition of 0.2 ml of chloroform per 1 ml of Trizol reagent. The tubes were shaken vigorously for 15

seconds by hand and incubate for 2-3 min at room temperature and centrifuged at 12,000 x g for 15 min at 4^{o}C. The upper aqueous phase was transferred to a fresh tube and 0.5ml of isopropyl alcohol per 1 ml of Trizol reagent. Sample was vortexed and incubated at room temperature for 10 min. Following centrifuging at 12,000 x g for 10 min at 4^{o}C, the supernatant was removed and the RNA pellet was washed with 75% ethanol, briefly air-dried and redissolved in RNase-free water.

4.2.2 RNAse protection assay

For RNase protection assay, 2 µg of isolated RNA was solubilized with 8 µl of hybridization buffer by vortexing for 3-4 minutes. The probe was diluted with hybrization buffer to appropriate concentration according to the technical data sheet and 2 µl of diluted probe was added to the RNA sample. After adding a drop of mineral oil to each tube, the samples were placed in a heat block pre-warmed to 90^{o}C and allowed the temperature to ramp down slowly to 56^{o}C. Following a 12–16 hour incubation, the samples were removed from the heat block and placed at room temperature for 15 min. 100µl of RNase cocktail was pipetted underneath the oil into the aqueous layer and the tubes were centrifuged for 10 sec followed by 45 min incubation at 30^{o}C. The RNase digests were transferred to eppendorf tubes containing 18 µl of Proteinase K cocktail and incubated for 15 min at 37^{o}C. Subsequently, 65µl Tris-saturated phenol and 65µl of chloroform:isoamyl alcohol (50:1) were added to the tube, vortex and centrifuged for 5 min at room temperature. The aqueous phase was transferred to a new tube and 120 µl of 4M ammonium acetate and 650µl of ice-cold 100% ethanol were added to the tube. The

solution was mixed and incubated for 30 min at -70 ^{o}C and centrifuged for 20 minutes at 4^{o}C. The supernatant was discarded and 100µl of ice-cold 90% ethanol was added and centrifuged for 10 min at 4^{o}C. The supernatant was removed, air dried, and resuspended in 1X loading buffer. Protected probes were separated in polyacrylamide gel and the bands were captured and quantified.

4.2.3 Measurement of chemokine secretion

The BD PharMingen cytometric bead array (CBA) was used to simultaneously assay for the chemokines IP-10, MCP-1, MIG, Ranter and IL-8. This flow cytometry-based measurement quantify soluble analytes in a particle-based immunoassay and was carried out according to the manufacturer's instruction. The lowest limit of detection for all cytokines in this assay was 20pg/ml.

4.2.4 Neutrophil chemotactic assay

Neutrophil migration was determined using Transwell chamber. Neutrophils were resuspended at 1 x 10^{7} cells / ml and 100 µl of the suspension were added to the transwell filter insert with the bottom well containing chemoattractant dilutions. After incubation, the filter inserts was removed and tapped against the edge of the well to remove any additional buffer. Following the pipetting of 10,000 Dynospheres bead (Bangs Laboratories) into each, the mixtures were mixed thoroughly and the number of cells migrated were counted using the FACS.

Results

4.3.1 Inhibition of LPS and CW-induced chemokine production by LT

Chemokines production is typically upregulated during infection to recruit other immune cells to specific sites. To determine if LT can influence chemokines production, cells were treated with LT and strong chemokine inducer such as LPS and *B. anthracis* cell wall (CW) component and supernatant were analyzed by CBA. Figure 4.1 shows both LPS and CW are strong producers of all chemokines tested when incubated with PMBC for 2 hrs. Among the various chemokines, MCP-1 represents the highest stimulation of up to 50 and 68 fold increments for CW and LPS treatment respectively as compared to untreated control. However, the presence of LT apparently abolished all chemokine inducing properties of CW and LPS in all the various types of chemokines to levels similar to untreated control. Next, individual component of LT were tested for their effects on chemokine productions. Surprisingly, PA showed a high induction of chemokine production in IP-10 and MIG, and moderate induction for MCP-1 and Rantes. PA alone has no impact on IL-8 production in PMBC cells. Similar to LPS and CW, the chemokine-inducing properties of PA were evidently suppressed when LF was introduced into the cell culture. In contrast to PA, the exposure of LF alone to PMBC has no observable changes in cellular chemokine production except for a slight 2 fold increase in MIG. The differences observed between PA and LF effects on chemokines production may be attributed to the binding of PA to cell surface receptors ANTRX 1 and ANTRX 2 [80, 81] which may trigger signal transduction for chemokines production. On

the other hand, LF is not known to bind to cell surface receptors and requires PA for translocation to the cell cytoplasm.

4.3.2 LT inhibits CW and LT-induced transcription of chemokines

To corroborate the findings of LT suppression of LPS-induced chemokine production in PMBC, the highly sensitive RNase protection assay was employed for detecting mRNA level in cells. The chemokine gene expression profile of Rantes, Mip 1α, Mip 1β, Gro and IL-8 of PBMC treated with various stimulus were determined after 2, 4 and 24 hours of incubation. Similar to results obtained from CBA analysis, both CW and LPS upregulated the expression of Mip 1α, Mip 1β, and IL-8 within 2 hours of exposure with the peak expression at 4 hours and eventually tapered off at 24 hours (Figure 4.2). MCP-1 upregulation by CW and LPS only occurred at 4 hours and peaked at 24 hours whereas Gro was only detectable at 24 hours in LPS-treated PBMC (Figure 4.2). The level of all chemokines expression for LT-treated PBMC remained unchanged as compared to untreated control for all time points tested.

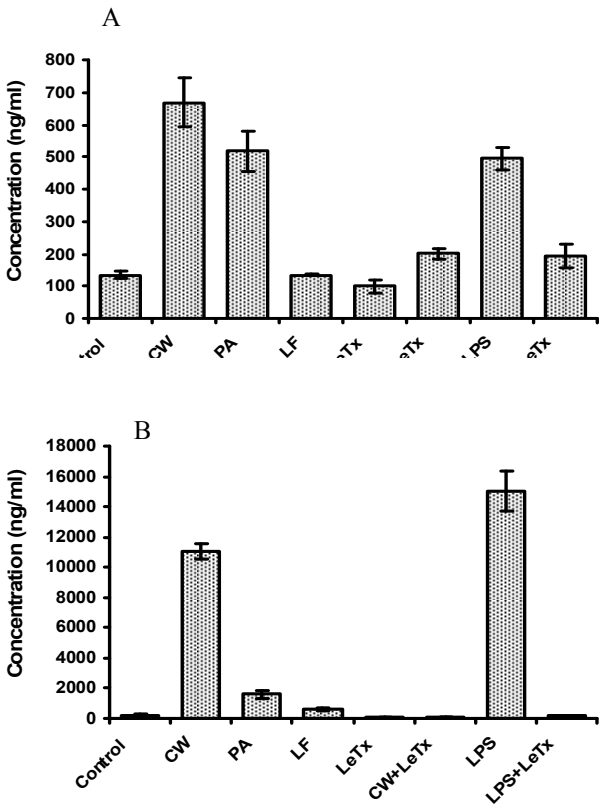
4.3.3 LT-treated PMBC does not induce neutrophils chemotaxis

The induction of chemokines as observed in CBA and RNase protection assay was verified by neutrophil chemotaxis assay. Blood was treated with LT, *B. anthracis* Sterne and *Staphylococcus aureus* for 4 and 6 hr and chemotaxis was subsequently assessed. Using Transwell system, neutrophils that migrate across a membrane filter in response to the presence of chemotactic factors were enumerated. As shown in Figure

4.3, both blood and plasma that were treated with LT for 4 and 6 hours did not promote neutrophils migration across the transwell membrane filter. The apparent lack of enhanced neutrophils migration indicates the absence of chemokines secretion by PBMC in both blood and plasma following LT treatment. Although LT failed to enhance chemokines production in PBMC, it also did not reduce the neutrophil migration in both the blood and plasma, thus implying that LT has no effect on the chemotactic activity of the chemokines that are already present in the plasma.

In contrast, the blood and plasma sample treated with *S. aureus*, a gram negative bacterium containing LPS, showed a considerable increase in neutrophils movement across the membrane. Consistent with the LPS and CW-induced chemokines production as determined by CBA and mRNA expression profile in figure 4.1 and figure 4.2 respectively, the increase in neutrophils chemotaxis demonstrated that the PBMC in the blood indeed produced chemokines in response to *S. aureus* infection. Interestingly, treatment of blood with *B. anthracis* Sterne strain which produces both PA and LF also displayed increase in neutrophils chemotaxis (figure 4.3), presumably due to the effect of CW from the vegetative cells. However, earlier experiment on chemokines detection with CBA demonstrated that LT was able to obviate CW-mediated chemokines production to almost that of the untreated control (figure 4.1). Although both experiments involved the use of CW, there were some relevant experimental variations that may account for the apparent differences in the results obtained. In neutrophils chemotaxis assay, culturing *B. anthracis* Sterne strain involved a certain lag time before it can begin producing PA and LF in the culture whereas LT was immediately present in the CBA experiment to exert its

chemokines suppression effect. The other probable reason could be attributed to the lower LT concentration present in the *B. anthracis* culture from the relatively short incubation time of 4-6 hour as compared to the CBA experiment where purified PA and LF were added directly into the media. Nevertheless, the suppression of chemokines by LT is still a clinically relevant finding as evidenced by the normal or slightly elevated PMN counts in inhalational anthrax patients [25, 260].



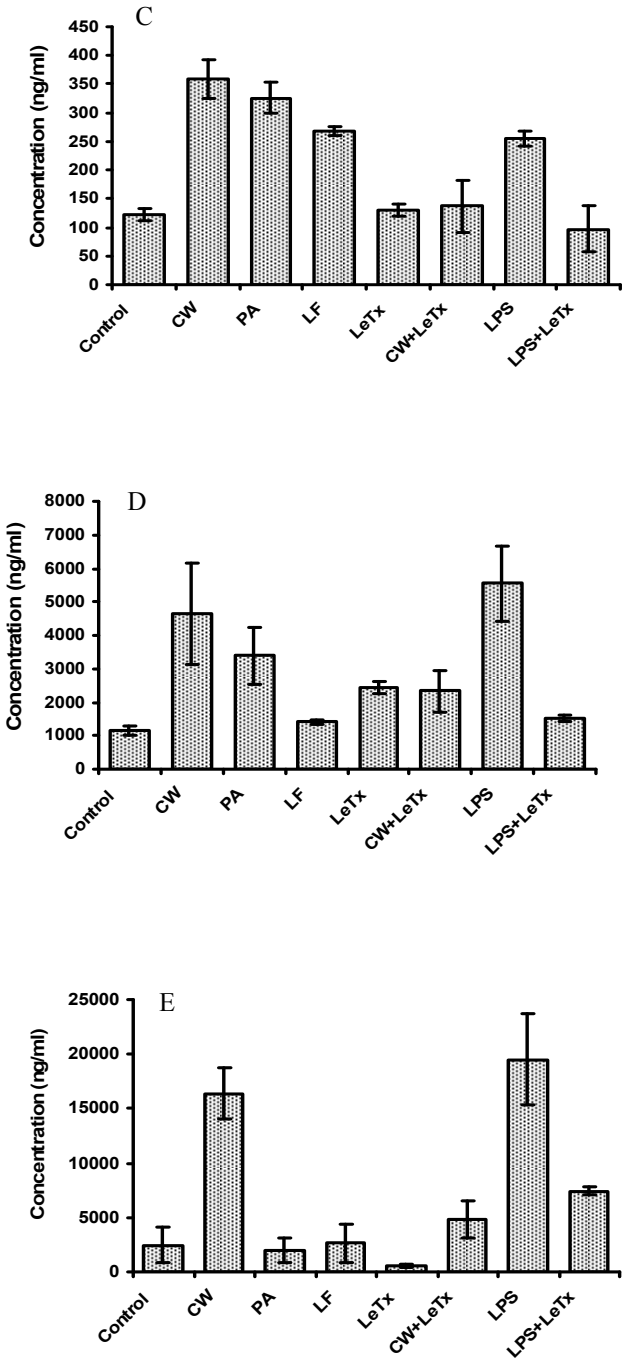


Figure 4.1: Chemokine secretion measurement with cytometric bead array.
A - IP10, B MCP-1, C – MIG, D – Rantes, E – IL-8

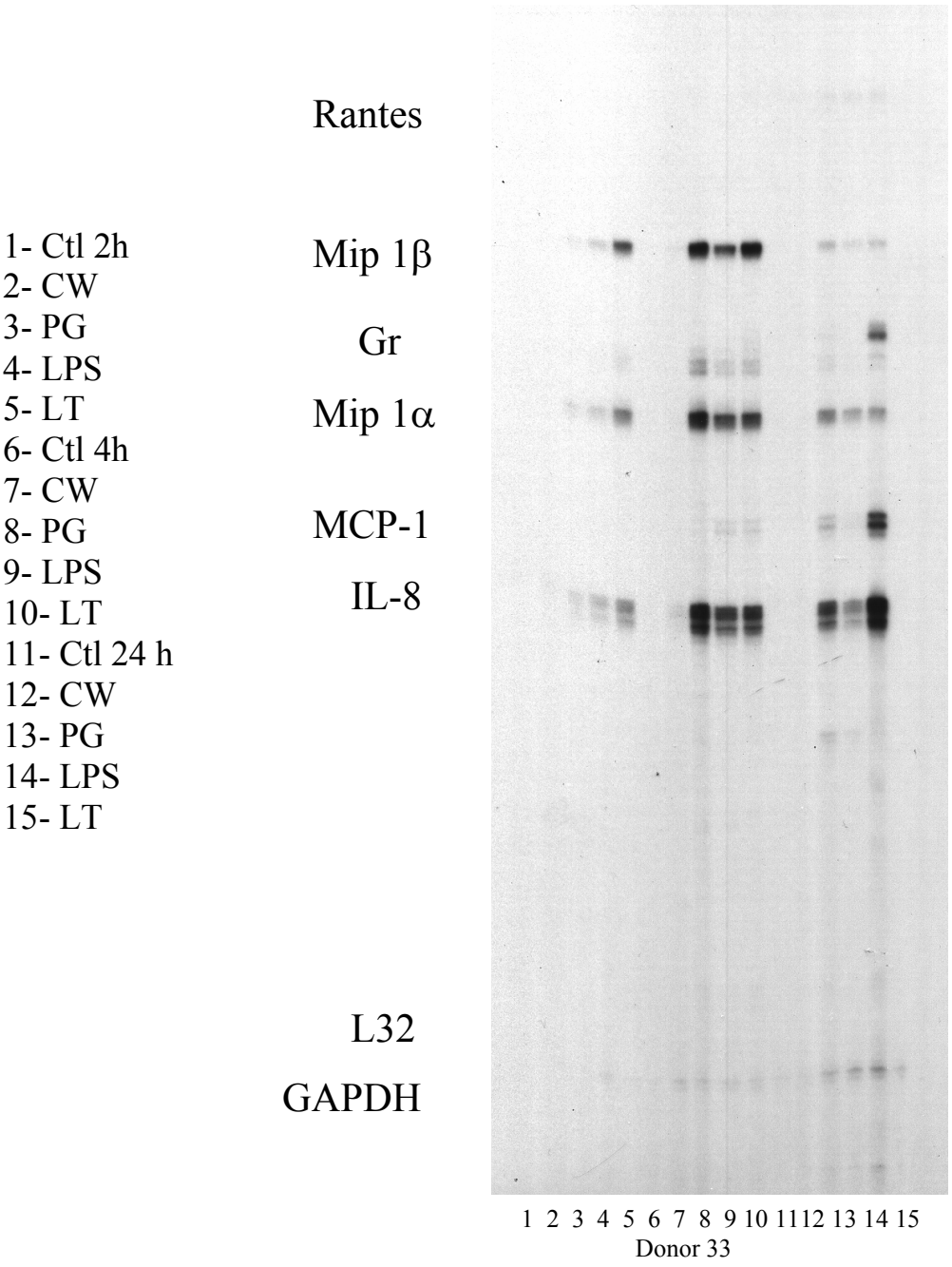
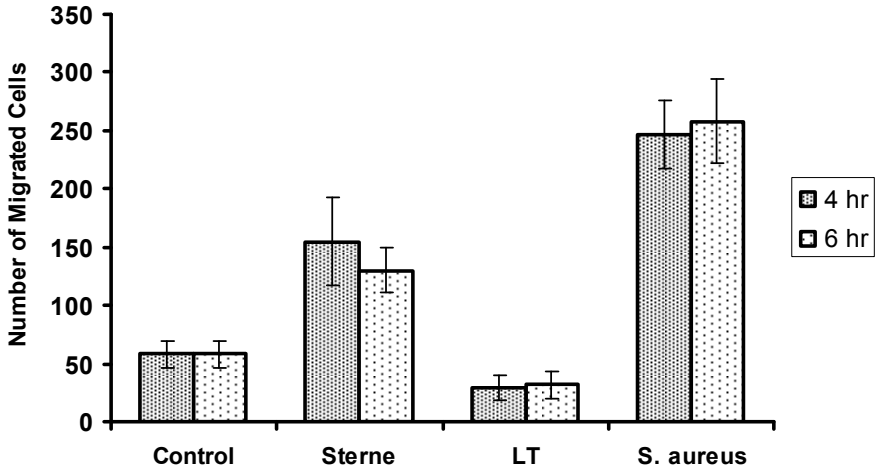


Figure 4.2: Chemokine gene expression in human PBMC after treatment with LT, CW, peptidoglycan (PG), and LPS for 2, 4, and 24 hours. Total RNA was purified and the gene expression was detected using RNAse protection assay.

A



B

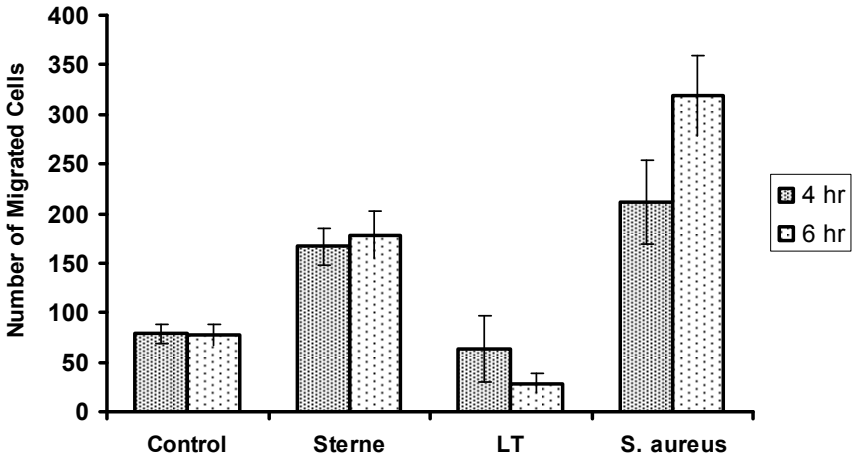


Figure 4.3: Effect of LT-treated blood and plasma on neutrophils chemotaxis. A) Blood was treated with LT, *B. anthracis* Sterne and *S. aureus* for 4 and 6 hr and subsequently used for chemotaxis analysis using transwell according to manufacturer's instruction. B) Plasma was derived from the blood treated in the same way as A and used for chemotaxis analysis

4.4 Discussion

Chemokines represent the largest family of cytokines and in most cases, defined by four conserved cysteine residues [263, 264]. It plays an important role in both innate and adaptive immunity and consequently, the impairment of chemokine production can lead to various disease states [265]. The role of the chemokines is to promote accumulation of the immune cells at the place where they are produced in response to certain stimulus such as infection or injury. Simulation of chemokine production by infectious organism can be mediated by direct interaction between microbial pathogen-associated molecular patterns (PAMP) and pattern recognition receptors (PRR) in host cells. This includes toll-like receptors (TLR) [266], or the nucleotide-binding site leucine-rich repeat proteins NOD1 and NOD2 [267]. In addition, endogenous molecules when stimulated by injury or infection such as fibrinogen, elastase, and defensins and many major inflammatory and immunomodulatory cytokines can also induce chemokines production[268].

In response to treatment with LPS and bacteria CW, certain cell types stimulate production of chemokines to attract other immune cells to sites of infection where they exert anti-bacterial activity [269]. IP-10 interacts with G-protein-coupled receptor CXCR3 expressed on Th1 lymphocytes and participates in various immune and inflammatory responses [270]. MCP-1 functions as a chemoattractant for recruiting monocytes, T cells and dendritic cells to sites of injury and infections [271, 272]. Both MIG and Rantes are involved in T-cells trafficking [273] and in addition, Rantes can also attract eosinophils and basophils [274]. The primary function of IL-8 is to induce

chemotaxis in neutrophils as well as exocytosis and respiratory burst [275]. In this study, LPS and bacteria CW greatly enhanced the production of chemokines in PBMC including IP-10, MCP-1, MIG, Rantes and IL-8 (Figure 4.1). However, treatment with LT almost completely abrogated the chemokine-inducing effects of LPS and CW. Due to the wide ranging effects of chemokines on many different types of immune cells, the inhibition of chemokine production in PBMC results in the inability to recruit other immune cells and therefore providing opportunity for the pathogen to establish itself in the host system.

Some of these chemokines not only play a role in immune response against bacteria infection, but also exhibit anti viral properties. It was demonstrated that Rantes, Mip 1α and Mip 1β also functions as major HIV and simian immunodeficiency virus (SIV) suppressive factors by binding and downregulating the viral co-receptor CCR5 [276]. Indeed, it was observed that the ability to maintain an adequate beta-chemokine production is an important parameter for preventing HIV patient from developing full blown AIDS as well as for controlling the natural course of HIV infection in uninfected persons [277].

Gene expression studies suggest that the upregulation of chemokines by LPS and CW is in part regulated at the transcriptional level as indicated by the increase in mRNA levels using RNase protection assay. Expectedly, the presence of LT did not cause any upregulation of mRNA levels for Rantes, Mip 1α, Mip 1β, Gro and IL-8 in PBMC. The findings from this highly sensitive method of determining chemokine gene expression profile are consistent with the chemokine levels that were released into the supernatant by

PBMC. This indicates that LT not only fail to elicit chemokine production in PBMC but also suppresses the chemokine-inducing properties of LPS and CW.

In addition to the inhibition of chemokine production that prevents neutrophils accumulation at the site of infection, LT also directly affects neutrophils chemotaxis function by impairing actin assembly in the cells [52]. Collectively, the concerted effects of both inhibiting chemokine production and impairing neutrophils actin cytoskeleton rearrangement have a profound impact on the functional ability of neutrophils in anthrax infection.

It is believed that LT induces overwhelming pro-inflammatory cytokines which are speculated to be responsible in part for sepsis and ultimately contributes to death [278, 279]. Correspondingly, it was also observed that the occurrence of sepsis is often associated with the failure of neutrophils migration to the site of infection [280-282]. However, it is not completely understood how neutrophils contribute to sepsis beyond its lack of participation to combat the pathogen at the infection sites.

Recent studies describe the inhibition of PA as an adjunct method to complement antibiotic treatment appears to be promising for the treatment of anthrax infection. Antibodies against PA have shown to be effective in preventing toxin entry into the cells in both *in vitro* and *in vivo* model [283-286]. Other types of PA-directed therapy includes mutant forms of PA that generate dysfunctional heptamer with wild-type PA [287, 288], cyclodextrin that may suppress PA pore formation [289, 290] and cisplatin that modify PA configuration [291]. As the presence of a functional PA is a pre-requisite for translocation of LF into cells, it is reasonable to speculate that effective PA-directed

therapies may conceivably block the downstream effect of LT intoxication which includes, among others, suppression of chemotaxis production and impairment of neutrophil migration.

Chapter 5: Concluding Remarks

The current treatment method for anthrax infection is essentially based on the administration of antibiotic and supportive therapy. However, it has shown to be insufficient as evidenced by the poor prognosis in inhalational anthrax. Inhalational anthrax occured in 2001 had a survival rate of only 55% despite intensive medical care and early administration of antibiotics. Recent studies showed that several PA-directed therapies provided protection in mice that were challenged with anthrax spores [292] and it is likely that future treatment for anthrax may possibly consists of lethal toxin inhibitory compounds in conjunction with antibiotic treatments.

The induction of autophagy by anthrax LT represents a novel effect amongst the many others that have been described in the literature. Although autophagy was initially discovered as a response to starvation, it has since been known to be associated with many physiological and developmental processes. In the scenario of infection, autophagy has been shown to be involved in toxin, bacteria, viruses as well as prions interactions with the host cells. Recent studies also suggest the involvement of autophagy in MHC class II presentation of intracellular antigens [191]. The results presented here are only the initial findings on the possible implication of autophagy in anthrax pathogenesis. Given the defensive role of autophagy, it is possible that LT may be in part degraded in the cytoplasm via autophagy pathway. A fuller understanding of the implication of autophagy in LT which may in turn open up new options for combating against anthrax

infection. For instance, autophagy has been showed to rescue cells from death by promptly removing damaged mitochondria [293]. Similarly, it would be useful to determine if the induction of autophagy can eliminate LT-mediated mitochondria damage before the leaking of its constituents into the cytoplasm which can initiate apoptosis. In addition, possible future studies should also investigate the effect of autophagy on spores germination, bacteria growth and toxin degradation.

Several recent studies have geared towards the direction of PA or LF-directed therapies. This seems to be a promising approach as toxin inhibition were shown to be beneficial even when administered later in the disease thus allowing a broader window of opportunity for treatment. Effective inhibition of PA or LF would also address other concerns such as the overproduction of ROS that may result in death and the suppression of chemotaxis by inhibiting chemokines production

With the discovery of more physiological effects of LT on host cells and the advancement of our understanding on anthrax pathogenesis, it is likely that the next approved treatment method would go beyond just antibiotic treatment. In essence, the findings in this study contribute to the elucidation of the convoluted nature of anthrax pathogenesis and provide new insights into the functions of anthrax toxin. The next phase would be to determine how best to apply these new knowledge in combination with conventional treatments for anthrax treatment.

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