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FACTORS INVOLVED IN CELLULAR SENSITIVITY TO ANTHRAX LETHAL TOXIN

A Dissertation APPROVED FOR THE DEPARTMENT OF BOTANY AND MICROBIOLOGY

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ABSTRACT

The lethal factor (LF) component of *Bacillus anthracis* lethal toxin (LeTx) cleaves mitogen activated protein kinase kinases (MAPKKs) in a variety of different cell-types, yet not all cells are susceptible to the toxin. Previous studies revealed that this toxin rapidly kills macrophages from specific genetic backgrounds whereas most other cell types are resistant. The reason for this selective killing is unclear, but suggests other factors may also be involved in LeTx intoxication. In the current study, DNA membrane arrays were used to identify broad changes in macrophage physiology after treatment with LeTx. Expression of genes regulated by MAPKK activity did not change significantly, yet a series of genes under glycogen synthase kinase-3-β (GSK-3β) regulation changed expression following LeTx treatment. Correlating with these transcriptional changes, GSK-3β was found to be below detectable levels in toxin-treated cells, and, an inhibitor of GSK-3β, LiCl, sensitized resistant IC-21 macrophages to LeTx. In addition, zebrafish embryos treated with LeTx showed signs of delayed pigmentation and cardiac hypertrophy; both processes are subject to regulation by GSK-3β. A putative compensatory response to loss of GSK-3β was indicated by differential expression of three motor proteins following toxin treatment, and *kif1c*, a motor protein involved in sensitivity to LeTx, increased expression in toxin-sensitive cells yet decreased in resistant cells following toxin treatment. Differential expression of microtubule associating

proteins and a decrease in the level of cellular tubulin were detected in LeTx-treated cells, both of which can result from loss of GSK-3β activity. In addition to examining the cellular impact of LeTx on macrophages, studies were performed in order to identify additional factors that govern LeTx sensitivity among different cell-types. Specifically, comparisons were made regarding the rate of toxin entry among macrophage and non-macrophage lines. These studies revealed differences in the rate of toxin entry among the cell lines tested, which, in turn, could contribute to the differences in susceptibility of these lines. Together, the data presented in this thesis provide new information on LeTx's overall influence on macrophage physiology, and suggest that loss of GSK-3β as well as changes in kinesin motor proteins and microtubule stability contributes to cytotoxicity.

INTRODUCTION

In this thesis I will be discussing anthrax, its causative agent, and the virulence factors required for pathogenesis. Special focus will be given to anthrax toxin and its role in disease and, more specifically, the role of anthrax lethal toxin. In addition, I will provide an overview of the host cell-types and signaling pathways implicated in disease as well as factors that govern host sensitivity. In line with this, I will highlight the gaps in our current understanding regarding lethal toxin and the mechanism by which it causes lethality in the host.

Due to its marked cytotoxic effects *in vitro* and *in vivo*, lethal toxin has been the focus of many studies related to anthrax. In spite of all that is known about lethal toxin and its involvement in mediating death in the host, many questions remain regarding its cellular activity as well as its overall role in disease. Therefore, my work focuses on studying the interaction of anthrax lethal toxin with mammalian cells in order to elucidate its mechanism of action.

LITERATURE REVIEW

Organism

The *Bacillus* genus encompasses a diverse group of Gram-positive, aerobic, spore-forming bacteria. Within this group, members considered to be medically relevant include *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides*. Of these, *B. anthracis* is the most prominent due to its history of causing widespread disease in animal and human populations. In contrast, the other pathogenic *Bacillus* are more commonly considered to be opportunistic and are associated with diseases that tend to be rare and non-fatal.

B. anthracis can normally be discriminated from other *Bacillus* species through phenotypic and genotypic comparisons. This bacterium's distinguishing characteristics include lack of motility, lack of hemolysis, and the presence of unique plasmid-encoded virulence factors that mediate this organism's pathogenicity (Turnbull 1991). *B. anthracis* is also unique with regards to the forms and severity of disease that it is associated with. As the causative agent of anthrax, *B. anthracis* has been linked to zoonotic disease, affecting herbivores such as cattle, sheep, and horses, although disease can occur in all mammals including humans (Mock and Fouet 2001). Disease in humans can manifest in one of three forms depending on the route of infection to include cutaneous, inhalational, and gastrointestinal. Although the prevalence of disease differs among the three forms, all have the potential to cause a fatal, systemic infection unless proper treatment is administered.

Due to the extreme difference in the pathogenicity of *B. anthracis* versus other members of the *Bacillus* genus, genetic comparisons have been performed in order to determine the relative amount of homology that exists. Interestingly, reports have indicated that the pathogenic *Bacillus* are virtually indistinguishable based on standard genotypic characteristics, including both the 16S and 23S rRNA sequences (Ash, C. et al. 1991; Ash, C. and Collins, M. D., 1992). The high degree of chromosomal similarity prompted the contention that *B. thuringiensis*, *B. mycoides*, *B. anthracis*, and *B. cereus* are varieties of a single species (Somerville and Jones 1972; Kaneko, Nozaki et al. 1978; Seki 1978). It has since been proposed that *B. thuringiensis*, *B. mycoides*, and *B. anthracis* are subspecies of *B. cereus* (Ash, Farrow et al. 1991; Ash and Collins 1992). Efforts remain focused on elucidating the unique virulence factors of each of the *Bacillus* members in order to better define the pathogenicity associated with each.

There is significant homology between *B. anthracis* and *B. cereus*. This has been of particular interest since *B. cereus* is the most significant pathogen within the *Bacillus* genus second to *B. anthracis*. *B. cereus* is most often associated with gastroenteritis (Kotiranta, Lounatmaa et al. 2000; Jensen, Hansen et al. 2003), ocular infections (Callegan, Kane et al. 2003; Chan, Liu et al. 2003), and opportunistic infections that occur in immunocompromised individuals (Tokieda, Morikawa et al. 1999; Ginsburg, Salazar et al. 2003; Girisch, Ries et al. 2003; Hilliard, Schelonka et al. 2003). *B. cereus*-related gastroenteritis develops after consuming contaminated food while the associated ocular infections and

opportunistic infections are both secondary to existing illnesses. *B. cereus*-related gastroenteritis is usually very mild and self-limiting while the ocular and opportunistic infections do require antibiotic treatment (Drobniewski 1993). The major factor involved in managing *B. cereus*-related infections is the incidence of multi-drug resistance due to the organism's production of β-lactamases (Coonrod, Leadley et al. 1971). In spite of this, these infections are usually successfully controlled with specific antibiotic therapy (Drobniewski 1993).

Due to the enhanced pathogenicity of *B. anthracis* and *B. cereus* compared to corresponding members of this species, studies have focused on identifying and characterizing potential virulence factors associated with each. Comparative genome hybridizations have been performed from which it was reported that almost all of the potential virulence genes contained on the *B. anthracis* chromosome, including hemolysins, phospholipases, iron-acquisition genes, and surface proteins, have homologues in *B. cereus* (Read, Peterson et al. 2003). Additionally, Read et al. reported that only 141 *B. anthracis* genes, whose functions are unknown, out of approximately 5508 chromosomal genes did not have *B. cereus* homologues. This study demonstrated the high degree of similarity that exists between these bacteria and further emphasized a common ancestral link. Additionally, these data suggests that the virulence capabilities associated with each organism are not necessarily related to chromosomally-encoded genes but are directed by unique plasmid-contained elements.

The homology between *B. anthracis* and *B. cereus* was further examined in a study that revealed an anthracis-like virulence plasmid in a confirmed *B. cereus* isolate. This particular isolate was found to contain a large plasmid, pBc10987, that shared 65% homology in protein, 50% synteny, and retained the transcriptional direction exhibited by the corresponding plasmid, pXO1, of *B. anthracis* (Rasko, Ravel et al. 2004). In contrast, this group reported only 7% homology between pBc10987 and a *B. thuringiensis* plasmid, pBtoxis. The distinguishing feature of pBc10987 when compared to pXO1 of *B. anthracis* was the absence of the pathogenicity island containing the toxin genes and the corresponding regulatory elements that mediate anthrax disease. In spite of this, Rasko et al. concluded that this particular *B. cereus* isolate was more closely related to the fully virulent Ames strain of *B. anthracis* than to another *B. cereus* isolate.

Recent studies performed by Hoffmaster *et al*. not only complement the findings of Rasko et al. but provide evidence to further complicate the task of making clinical distinctions between *B. anthracis* and *B. cereus*. This group identified a *B. cereus* isolate, G9241, that not only contained an *anthracis*-like plasmid but also possessed the toxin genes associated with anthrax disease (Hoffmaster, Ravel et al. 2004). This bacterium was isolated from a patient with severe pneumonia whose clinical history and disease symptoms mimicked those described in previous cases of inhalational anthrax. The phenotypes of *B. cereus* G9241, *B. anthracis*, and another *B. cereus* isolate were examined and compared on the basis of characteristics including hemolysis, motility, and antibiotic resistance. G9241

displayed a non-*anthracis* phenotype in that it was hemolytic, motile, and penicillin resistant. However, genomic comparisons revealed G9241 to be more closely related to *B. anthracis* than the other *B. cereus* isolate based on the existence of a pXO1-like plasmid that contained the anthrax toxin genes. From this, it can be concluded that *B. anthracis* and *B. cereus* exhibit an exceptional degree of homology and can no longer be distinguished solely on the basis of standard phenotypic comparisons or on the severity of the associated disease. These findings support the need to further characterize the unique virulence factors that contribute to the varied degree of pathogenicity demonstrated among the *Bacillus* members and to identify the factors that determine the type of disease that ensues.

Historical Significance of *B. anthracis*

The long-standing history between *B. anthracis* and medical research has laid the foundation for the fields of microbial pathogenesis and immunology. Work with this organism began in the mid-1800s in response to devastating outbreaks of anthrax that plagued animals and humans throughout Europe (Bryskier 2002). Aloys Pollender made one of the first reports in 1845 after studying animals that had died of anthrax. At that time, the "stick-like bodies" observed in the blood of diseased animals were not characterized as bacteria but were instead referred to as "plants" (Munch 2003). Later, in 1876, Robert Koch identified the organisms he observed as "Bacteridien" during his studies on anthrax (Munch 2003). After transmission to rabbits, Koch was able to isolate these organisms from the blood of the infected animals. Koch considered the organisms to be living and was able

to support this by cultivating them. Taken together, these findings enabled Koch to specifically link this bacterium to anthrax disease (Koch 1876; Jay 2001). In doing so, Koch's postulates were established and have since remained fundamental to microbial pathogenesis (Jay 2001).

Research on anthrax continued and in 1881, *B. anthracis* was the first organism to be used in a live, attenuated bacterial vaccine developed by Louis Pasteur. This came in response to a widespread outbreak of anthrax among domestic livestock and was considered to be of great success**.** In 1905, *B. anthracis* was central to studies performed by Metchnikoff following his discovery of macrophages and phagocytosis. Specifically, Metchnikoff studied the uptake of anthrax bacilli by macrophages in order to examine variation in susceptibility to phagocytosis based on the organism's level of virulence (Hirsch 1959). Evidence revealed by these studies not only served as a foundation for the field of cellular immunology but also provided insight into anthrax pathogenesis, which remains under intense focus today.

Anthrax

The first reports of anthrax date back to 15th century B.C. when it was first described in the book of *Genesis* as one of the Egyptian plagues (Oncu and Sakarya 2003). It is also believed to be the disease associated with the "Black Bane" that plagued Europe during the Middle Ages, having caused significant death among animals and humans (Bryskier 2002). Anthrax has also been linked to widespread disease that occurred during the industrialization of Europe,

resulting in the death of more than half of the sheep throughout Europe by the mid-1800s (Bryskier 2002). The prominence and devastation of disease are what first prompted intense focus on anthrax research and led to the notable scientific discoveries that began in the mid-1800s. In modern day, anthrax remains endemic to developing countries of the world that do not vaccinate domestic livestock and primarily affects people who come into contact with infected animals or their products (Oncu and Sakarya 2003).

Aside from reports of naturally occurring disease, anthrax has a long history with biological warfare. The first account describing the use of anthrax as a biowarfare agent dates back to Moses (Bryskier 2002). The use or misuse of *B. anthracis* became especially prevalent in the 20th century, beginning with Germany during World War I. Additional reports describing the use of anthrax in biowarfare involved Japan and Great Britain during the early 1930's and the mid-1940s. During that time, *B. anthracis* was deliberately used to contaminate food and water supplies while sporulated forms of this organism were aerosolized and tested for use with bombs. The most notable, accidental outbreak of anthrax occurred in Sverdlosk in 1979 after an explosion in a Soviet biological weapons laboratory. This explosion caused the release of aerosolized *B. anthracis* spores, leading to a severe outbreak of inhalational anthrax and resulting in 64 deaths (Meselson, Guillemin et al. 1994). Prior to the 9/11 attacks of 2001, the most recent account of anthrax and bioterrorism involved a Japanese terrorist group that released *B. anthracis* spores in Tokyo subway stations in 1995, fortunately to no avail. The anthrax attacks in the U.S. following 9/11 caused 11 cases of

inhalational anthrax, resulting in 5 deaths, as well as 11 cases of cutaneous anthrax (Spencer 2003). This event renewed focus on *B. anthracis* due to its potential as a biological weapon and its ability to cause severe disease in humans.

Disease Cycle

Disease is initiated by host contact with spores at which point spores are engulfed by tissue-resident macrophages at the site of infection. Once the spores have been phagocytosed, they are transported to regional lymph nodes (Ross 1957; Lincoln, Hodges et al. 1965). Germination occurs within host macrophages during transport to regional lymph nodes (Ross 1957; Guidi-Rontani, Weber-Levy et al. 1999; Guidi-Rontani, Levy et al. 2001; Welkos, Little et al. 2001; Guidi-Rontani 2002; Welkos, Friedlander et al. 2002). Replication of vegetative bacilli soon follows, and as the regional lymph nodes become overwhelmed, vegetative cells spread to surrounding tissues and into the bloodstream, ultimately causing systemic infection (Ross 1957; Lincoln, Hodges et al. 1965). Finally, upon death of the host, sporulation of the vegetative cells occurs and is followed by the return of spores to the soil environment (Mock and Fouet 2001). These spores exist in a dormant state until the disease cycle is initiated once again.

Clinical Disease

Anthrax disease manifests in one of three forms: cutaneous, inhalational, and gastrointestinal. Cutaneous anthrax is the most common form of human disease (Inglesby, Henderson et al. 1999) and is easily recognized and treatable. Inhalational and gastrointestinal anthrax are more serious forms of disease and are

therefore associated with relatively high mortality rates, particularly with infections that are not treated within the early stages. In these cases, disease results from the inhalation of spores or the consumption of contaminated meat, respectively. Although less common, these forms pose a greater threat since diagnosis is difficult as early symptoms of disease are non-specific.

All three forms of anthrax disease have the potential to cause a fatal, systemic infection in the host. Systemic anthrax occurs unless proper treatment is administered during the early stage of disease. Once the disease progresses to the later stage, treatment is often ineffective. During the later stage of anthrax, it has been reported that there are $10^7 - 10^9$ organisms per ml of blood (Smith and Keppie 1954). Furthermore, early studies revealed that sterile-filtered serum obtained from infected guinea pigs could induce death when injected intravenously (Smith and Keppie 1954). This evidence prompted researchers to identify key virulence factors produced by this organism. Studies have since shown that anthrax toxin, one of two major virulence factors produced by *B. anthracis*, is central to the disease process. In addition to the high number of organisms contained in the blood at the later stage of disease, high levels of toxin contribute to death of the infected host (Keppie 1955). The severity of systemic disease emphasizes the importance of elucidating the specific virulence mechanisms that facilitate the progression of anthrax infections.

Treatment and Prevention of Disease

In recent years, *B. anthracis* has gained increasing recognition for its potential use as a biological weapon. The U.S. mandated an anthrax vaccine for all military personnel although the long-term effectiveness of this vaccine in response to exposure has not been determined. In addition, current vaccine supplies are limited and therefore do not benefit the civilian population. In the event of a biological attack, antibiotic therapy would be used to treat disease. However, this

organism's natural resistance to a variety of antibiotics, particularly cephalosporins and trimethoprim-sulfamethoxazole, limits the number of effective drugs that can respond to infection (Doganay and Aydin 1991; Odendaal, Pieterson et al. 1991). Additionally, recent studies revealed the presence of two beta-lactamases within the genome of *B. anthracis* (Chen, Succi et al. 2003). This finding further heightens the concern regarding the ability of this pathogen to circumvent current drug therapies. Problems in determining the most effective drug treatments are compounded by the fact that, in order to be effective against severe forms of disease, administration of drugs must begin during the early stages of infection. This is based on reports showing that death occurred in experimental animals once anthrax disease had progressed to a later stage despite having achieved sterility of the blood with antibiotic treatment (Keppie 1955). The limitations of current preventative and therapeutic measures support the need to further elucidate the role of key virulence factors in order to minimize the efficacy of *B. anthracis* within the host.

Pathogenesis

There are two major virulence factors involved in anthrax pathogenesis, each of which is encoded on a separate plasmid carried by *B. anthracis*. The first major virulence factor is a tripartite toxin, encoded by pXO1, which consists of protective antigen (PA), lethal factor (LF), and edema factor (EF). This toxin acts in binary combination whereby the binding domain (PA) facilitates the uptake and entry of the enzymatic domain (LF or EF) into the cytosol of host cells (Brossier and Mock 2001). These binary combinations yield two distinct toxins, edema

toxin and lethal toxin, which cause edema and death. The second major virulence factor produced by *B. anthracis* is an anti-phagocytic capsule composed of poly-D-glutamic acid and is encoded by pXO2. The capsule is thought to facilitate the disease process by inhibiting phagocytosis of vegetative bacilli by host cells (Makino, Uchida et al. 1989; Welkos 1991).

Fully virulent strains of *B. anthracis* possess both virulence plasmids; strains lacking either of the plasmids exhibit a marked reduction in virulence. Specifically, strains lacking pXO2 have exhibited a 10^5-fold decrease in virulence compared to wild-type strains (Ivins, Ezzell et al. 1986; Welkos and Friedlander 1988) while strains deficient in pXO1 are reportedly avirulent (Ivins, Ezzell et al. 1986; Uchida, Hashimoto et al. 1986). Because the detrimental effects associated with *B. anthracis* infections are attributed to pXO1-encoded factors, preferential focus has been given to studying anthrax toxin in order to better understand its role in disease.

The requirement for this virulence factor reportedly begins during disease establishment. Upon host contact with *B. anthracis* spores, rapid and efficient phagocytosis by macrophages occurs at the initial site of infection. Following uptake, studies have revealed that germination of spores occurs rapidly within host macrophages (Guidi-Rontani, Weber-Levy et al. 1999). During this intracellular step, it has been reported that the vegetative bacilli display an early onset of virulence gene expression, specifically that of the toxin-encoding genes (Guidi-Rontani, Weber-Levy et al. 1999; Dixon, Fadl et al. 2000). Subsequent

comparisons of *B. anthracis* strain variants, differing in pXO1 plasmid content, revealed that strains devoid of anthrax toxin do not survive in host macrophages after germination (Guidi-Rontani, Weber-Levy et al. 1999). This suggests that the early onset of toxin gene expression mediates the survival of vegetative bacilli during this intracellular step. Separate from this is the contention that the toxin facilitates the release of the vegetative bacilli from host macrophages into the extracellular milieu. This is based on studies that revealed a loss of membrane integrity among *B. anthracis*-infected macrophages (Dixon, Fadl et al. 2000; Guidi-Rontani, Levy et al. 2001). The most notable change in membrane integrity occurred in macrophages infected with a strain containing functional pXO1. In contrast, macrophages infected with strains deficient in pXO1 exhibited minimal change in membrane integrity. Taken together, these data suggest that early onset of toxin gene expression is essential for the progression of disease. Beyond that, anthrax toxin is thought to play a vital role throughout the course of infection as it mediates edema and death in the host, as described in following sections.

Virulence Plasmids

Early studies revealed that anthrax toxin is encoded by a large plasmid that confers toxigenic characteristics (Mikesell, Ivins et al. 1983). Preliminary analysis indicated that strains lacking this extrachromosomal element did not produce EF, LF, or PA. Incubation at 42.5°•C cured toxigenic strains of the plasmid and completely eliminated toxin production. In addition to discovery of the toxin-encoding plasmid, this work also explained Pasteur's early observations

regarding the loss of virulence when *B. anthracis* was incubated at 43°•C. Subsequent work found that the toxin structural genes, *cya*, *lef*, and *pag* (which correspond to EF, LF, and PA, respectively), are all encoded on this plasmid, thereby making a direct link between toxin production and this extrachromosomal element. pXO1 has now been found in all toxigenic strains of *B. anthracis* and there is currently no evidence of strains harboring toxin genes within the chromosome.

Following discovery of pXO1, Leppla and colleagues subsequently cloned *pag* with cloning of *lef* and *cya* following soon thereafter. Each of these genes were confirmed to be encoded by pXO1 as this plasmid was used for cloning each of these elements (Vodkin and Leppla 1983; Robertson and Leppla 1986; Mock, Labruyere et al. 1988; Tippetts and Robertson 1988). Sequence comparisons revealed homology within the 5'end of *lef* and *cya*, suggesting this could be a common region of PA binding between these two enzymatic components (Bragg and Robertson 1989). This possibility was subsequently confirmed by numerous functional and biochemical experiments over the next decade. Each of these genes was found to share AT base composition similar to the genome of *B. anthracis* indicating co-evolution of these genes with this organism or one closely related.

Okinaka and colleagues reported the complete sequence of pXO1 in 1999 and the plasmid was found to contain 181,654 bp of DNA encoding 143 ORFs (Okinaka, Cloud et al. 1999). The genes of *cya*, *lef*, and *pag* are located within a

pathogenicity island, approximately 44.8 kb in size, and flanked by exact inverted repeats of IS*1627* elements (Okinaka, Cloud et al. 1999). This pathogenicity island also contains the regulators *pagR* and *atxA*. Of the 31 ORFs within the pathogenicity island, Okinaka and colleagues reported that 15 have no assignable function. Interestingly, three spore germination responsive elements, *gerX-A,B,C*, are present on the pathogenicity island of pXO1. Additionally, pXO1 encodes a type 1 topoisomerase, topA; a resolvase, and a transposase (Okinaka, Cloud et al. 1999). The G + C content of pXO1 is similar to that of the *B. anthracis* chromosome, both of which are approximately 33% G + C (Mock and Fouet 2001).

Anthrax Toxin Genes

pagA. The gene encoding PA (*pagA*: accession number M22589) is found at region 133161 to 135455 on pXO1 and is encoded on the negative strand of DNA (Okinaka, Cloud et al. 1999). Early literature refers to *pagA* as *pag*; but, as described below, *pag* is encoded as a part of a bicistronic message with a regulatory element *pagR*. Thus, the more current literature correctly refers to the gene encoding PA as *pagA*. The DNA sequence of *pagA* encodes a protein 764 amino acids in size with a molecular weight of 83 kDa (Okinaka, Cloud et al. 1999). The overall G+C content of PA is 31%. *pagA* shares sequence homology with other known binary toxins, such as *Clostridium perfringens* iota toxin Ib and *Clostridium spiroforme* Sb (Lacy and Collier 2002). These homologous proteins function in a similar fashion to PA, acting as cell entry components of binary toxins. PagA has been cloned and expressed in a variety of recombinant systems,

and like the other two components of anthrax toxin, the three dimensional structure of this protein has been solved.

lef. The gene encoding LF (*lef*: accession number M29081 and M30210) is located upstream of *pagA* with the intervening sequence containing the *pagR* region. LF is encoded on the positive strand of pXO1 in the opposite direction from the *pagA*/*pagR* bicistronic element. The open reading frame of *lef* lies at regions 127442 to 129871 on pXO1 and encodes a protein 809 amino acids in size (Okinaka, Cloud et al. 1999). The mature protein consists of 776 residues following the cleavage of a 33-residue signal peptide (Duesbery and Vande Woude 1999). Based on this sequence, the predicted size of LF is 90.2 kDa (Duesbery and Vande Woude 1999). LF shares sequence homology with EF across the first 255 residues which reflects a common region of PA-binding (Collier and Young 2003). With the exception of homology with EF, LF does not have any known protein neighbors.

cya. The gene encoding EF (*cya*: accession number M23179 and M24074) is located downstream of both *lef* and *pagA* at region 154224 to 156626 and is encoded on the negative strand of pXO1 (Okinaka, Cloud et al. 1999). *cya* encodes an 800 amino acid protein, with a 767 amino acid mature protein following cleavage of the secretion signal peptide (Duesbery and Vande Woude 1999). The final predicted molecular weight of the mature protein is 88.8 kDa (Duesbery and Vande Woude 1999). EF shares significant sequence homology

with the adenylate cyclase produced by *Bordetella pertussis* and with the amino-terminal region of LF (Escuyer, Duflot et al. 1988).

Regulation of Anthrax Toxin by Environmental Factors

Anthrax toxin expression is inducible and responsive to environmental signals such as CO_2 and temperature (Leppla 1988; Sirard, Mock et al. 1994). Furthermore, growth of *B. anthracis* in defined media leads to increased expression of PA, LF, and EF (Leppla, 1988). Expression of *pagA*, *cya*, and *lef* is coordinated in response to these environmental signals, yet the genes are not organized within an operon.

Growth of *B. anthracis* in the presence of bicarbonate (0.8%) provides CO_2 levels adequate for the induction of toxin expression (Sirard, Mock et al. 1994). In conjunction with CO_2 levels, toxin expression is impacted by medium composition. Bartkus and Leppla observed that toxin production was enhanced when *B. anthracis* was grown at 37° C in R minimal medium containing 0.1M Tris hydrochloride (pH 8.0) supplemented with uracil (40 μg/ml), sodium bicarbonate (0.8%), horse serum (5%), and streptomycin (500 μg/ml) compared to *B. anthracis* grown in brain heart infusion broth (Bartkus and Leppla 1989). Finally, under these growth conditions, toxin expression is also influenced by temperature (Sirard, Mock et al. 1994). Using a *lacZ* reporter system, Sirard and colleagues found a four to six-fold higher expression level (based on β-galactosidase activity) when *B. anthracis* was grown at 37° C compared to 28° C.

It is important to note that, despite the coordinated expression of *pagA*, *cya*, and *lef* in response to the environmental signals, the corresponding proteins do not accumulate at similar levels. Results from *lacZ* fusion/reporter assays indicate *pagA* is transcribed at higher levels than *lef* and *cya*, with a ratio of about 5:1 for *pagA*:*lef* and 10:1 for *pagA*:*cya* (Sirard, Mock et al. 1994). This corresponds with and explains the fact that protein levels of PA exceed LF and LF exceeds that of EF. Reportedly, PA, LF, and EF are produced in relative levels of 20 •g PA, 5 •g LF, and 1 •g EF (Leppla 1988).

Genetic Regulation of Virulence Factors

Expanding on the initial observations of regulation of toxin production, Koehler and colleagues further demonstrated that regulation of *pagA* was modulated by CO_2 through a specific trans-activating element (Koehler, Dai et al. 1994). Screening a transposon mutant library for the absence of toxin production led to identification of a site 13 kb upstream of *pagA*, proximal to the 3' end of *cya*, which was important for toxin expression (Koehler, Dai et al. 1994). This region corresponded to a trans-activator element termed AtxA (~55.6 kDa), which had recently been reported by Uchida and colleagues (Uchida, Hornung et al. 1993).

AtxA is now known to control expression of both the pXO1-encoded toxin genes as well as the genes involved in capsule synthesis that are located on pXO2 (Dai, Sirard et al. 1995; Guignot, Mock et al. 1997; Uchida, Makino et al. 1997). Consequently, *atxA*-null mutant strains have been shown to be avirulent in a

mouse model (Dai, Sirard et al. 1995). Studies conducted with *B. anthracis* strain variants, differing in plasmid components, suggest that *atxA*-mediated expression is required to direct the release of vegetative bacilli from macrophages during disease establishment (Dixon, Fadl et al. 2000). This is based on observations with an *atxA*-null mutant strain that was incapable of escaping from the infected macrophages.

Recent studies demonstrated that, in addition to controlling synthesis of the toxin and capsule genes, AtxA controls the expression of numerous other genes on both plasmids and the chromosome (Bourgogne, Drysdale et al. 2003). For this reason, AtxA is now believed to be a major global regulator of virulence, controlling the expression of capsule, S-layer, and toxin. An overview of AtxA-mediated virulence regulation is shown in Figure 1.

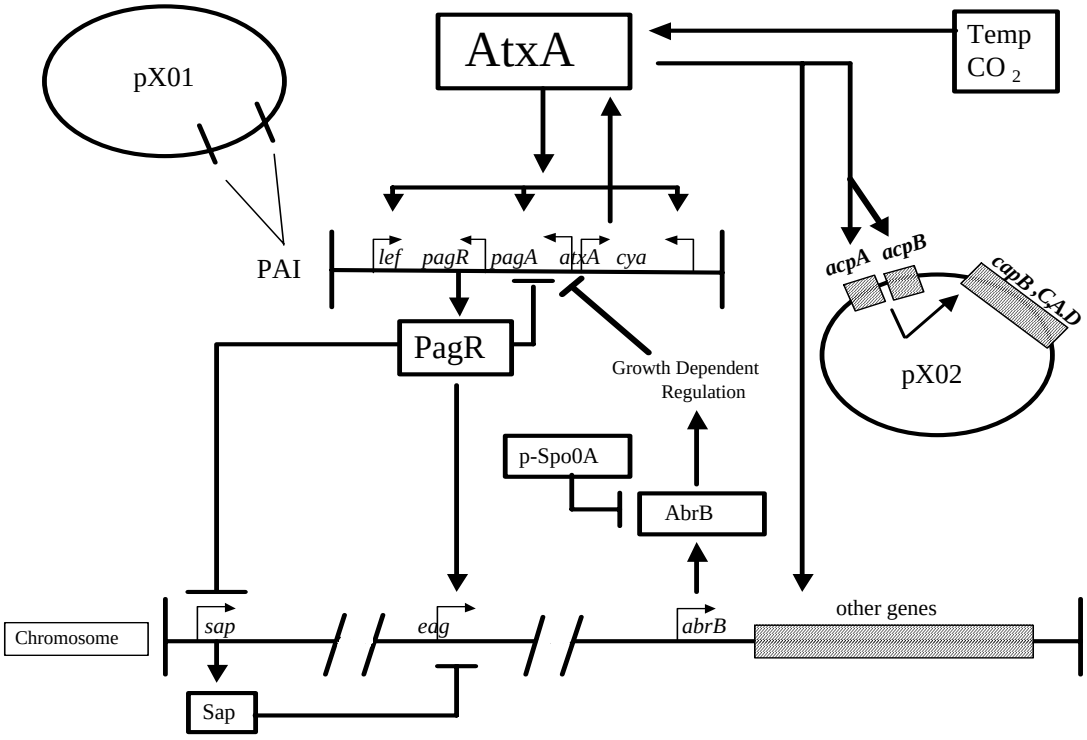


Fig. 1. **Regulatory Network Modulating Anthrax Toxin Expression** In response to environmental signals, AtxA induces expression of *cya*, *pag*, and *lef*. A bicistronic operon of *pagApagR* encodes the negative regulator *pagR*, which represses *pagA* expression and modulates levels of S-layer components Sap and Eag. In addition to regulation of toxin production, AtxA modulates capsule synthesis genes on pXO2 as well as several genes on the chromosome. AbrB functions as a growth-phase regulator of toxin production by repressing expression in lag and early exponential phase growth. AbrB is subject to repression by phosphorylated Spo0A, which accumulates near stationary phase and allows increased toxin production and steady state levels during late stages of growth.

Once the AtxA-responsive region of *pagA* had been defined, it was also found that initiation of *pag* transcription occurred at 2 promoter binding sites termed P1 and P2 (Koehler, Dai et al. 1994). Koehler and colleagues reported that AtxA modulates P1, which lies 58 bp upstream of the pag start codon. Initiation from P2 is minimal but does not require CO_2 or AtxA (Koehler, Dai et al. 1994). As expected, further studies found that in addition to *pag*, *lef* and *cya* expression are also modulated by AtxA (Dai, Sirard et al. 1995).

The most compelling evidence supporting the role of AtxA as a major global regulator comes from recent experiments of Bourgogne and colleagues which used transcriptional profiling to identify genes subject to regulation by AtxA (Bourgogne, Drysdale et al. 2003). Comparisons of mRNA profiles between wild-type organism and *atxA*-null mutants revealed several chromosomal genes regulated by AtxA. Furthermore, this group reported that of the 38 plasmid-encoded genes expressed under specific growth conditions, 18 were subject to regulation by AtxA (Bourgogne, Drysdale et al. 2003).

As one might expect, regulation of toxin production does not occur independently of other factors within the cell. Coordinated regulation of multiple factors important to virulence works to the advantage of *B. anthracis*. Thus, it is important to briefly mention the influence toxin regulation has on other expression events within *B. anthracis*. AtxA regulates the expression of capsule through two regulators, *acpA* and *acpB* (Drysdale, Bourgogne et al. 2004). (This is depicted in Fig. 1 which provides an overview of AtxA-mediated gene

expression.) Thus, pXO1$^-$ strains and *atx*A-null mutants are defective in capsule production. Since the capsule encoding operon *capBCAD* is located on pXO2, it is evident that factors from pXO1 can influence pXO2. To date, there are no reports of pXO2-dependent regulation by pXO1. Finally, it is worth noting that the attenuation of pXO1$^-$ mutants is often attributed to loss of toxin production; however, given the influence of this plasmid and AtxA on expression of numerous genes, the possible involvement of other pXO1-regulated factors in virulence cannot be excluded.

AbrB, Growth Phase-dependent Regulator of Anthrax Toxin

Expression of *pagA*, *lef*, and *cya* and corresponding toxin production reaches maximal levels during late log phase when *B. anthracis* is grown in laboratory medium, and is retained at steady state into stationary phase of growth (Leppla 1988; Koehler, Dai et al. 1994; Sirard, Mock et al. 1994). There is limited production of anthrax toxin during early exponential phase of growth. This growth phase-dependent expression of anthrax toxin is modulated by a negative regulator, AbrB (Saile and Koehler 2002). Studies showed that AbrB is a growth phase-dependent regulator of anthrax toxin synthesis with a predominant impact on *pag* activation. Orthologues of AbrB are encoded on the chromosome and pXO1; however, the plasmid-encoded element does not encode the first 27 residues of *abrB* and isogenic strains lacking this gene are not altered in toxin production (Saile and Koehler 2002). Conversely, chromosomal *abrB* encodes full-length protein and disruption of this gene leads to altered toxin production. In the absence of chromosomal AbrB, toxin production is substantially increased

during early and mid-exponential phase growth (Saile and Koehler 2002). AbrB apparently modulates toxin expression, at least in part, by the repression of AtxA. Interestingly, AbrB has a predominant impact on *pagA* expression with limited influence on *cya* and *lef*. (Refer to Fig. 1 which illustrates the role of AbrB with regards to AtxA-mediated gene regulation.)

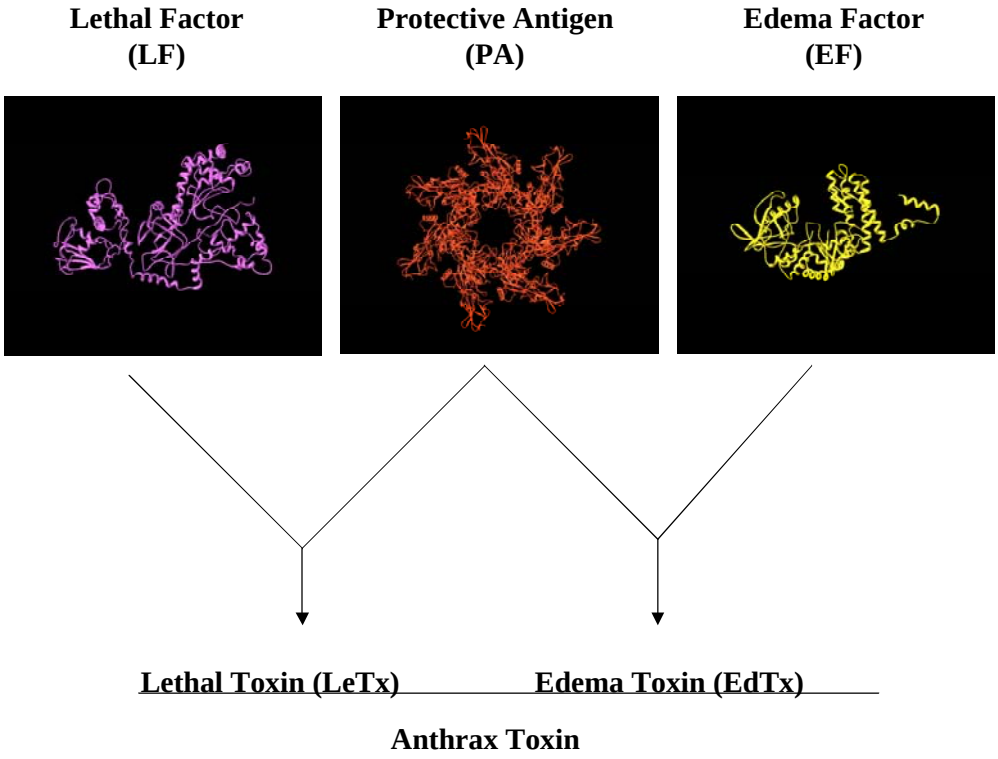
Anthrax Toxin

Although successful disease requires both major virulence factors produced by *B. anthracis*, there is substantial evidence suggesting that anthrax toxin serves as a critical mediator of pathogenesis. First, it has been shown that although complete clearance of the organism can be achieved for septic infections, the host still succumbs to death (Keppie et al., 1955). This observation is attributed to the high level of toxin presumed to be present at the later stage of disease in addition to the high bacterial cell count. Secondly, reports have indicated that highly purified preparations of the toxin cause death and edema in animals (Pezard, Berche et al. 1991). Furthermore, it has also been demonstrated that strains deficient in pXO1 or in the individual toxin genes exhibit a marked reduction in virulence (Pezard, Berche et al. 1991). Finally, there is evidence that immunization against the toxin components, particularly PA, protects against disease (Friedlander, Welkos et al. 2002). Based on these findings, efforts have been directed towards defining the mechanism of action of anthrax toxin in order to better understand its overall contribution to disease.

Components

Anthrax toxin is comprised of three polypeptides: protective antigen (PA; 83kDa), lethal factor (LF; 90 kDa), and edema factor (EF; 89 kDa). This toxin belongs to the family of AB toxins in which a cell-binding (B) moiety facilitates the entry of an enzymatic (A) moiety into target cells. However, two features make anthrax toxin unique among other AB toxins. First, the A and B moieties interact only after being secreted from the bacteria. Second, the toxin has two distinct A moieties (LF and EF) that interact with a single B moiety (PA) (Collier and Young 2003). During intoxication, PA works in combination with EF to form edema toxin (EdTx) and with LF to form lethal toxin (LeTx). EdTx functions as a calmodulin-dependent adenylate cyclase (Leppla 1982) while LeTx acts as a zinc-dependent metalloprotease (Klimpel, Arora et al. 1994; Kochi, Schiavo et al. 1994). These binary combinations act intracellulary to yield two separate toxic effects, edema and lethality, as suggested by each of the toxin's names. The components of anthrax toxin are shown in Figure 2.

Fig. 2. **Components of Anthrax Toxin**



The three dimensional structure has been resolved for each of the toxin components. Studies have revealed that PA consists of 4 distinct functional domains (Petosa, Collier et al. 1997). Domain 1 contains the proteolytic cleavage site that mediates initiation of the intoxication process (Petosa, Collier et al. 1997; Gao-Sheridan, Zhang et al. 2003). Domain 2 is involved in pore formation, which is required for the delivery of LF and EF into the cytosol of host cells (Petosa, Collier et al. 1997; Benson, Huynh et al. 1998). Domain 3 functions in the self-assembly of PA monomers into active heptameric complexes that are capable of binding individual molecules of LF and EF prior to intoxication (Petosa, Collier et al. 1997). Lastly, Domain 4 mediates receptor binding to host cells (Singh, Klimpel et al. 1991).

The structure of LF was first solved in the presence of one of its major cellular substrates and has been shown to consist of four distinct domains (Pannifer, Wong et al. 2001). Domain I consists of the N-terminal portion of LF, also referred to as LFn, that contains the PA binding site (Lacy, Mourez et al. 2002). Domain II has an ADP-ribosyltransferase fold resembling that of a related toxin from *B. cereus*. However, the active site has been mutated in LF. Instead, this region is thought to enhance substrate recognition. Domain III is a small α-helical bundle that contains tandem repeats of a structural element of Domain II. This region is actually inserted into Domain II and it is thought to have functional significance involving substrate recognition. Domain IV, the C-terminal domain, contains the zinc-binding site that mediates the activity of this protein. Domains II-IV are closely associated with each other, possibly acting as a single folding

unit. Together, these domains form the protein substrate-binding site (Tonello, Ascenzi et al. 2003; Turk, Wong et al. 2004).

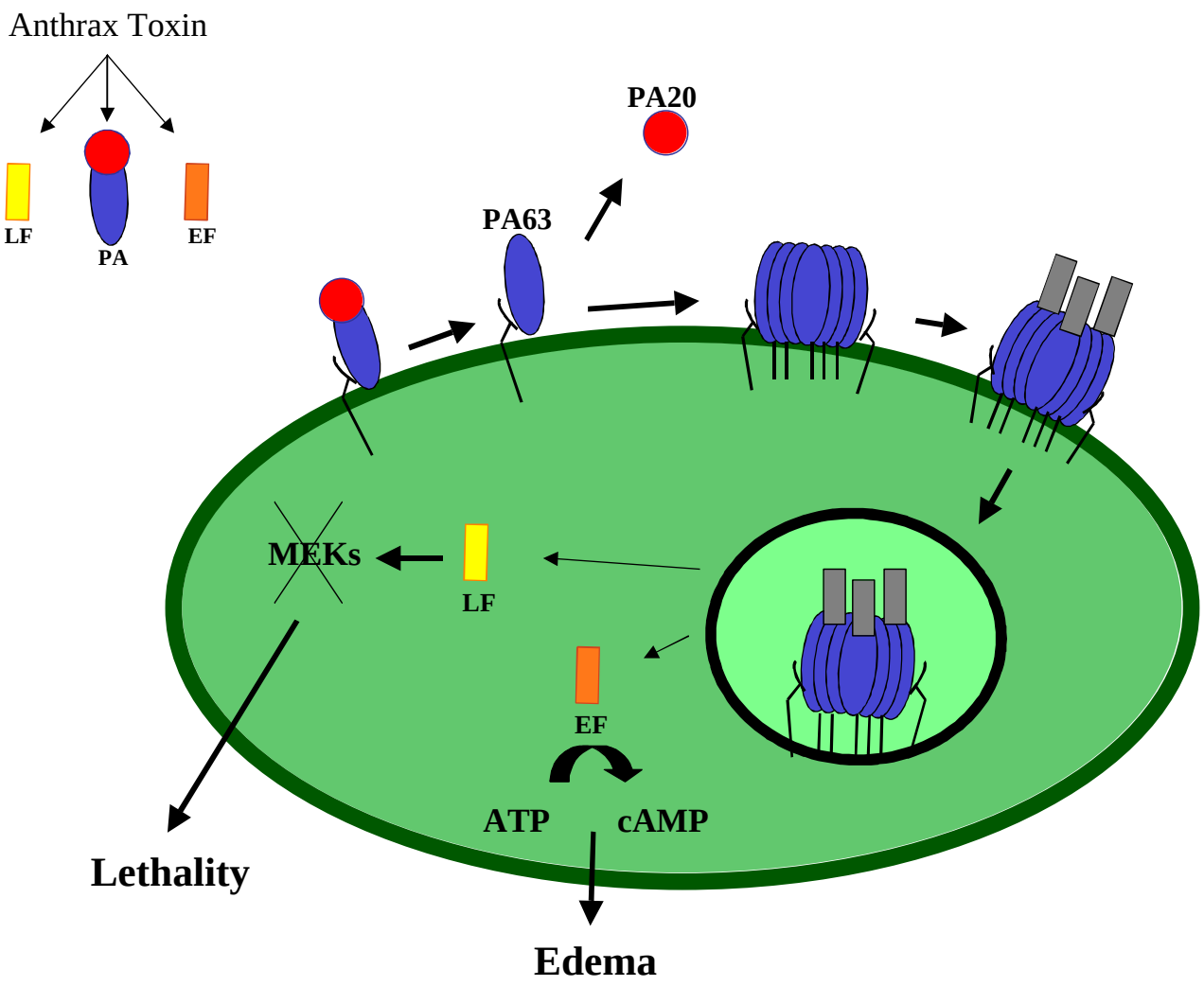
Mechanism of Cell Entry

An overview of anthrax toxin's mechanism of cell entry is illustrated in Figure 3. In the first step of cell entry, PA binds to the cell surface receptors ATR (anthrax toxin receptor), encoded by tumor endothelial marker 8 (TEM8), and capillary morphogenesis protein 2 (CMG2) (Bradley, Mogridge et al. 2001; Scobie, Rainey et al. 2003). Following receptor binding, PA is cleaved by furin-like proteases causing the release of a ~20 kD from the amino-terminus (Klimpel, Molloy et al. 1992). This cleavage event yields a form of PA termed PA_{63} with sites exposed for LF and EF binding. In the next stage of intoxication, PA_{63} oligomerizes into a heptamer (Milne, Furlong et al. 1994), forms a pre-pore complex (Miller, Elliott et al. 1999), and binds 3 molecules of EF and/or LF (Mogridge, Cunningham et al. 2002). A homologous N-terminal region (Arora and Leppla 1993) mediates the binding of LF and EF to the PA_{63} heptamer and the complex is endocytosed via lipid rafts (Abrami, Liu et al. 2003). Following acidification of the endocytic vesicle, the heptamer fully inserts into the membrane by formation of a 14-strand beta-barrel to create an acid-pH dependent channel (Collier and Young 2003). This insertion and channel formation then leads to LF and EF exposure to the cytosol, although this process remains poorly defined. There is evidence suggesting that upon translocation, LF is released into the cytosol of host cells while EF remains membrane bound (Guidi-Rontani, Weber-Levy et al. 2000).

Regardless, both EF and LF have a dramatic impact on cell physiology upon exposure to the cytosol.

Fig. 3. **Mechanism of Cell Entry** PA binds to cell surface receptor, ATR or CMG-2, and undergoes proteolytic cleavage by furin which releases a 20 kDa fragment (PA20). PA63 remains bound and undergoes heptamerization. The PA heptamer is then capable of binding LF and EF at which point the complex undergoes receptor-mediated endocytosis. The complex is contained within an endosome until pH triggers the release of LF and EF into the cytosol. Once in the cytosol, LF acts as a metalloprotease with specificity for members of the Mitogen Activated Protein Kinase (MAPK) cascade, specifically MEKs. EF is an adenylate cyclase and as such it generates high levels of cAMP within the cell. The resultant phenotypes are lethality and edema.

Fig. 3. **Mechanism of Entry for Anthrax Toxin**



Lethal Toxin

Studies have revealed that LeTx provides a selective advantage to the anthrax disease process. *B. anthracis* strains lacking LF exhibit a 100-fold decrease in virulence compared to EF deletion strains (Pezard, Berche et al. 1991), emphasizing the importance of this toxin's contribution to lethality in the host. As a result, preferential focus has been given to LeTx in order to elucidate this toxin's mechanism of action.

LF is a zinc-dependent metalloprotease that cleaves mitogen activated protein kinase kinases (MAPKK), which are described in the following section (Duesbery, Webb et al. 1998). MAPKKs were first identified as the specific substrate of LF when it was revealed that LeTx had a similar effect on tissue culture cells compared to the MAPKK inhibitor PD 98059, as described in the following section (Duesbery, Webb et al. 1998). Additionally, evidence from a yeast two-hybrid assay identified MEK-2 as the specific prey for an LF mutant that was used as bait (Vitale, Pellizzari et al. 1998).

In vitro, LF has been shown to cleave 6 MAPKKs including MEK (1,2) and MKK (3,4,6,7) (Vitale, Bernardi et al. 2000). These substrates share common amino-terminal sites that are cleaved by LF. Cleavage by LF may prevent MAPKK interaction with subsequent kinase targets since proteolysis removes an important MAPKK/MAPK docking site. Given the known substrate targets of LF, the toxin has the potential to modulate extracellular signal regulated kinases 1 and 2 (ERK1,2), c-jun N-terminal kinases (JNK), and p38 signaling pathways.

Based on the reported specificity of LF for certain MAPKKs, the impact of LF on MEK-regulated tumors has been examined. Studies have revealed that LF inhibits growth in V12 H-ras-transformed NIH 3T3 cells (Duesbery, Resau et al. 2001). Additionally, LF reportedly causes apoptosis in human melanoma cells (Koo, VanBrocklin et al. 2002). These observations regarding tumor inhibition and apoptosis were based on studies performed *in vivo*. Hence, these findings provide additional evidence with regards to the impact of LF on MAPK signaling.

Although MAPKKs have long been the only reported substrate for LF, cleavage of MAPKKs alone does not govern susceptibility to LeTx-induced cytotoxicity. Evidence to support this revealed that LF proteolysis of MAPKKs occurrs in cells resistant to LeTx (Watters, Dewar et al. 2001) and, at sub-cytotoxic doses in cells sensitive to the toxin (Pellizzari, Guidi-Rontani et al. 1999). Furthermore, treatment of RAW 264.7 macrophages with inhibitors of the ERK signaling pathway does not cause cell death, suggesting direct inactivation of this pathway is not the sole contributor to cytotoxicity. Recent studies by Park and colleagues indicated p38 prevents synergistic expression of NF-kappaB genes necessary for anti-apoptotic effects in activated macrophages (Park, Greten et al. 2002). Collectively, these studies indicate that while cleavage of MAPKK may be part of LeTx activity, a combination of events lead to cell death.

MAPK Signaling

Cells must be able to detect extracellular stimuli and respond appropriately in order to regulate proliferation and differentiation. The enzymatic activity required for regulating these events is governed by phosphorylation. All eukaryotic organisms are subject to regulation via phosphorylation cascades. These cascades respond to a variety of extracellular signals including mitogens, TNF-alpha, interleukins, and environmental stressors such as UV. In response to such stimuli, a series of phosphorylation events begins with an upstream kinase binding to and phosphorylating a downstream effector. This intermediate kinase subsequently activates a third kinase which then initiates the transcription of specific genes. The importance of phosphorylation is evident due to the number of protein kinases that have been identified in eukaryotes. In fact, yeast have greater than 120 kinases and humans have over one thousand to date.

The mitogen-activated protein kinase (MAPK) phosphorylation cascade has been implicated in the regulation of multiple cellular events in all eukaryotes. The MAPK cascade comprises three distinct signaling pathways; one of which mediates responses to mitogenic and differentiation signals while the other two respond primarily to stress and inflammatory cytokines. In addition to the extracellular stimuli associated with each, these pathways are defined by their MAPK component and are named accordingly. The three MAPK pathways include the extracellular regulated kinase (ERK) pathway, the c-Jun N-terminal kinase (JNK) or stress activated protein kinase (SAPK) pathway, and the p38 pathway. An overview of the MAPK pathways is illustrated in Figure 4. These pathways can operate independently or in combination to trigger gene expression.

However, the extent to which cross-talk occurs between these pathways is poorly defined as the efficiency of phosphorylation varies among the MAPKs. Additionally, it has been shown that different kinases associated with each pathway are localized in different cellular compartments, further complicating the understanding of the extent to which these proteins interact.

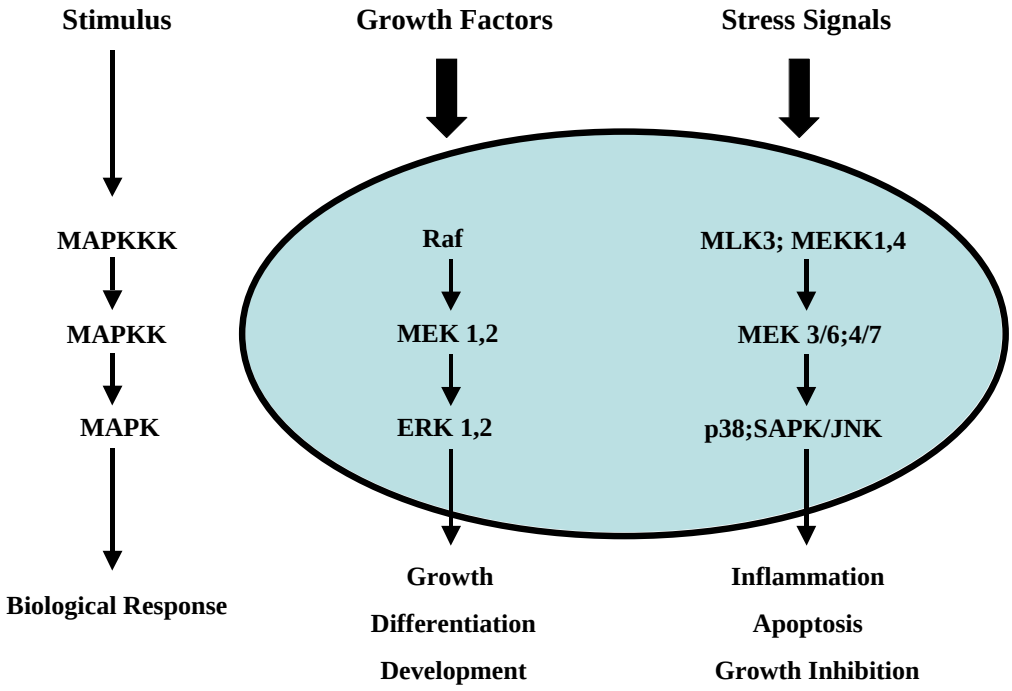


Fig. 4. **Mitogen Activated Protein Kinase (MAPK) Signaling Pathways** Activation of the MAPK pathways occurs in response to a variety of extracellular stimuli. Together, these signaling networks mediate cell growth, differentiation, development, inflammation, and apoptosis. There are three major MAPK pathways (ERK, p38, and JNK) which are distinguished based on the terminal kinase associated with each as well as the resultant biological response. Each pathway involves a phosphorylation cascade in which an upstream kinase (MAPKKK) is activated, thereby phosphorylating a downstream kinase (MAPKK), which then phosphorylates a third kinase (MAPK) which translocates the nucleus and initiates transcription.

Identification of Cellular Target of Lethal Toxin

The link between MAPK signaling and anthrax pathogenesis was first revealed by a study comparing the cellular impact of a chemical inhibitor of the ERK pathway to that of anthrax lethal toxin (Duesbery, Webb et al. 1998). Specifically, the impact of PD 098059 was compared to that of LF when tested on *Xenopus* oocytes, the maturation of which is strictly dependent on MAPK activation. It was shown that complete inhibition of oocyte maturation could be achieved when LF was injected directly into the oocytes. LF was presumed to somehow cause direct inhibition of the activation of MAPK; thus, prompting studies aimed at identifying its impact on upstream kinases. As a result, LF-treated oocyte lysates were probed with antibodies specific for the COOH-terminus and the NH_2-terminus of MAPKK1 (MEK). The resultant COOH-terminus immunoblot revealed a band at increased mobility for lysates containing LF while the NH_2-terminus immunoblot showed no reactivity. Together, these data suggested that LF cleaved MEK, thereby causing the observed mobility shift with the C-terminal antibody and the lack of reactivity with the N-terminal antibody.

This group further examined LF-mediated cleavage of MEK in NIH 3T3 cells in which the MAPK pathway is constitutively activated. Immunoblots of these cell lysates revealed that LF inhibited MAPK activation/phosphorylation. Additionally, LF caused an increase in the mobility of MEK when probed with a COOH-terminus antibody and a loss of MEK when probed with an NH_2-terminus antibody. These results further supported the notion that LF inhibited MAPK signaling via cleavage of MEK. Finally, an *in vitro* phosphorylation assay was

performed in the presence of LF from which it was shown that the presence of LF blocked phosphorylation of MAPK. Together, these findings demonstrated LF's ability to inhibit MAPK signaling in a variety of cell types and identified MEK as the first known substrate of anthrax lethal toxin. Hence, these data laid the foundation for elucidating the cellular mechanism of action of anthrax lethal toxin.

Cellular Factors That Govern Sensitivity to LeTx

Mouse macrophages from specific genetic backgrounds, such as A/J and C57BL/6 mice, are resistant to LeTx (Friedlander, Bhatnagar et al. 1993) and provide useful reagents to better understand sensitivity to this toxin. A comparative genetics approach by Watters and colleagues (Watters, Dewar et al. 2001) found that macrophage sensitivity is linked to single nucleotide polymorphisms in the gene encoding for the kinesin-like motor protein Kif1C. Kif1C is also known to localize to the Golgi apparatus and is important for trafficking cargo between this site and the endoplasmic reticulum. In the same study, this group showed that disruption of the Golgi apparatus with brefeldin A resulted in susceptibility to LeTx in otherwise resistant cells, suggesting localization of Kif1C is important for resistance to the toxin. While the role of Kif1C in LeTx intoxication has not been determined fully, these data further suggest that events outside of direct inactivation of MAPKKs are involved in cytotoxicity.

Studies have revealed that proteasome activity is an important factor in governing sensitivity to LeTx among macrophage cell lines. Specifically, it was shown that sensitive (RAW 264.7) macrophages pretreated with proteasome inhibitors were protected from LeTx-mediated cytotoxicity (Tang and Leppla 1999). In this study, it was also revealed that the proteasome inhibitors did not prevent LeTx-mediated cleavage of MEK; thereby suggesting that the requirement of the proteasome is independent of LF's catalytic activity and is subsequent to cleavage of MEK. In examining whether LeTx specifically activated the proteasome to disrupt homeostasis within macrophages, the investigators did not observe an increase in proteasome activity in the presence of LeTx. Finally, it was shown that LeTx-induced cytotoxicity was inhibited by concentrations of proteasome inhibitor comparable to those that actually blocked intracellular activity of the proteasome. From this, it was concluded that LeTx requires functional proteasomes to induce cytotoxicity in sensitive macrophages.

The requirement of the proteasome in LeTx-mediated cytotoxicity was later linked to a study reporting the induction of transient resistance in macrophages that are normally sensitive to the toxin. In this study, RAW macrophages were pretreated with a sublytic dose of LeTx prior to challenge with high dose LeTx. Cells subjected to this pretreatment were protected from the high dose challenge whereas non-pretreated cells underwent rapid lysis in response to the toxin (Salles, Tucker et al. 2003). This phenotype was termed toxin-induced resistance (TIR). In examining cellular events that contributed to TIR, proteasome activity was investigated based on its reported association with LeTx sensitivity. From

this, it was found that both TIR macrophages and naturally resistant (IC-21) macrophages had a sustained level of ubiquitinated proteins after treatment with high dose LeTx whereas sensitive (non-TIR), RAW macrophages showed a marked reduction in ubiquitinated proteins. As in the studies performed by Tang and Leppla (1999), no increase in proteasome activity was detected in association with TIR. Instead, a direct correlation was made between normal levels of ubiquitinated proteins and cell survival in response to high dose LeTx. This data further implicates proteasome activity in conferring resistance to LeTx in macrophages.

In addition to the link between proteasome activity and TIR, the impact of LeTx on MAPK signaling was also examined in TIR and non-TIR RAW macrophages. MEK cleavage occurred in both TIR and non-TIR RAWs following high dose treatment of LeTx. This suggested that susceptibility might be governed by changes that occurred downstream of this cleavage event. It was shown that there was a sustained reduction in the levels of full-length MEK and diphosphorylated ERK for 24h following high dose LeTx treatment of TIR macrophages. These cells showed an increase in monophosphorylated ERK within that same time-frame. Interestingly, 48h after high-dose treatment of TIR macrophages, levels of both monophosphorylated and diphosphorylated ERK were comparable to control while full-length MEK remained undetectable. This suggests that these cells used a compensatory mechanism to regain activity of the ERK pathway. In contrast, non-TIR macrophages showed no change in the level of monophosphorylated or diphosphorylated ERK in response to high-dose LeTx

although rapid cleavage of MEK was observed. Thus, although MEK cleavage is not unique to LeTx-sensitive cells, the impact on this pathway and subsequent changes in the levels of downstream effectors may contribute to cell survival.

Macrophages

Macrophages play an essential role in host immune defense. These cells are antigen presenting cells (APCs) that activate other macrophages, B cells, or T cells in response to bacterial and viral infections. Macrophages are found in all tissues of the body and have unique receptors that recognize specific conserved motifs on pathogens that are not found on higher eukaryotes (Aderem 2002). As phagocytic cells, their basic functions include phagocytosis, antigen presentation, and secretion of cytokines to activate both innate and adaptive immune responses. Additionally, macrophages also serve an important role in the elimination of senescent cells and in embryonic development (Henson, Bratton et al. 2001).

As the first line of defense against bacterial and viral infections, macrophages rapidly recognize and ingest microbes. Upon phagocytosis, the phagosome fuses with the lysosome to form the phagolysosome. Ingested microbes are then destroyed within the phagolysosome by oxidative burst, acidification, nutrient starvation, and lysosomal enzymes. Antigens from degraded bacteria are then presented at the macrophage cell surface to recruit other immune cells to the site of infection. In addition, macrophages secrete cytokines that stimulate other immune responses to help clear infections. Despite the array of anti-microbial activities used to defeat infectious agents, several bacterial pathogens such as *B.*

anthracis have adapted mechanisms that promote survival within host macrophages. Interacting with host macrophages allows the pathogen to avoid the host's immune system and ensures systemic spread. In the case of *B. anthracis,* the macrophage reportedly provides a suitable environment for germination and commencement of the vegetative cell cycle (Guidi-Rontani, Weber-Levy et al. 1999; Dixon, Fadl et al. 2000). Not only does this provide evidence of this pathogen's ability to circumvent the host's immune system, it has been proposed that this intracellular step is essential in order for disease to ensue (Guidi-Rontani, Weber-Levy et al. 1999).

Macrophage Involvement in Anthrax Pathogenesis

The specific host cell-types involved in anthrax disease have yet to be determined, particularly those targeted by anthrax toxin. However, there is substantial evidence implicating macrophages as mediators of the disease process. To begin, macrophages are the first host cells to contact *B. anthracis* spores during infection; upon which, spores are phagocytosed and transported to regional lymph nodes (Ross 1957; Lincoln, Hodges et al. 1965). In addition, there have been several reports indicating that germination of spores occurs within macrophages following uptake (Ross 1957; Shafa, Moberly et al. 1966; Guidi-Rontani, Weber-Levy et al. 1999). Furthermore, studies have revealed that expression of toxin genes occurs in host macrophages as the vegetative life cycle begins (Guidi-Rontani, Weber-Levy et al. 1999; Dixon, Fadl et al. 2000). Although the role of anthrax toxin at this stage of disease is unknown, it has been proposed that the toxins facilitate the release of vegetative bacilli from macrophages into the

extracellular milieu, thus allowing systemic disease to ensue (Guidi-Rontani, Weber-Levy et al. 1999). Taken together, these data suggest that macrophages provide *B. anthracis* with a suitable environment that allows for germination and commencement of the vegetative cycle.

Based on the evidence described above, it has been proposed that an intracellular step within host macrophages is essential to the progression of disease. With regards to this intracellular step, studies have been directed towards examining the potential role for LeTx at this stage of disease. As described previously, reports indicate that toxin gene expression occurs within host macrophages during commencement of vegetative growth (Guidi-Rontani, Weber-Levy et al. 1999; Dixon, Fadl et al. 2000). Additionally, it has been shown that non-toxinogenic strains of *B. anthracis* are unable to survive within the macrophages following germination (Guidi-Rontani, Weber-Levy et al. 1999; Dixon, Fadl et al. 2000). These studies reported a correlation between the survival of toxigenic *B. anthracis* strains to changes in the membrane integrity of infected macrophages; thereby suggesting a mechanism to mediate the release of vegetative bacilli into the extracellular environment. Based on these findings, efforts have been directed towards identifying a potential role for LeTx during the establishment of disease.

In addition to mediating the release of vegetative bacilli from infected macrophages, another possible role exists for LeTx with regards to macrophages and disease establishment. Toxin produced during the preliminary stages of disease could serve as a protective measure for *B. anthracis* against resident

macrophages so as to allow maximal germination and growth prior to the spread of vegetative bacilli. This is based on the contention that the events surrounding spore uptake, germination, commencement of vegetative growth, and escape from host macrophages likely occur over time rather than simultaneously. As a result, a protective measure would be required after the initial release of vegetative bacilli into the extracellular environment so as to allow continued germination and vegetative growth of *B. anthracis* still contained in host macrophages. One such protective measure is supported by the toxin-induced resistance (TIR) phenomenon in which transient resistance to high dose treatment of LeTx is induced in macrophages that have been pretreated with a non-lethal dose of LeTx (Salles, Tucker et al. 2003). TIR could represent a mechanism to ensure that toxin produced by vegetative bacilli first released into the extracellular environment does not disrupt surrounding macrophages prior to completion of germination and growth. This in turn, would allow the bacilli to achieve maximal growth before engaging the host's immune system. Although the role of anthrax toxin at the early stage of disease is still speculative, there is sufficient evidence to suggest that *B. anthracis* is uniquely suited to interact with macrophages such that optimal growth and persistence of disease is achieved.

In spite of the evidence described above, the exact triggers for germination along with the host cell types involved during the establishment of disease have yet to be determined. Recent reports dispute the role of macrophages based on evidence showing that germination of *B. anthracis* spores can occur independently of host cells, in an *in vivo* model (Cote, Rossi et al. 2005). Additionally, it has been

reported that mice depleted of macrophages prior to infection with *B. anthracis* spores are more susceptible than mice with intact macrophages (Cote, Rea et al. 2004). The authors contend that although macrophages are involved in the disease process with regards to mediating the host's defense mechanisms, these cells may not be uniquely suited for providing the necessary triggers for germination and outgrowth.

Macrophages & Other Cell Types Implicated in LeTx Sensitivity

Aside from the proposed role of macrophages during disease establishment, there is also evidence suggesting that these cells govern host sensitivity to LeTx. The link between LeTx and macrophages was first made when it was shown that these cells were uniquely sensitive to the toxin compared to a variety of other cell lines tested (Friedlander 1986). In this case, it was shown that macrophages began to lyse 2 h after treatment with LeTx. Additionally, it was shown that the resistance of certain mice strains to LeTx-induced lethality correlated with the resistance of their macrophages to the toxin (Welkos, Keener et al. 1986). Later, it was reported that mice that had been depleted of their macrophages via silica injections had a 100% survival rate following challenge with LeTx. Furthermore, sensitivity to LeTx was restored in silica-treated mice by co-injection of cultured macrophages (Hanna, Acosta et al. 1993). Taken together, these findings suggest that macrophages play a pivotal role in anthrax pathogenesis with regards to their interaction with LeTx. However, the link between LeTx-induced cytotoxicity in these cells and LeTx-mediated death of the host remains to be determined.

Initially it was thought that death of the host resulted from cytokine-induced shock. This contention was derived from a study in which macrophages treated with sublytic doses of LeTx released interleukin-1 β and Tumor necrosis factor (TNF-α) (Hanna, Acosta et al. 1993). Furthermore, it was shown that treatment of mice with anti-IL-1 β and anti-TNF-α sera provided protection against the toxin (Hanna, Acosta et al. 1993). Hence, it was proposed that LeTx-treated macrophages experienced a build up of cytokines that were then released as these

cells began to lyse. This, in turn, caused shock-like death in the host. However, reports have since shown that LeTx treatment actually suppresses cytokine production in macrophages (Erwin, DaSilva et al. 2001). Additionally, it has been reported that TNF-α and iNOS (inducible nitric oxide synthase) knockout mice are not protected from treatment with *B. anthracis*, thereby discounting the impact of cytokines on lethality (Kalns, Scruggs et al. 2002). Finally, a recent study examined cytokine production and histopathology in mice following intravenous injection of LeTx. This report indicated that death was not due to cytokine-induced shock but instead resulted from hypoxia-induced liver failure (Moayeri, Haines et al. 2003). It should be noted that the discrepancies in the above findings could be attributed to the fact that each group used differing amounts of toxin in their studies. Based on the reported stoichiometry of anthrax toxin, differing amounts of the toxin subunits impacts the overall effective dose of LeTx, which, in turn, would have a dramatic impact on the observed phenotypes. Regardless, the conflicting reports on LeTx-mediated death necessitate a better understanding of the response of macrophages to the toxin and the significance of this interaction during disease.

Current knowledge regarding the cellular impact of LeTx does not support the contention that macrophages are solely responsible for mediating cytotoxicity. It has been shown that proteolytic cleavage of MEKs, the primary cellular targets of LF, occurs in both sensitive and resistant macrophages (Watters, Dewar et al. 2001). Additionally, recent evidence discounts a direct correlation between the sensitivity of animals to LeTx and that of their macrophages**.** In fact, it has been

shown that different strains of mice are susceptible to treatment with LeTx regardless of whether they harbor sensitive or resistant macrophages (Cui, Moayeri et al. 2004). Furthermore, some resistant macrophage lines are derived from species, including rats and humans, sensitive to both LeTx and anthrax (Popov, Villasmil et al. 2002; Kim, Jing et al. 2003). Overall, this evidence does not disregard the involvement of macrophages in anthrax disease but it has prompted further investigation to elucidate the cellular impact of LeTx.

In spite of the unique sensitivity demonstrated by macrophages, recent studies have examined the impact of LeTx on non-macrophage cell lines. One report of this type describes the treatment of dendritic cells with LeTx; the result of which prevented their interaction with T cells. Normally, the primary function of dendritic cells is to stimulate cellular immunity, thereby initiating an inflammatory response, antibody production, and differentiation of B and T cells into memory cells (Mourez 2004). Although it is not known if dendritic cells actually have a role in controlling the progression of anthrax disease, this evidence implicates an additional mechanism, separate from interacting with macrophages, of LeTx directed at down-regulating the host's immune response.

It is plausible that additional cell types are susceptible to LeTx due to the fact that the only known cellular target of LF is central to the physiology of a variety of cells and that the receptors for anthrax toxin are ubiquitously expressed (Mourez 2004). Thus, investigators have begun to examine the impact of LeTx on cells types that are not strictly related to the immune response. In line with this, it has

recently been reported that LeTx is cytotoxic to endothelial cells (Kirby 2004). Specifically, LeTx induced caspase-dependent apoptosis in endothelial cells, which also revealed evidence of MEK cleavage and subsequent inhibition of phosphorylation of ERK, p38, and JNK. Based on these findings, it has been proposed that LeTx-mediated cytotoxicity in endothelial cells contributes to the vascular damage, including tissue hemorrhages and gastrointestinal bleeding, that has been reported in clinical cases of systemic anthrax (Kirby 2004). Collectively, these studies demonstrate the ability of this toxin to impact a variety of cell types. However, this does not discount the significance of host macrophages in mediating the disease process.

Wnt Signaling and GSK-3β-related Activities

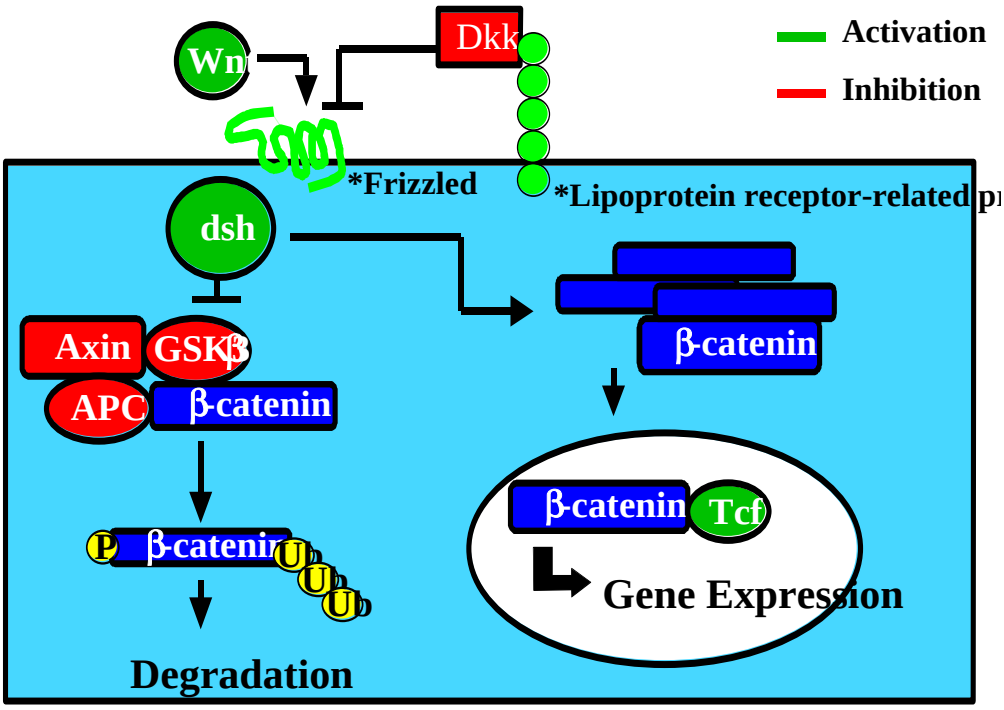
In examining the effects of LeTx on RAW 264.7 macrophages, my preliminary data revealed changes in GSK-3β and its related activities. These changes prompted further investigation as to whether or not the activity of this protein contributed to LeTx sensitivity. Hence, this protein became a focal point for my research; data derived from those studies is presented in the following sections.

Glycogen synthase kinase 3 (GSK-3) was first associated with insulin-related signaling based on its ability to phosphorylate and inhibit glycogen synthase (Ding, Chen et al. 2000). It is a serine/threonine kinase that recognizes a specific target sequence contained not only by glycogen synthase but many other proteins including β-catenin. GSK-3 has two isoforms, alpha and beta, and is highly conserved among mammalian cell types. In addition to controlling the cellular

response to insulin, GSK-3 has been implicated in a variety of biological processes including the phosphorylation of microtubule-associated proteins. This, in turn, links GSK-3 to microtubule polymerization and stability (Wang, Liu et al. 2003). Additionally, GSK-3β is well known for its role in Wnt signaling, acting as a negative regulator of this pathway.

Wnt signaling is best known for its role in embryonic development and for its association with certain cancers. The Wnt pathway is critical for regulating growth and cell fate during early stages of development (Cadigan and Nusse 1997; Willert and Nusse 1998). In adults, improper activation of this pathway resulting from deregulation of the essential mediator, β-catenin, has been linked to certain cancers (Polakis 2000). In either case, activation of the Wnt pathway leads to accumulation of cytosolic β-catenin via inhibition of GSK-3β. This, in turn, allows β-catenin to translocate to the nucleus where it initiates transcription of *wnt* target genes (Schneider, Finnerty et al. 2003). However, in the absence of Wnt activators, GSK-3β acts as a member of a multiprotein complex, including Axin and adenomatous polyposis coli (APC), to inhibit this pathway by preventing the accumulation of β-catenin and its subsequent translocation to the nucleus. GSK-3β achieves this by phosphorylating β-catenin, which targets the protein for ubiquitination and subsequent degradation via the proteasome (Aberle, Bauer et al. 1997). As a result, GSK-3β-mediated phosphorylation effectively blocks β-catenin-mediated transcription. In addition to its role in Wnt signaling, β-catenin is involved in cadherin-mediated cell-cell adhesion. In either case, the

activity of this protein is dependent on its phosphorylation state. Unphosphorylated β-catenin accumulates in the cytosol at which point it either translocates to the nucleus to initiate *wnt*-related gene expression, or it binds to cadherins at the plasma membrane (Giarre, Semenov et al. 1998). An overview of the Wnt pathway is illustrated in Figure 5.



(Nelson and Nusse 2004)

Fig. 5. **Overview of Wnt Signaling Pathway** Proteins involved in the activation of this pathway are highlighted in green and together, their activity results in the accumulation of cytosolic β-catenin. In this case, β-catenin translocates to the nucleus where it activates transcription. In contrast, proteins involved in the inhibition of this pathway are depicted in red. Their activity prevents the accumulation of β-catenin and subsequent activation of transcription. This is achieved by tagging β-catenin for proteasome-mediated destruction.

It has been shown that differential regulation of GSK-3β occurs according to the signaling pathway involved, with regards to insulin and Wnt serving as the extracellular signals (Ding, Chen et al. 2000). In this case, it was reported that although both insulin and Wnt triggered a decrease in GSK-3β activity, inhibition by each signal led to different downstream events. Specifically, Ding et al. reported that insulin did not result in the accumulation of cytosolic β-catenin and that Wnt did not increase glycogen synthase activity. Hence, it was proposed regulation of GSK-3β differs among the signaling pathways involved (in this case, insulin and Wnt). Additional studies revealed that GSK-3β activity can be artificially inhibited by LiCl, and that this inhibition mimics Wnt signaling (Stambolic, Ruel et al. 1996). For our purposes, we used LiCl to implicate the involvement of GSK-3β in governing susceptibility to LeTx. Data derived from those studies is presented in the following sections.

Kinesins

Kinesins were first associated with anthrax LeTx when it was reported that macrophage sensitivity is linked to single nucleotide polymorphisms in the gene encoding for the kinesin-like motor protein Kif1C (Watters, Dewar et al. 2001). Kif1C is also known to localize to the Golgi apparatus and is important for trafficking cargo between this site and the endoplasmic reticulum. In the same study, this group showed that disruption of the Golgi apparatus with brefeldin A resulted in susceptibility to LeTx in otherwise resistant cells, suggesting localization of Kif1C is important for resistance to the toxin.

Our analysis of LeTx-treated macrophages revealed changes in kinesin-related proteins. Due to the findings of Watters et al., these changes were of immediate interest and prompted further investigation. As described in following sections, our data revealed a distinction in the expression of a particular kinesin that could be related to differences in LeTx sensitivity.

Kinesins comprise one of two major groups of motor proteins that mediate microtubule-based intracellular transport. These motor proteins use ATP hydrolysis to drive movement along microtubules as they transport various cargoes, including vesicles and organelles, throughout the cell. Although kinesins were discovered two decades after the other major group of motor proteins, members of the dynein family, it has since been shown that they serve as the major molecular motors (Nakajima, Takei et al. 2002). A total of 45 kinesins have been identified in mice (Miki, Setou et al. 2001), each of which is comprised of a globular head domain, which mediates microtubule binding and ATP hydrolysis, and a tail domain, which mediates cargo specificity. Although the head domain is highly conserved, there is great variation in the tail domain which, reflecting broad specificity for various cargoes (Nakajima, Takei et al. 2002).

RATIONALE

One of the major virulence factors produced by *B. anthracis* is a tripartite toxin. This toxin acts in binary combination as edema toxin or lethal toxin to cause edema and lethality in the host. Although both toxins are required for full virulence, preferential focus has been given to LeTx based on its ability to cause death in animal models and because of its reported toxicity in mouse macrophages. However, the mechanism by which LeTx elicits these effects is yet to be fully determined.

LF is a zinc-dependent metalloprotease, which impacts MAPK signaling. In fact, MAPKKs are the only known cellular target of LF to date. However, inhibition of MAPK signaling alone does not cause cell death, as shown by studies that used a chemical inhibitor of MEK, PD 98059. Additionally, LF-mediated cleavage of MAPKKs has been shown to occur in a variety of cell types yet not all cells are sensitive to LeTx. Thus, although MAPKK cleavage is associated with the cellular activity of LF, events separate from this lead to cell death.

In this study, we sought to identify unique events that occur in LeTx-sensitive macrophages in response to the toxin. Specifically, we wanted to identify changes in macrophage physiology that occur separately or downstream of MAPKK cleavage. In doing so, we hoped to identify distinguishing factors that

contribute to the death of these cells and account for differences in LeTx sensitivity among various cell types.

MATERIALS AND METHODS

Cell culture and viability assay

Cell culture media and additives were purchased from Invitrogen. The cell lines RAW 264.7 (BALB/c mouse macrophage derived Abelson leukemia virus induced tumor macrophages) and IC-21 (C57BL/6 mouse macrophage derived SV-40 transformed peritoneal macrophages) were obtained from the American Type Culture Collection and maintained in RP-10 medium supplemented with 10% fetal bovine serum. The cell lines were grown at 37°C in a humidified atmosphere of 6% CO_2.

Cell viability was determined by visual observation for cell rounding and quantified using the Cell Counting Kit-8 (CCK-8; Dojindo), which determines cell viability via detection of cell dehydrogenase reduction of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). Percent viability was determined using the formula 100* ($A450_{test}$- $A450_{background}$) / ($A450_{control}$- $A450_{background}$), wherein test= toxin-treated samples, control=buffer alone and background=untreated cells. Viability assays were performed in triplicate.

Purification of recombinant PA and LF

Recombinant forms of PA and LF were purified from *E. coli*/BL-21 (DE3) (Novagen) using His-tagged affinity chromatography according to the

manufacturer's protocol. Purified proteins were desalted, passed over a polymyxin B agarose column, and eluted according to manufacturer's instructions (Sigma). Purified proteins were assayed for endotoxin using the Limulus Amebocyte Lysate (LAL) kit supplied by Biowhittaker, which has a detection minimum of 0.03 endotoxin units/ml.

LeTx-treatment of RAW 264.7 macrophages for DNA array analysis

For mRNA profiling, RAW 264.7 cells were grown in 75 cm^2 tissue culture-treated flasks as a confluent monolayer (approximately 1 X 10^7 cells per flask) and treated with 2 μg/ml LF and 2 μg/ml PA in a volume of 10 ml. RNA and protein were isolated from cells 30 min and 90 min following treatment with LeTx. Control cells were treated with an equal volume of endotoxin-free 20 mM Tris, pH 8.0 and subjected to the same incubation times.

RNA isolation and cDNA probe synthesis

Total RNA was isolated using Trizol according to manufacturer's instruction (Invitrogen Life Technologies). After the initial extraction, RNA samples were subjected to treatment with DNase (1 U) for 30 min. This reaction was terminated by the addition of 1 ml of Trizol at which point the samples underwent a second round of extraction. The final RNA samples were solubilized in RNase-free water and immediately processed for cDNA synthesis. The yield of total RNA for each sample was determined spectrophotometrically (OD_{260}) and the quality of RNA was confirmed by analysis on a 0.6 % formaldehyde agarose gel. Approximately 25 μg of total RNA was used for the synthesis of each cDNA

probe. For each sample, an initial annealing reaction was carried out in a 23 µl volume wherein RNA was combined with 1 µl of dATP, 1 µl of dTTP, 1 µl of dGTP (10mM stocks), 2 µl primer mix (Clontech), 6 µl 5X First Strand Buffer (Invitrogen Life Technologies), and 2 µl RNase-free water (Ambion) and incubated at 90° C for 2 min and 42°C for 20 min. At the completion of the annealing reaction, 2 µl [^{33}P]dCTP (10 mCi/ml; New England Nuclear), 3 µl 0.1 M DTT, 1 µl SuperScript II reverse transcriptase (200 U/µl), and 1 µl (40 U/µl) Ribonuclease inhibitor (Invitrogen Life Technologies) was added to the reaction and the sample incubated an additional 2 h at 42°C. Finally, each probe reaction was incubated at 94°C for 5 min in the presence of 5 µl 10 X denaturation solution (1 M NaOH, 10 mM EDTA) and 50 µl 2 X neutralization solution (1 M NaH_2PO_4, pH 7.0). NucAway spin columns (Ambion) were used according to manufacturer's instructions to remove unincorporated nucleotides.

Hybridization, image captures and data analysis

Each Mouse Atlas™ 1.2 expression array underwent a prehybridization reaction by incubating with 70 µl heat-denatured salmon sperm DNA (10.0 mg/ml) and 5.0 ml ExpressHyb solution (Clontech) at 68°C for 2.0 h. The purified cDNA probe was then hybridized to the Atlas™ array membrane at 68°C for 18 h. Following hybridization, membranes were washed 2 times with 50.0 ml 2 X SSC, 1.0% SDS for 20 min, followed by 2 washes with 50.0 ml 0.1X SSC, 0.5% SDS for 20 min. The arrays were then exposed to a storage phosphor screen (Molecular Dynamics-Amersham Pharmacia Biotech) for ~48 h. Images were

acquired by scanning on a Storm phosphorimager (Molecular Dynamics-Amersham Pharmacia Biotech) and intensities were quantified with ArrayVision software (Incyte Genomics, Inc.). Data analysis was performed using an associative analysis approach as previously described by Dozmorov and Centola (Dozmorov and Centola 2003).

Analysis of MEK-2 and Raf-1

Protein extracts were taken from the phenolic phase of a Trizol extraction (Invitrogen Life Technologies) of RAW 264.7, IC-21, and NIH-3T3 cells treated with LeTx or control as described in the above section. The protein samples were solubilized with 1% SDS and the relative concentrations were determined by the Bronsted-Lowry method (Lowry, Rosebrough et al. 1951). For each sample subjected to analysis of MEK-2 or Raf-1, 10 μg of protein was separated by SDS-PAGE and then electro-transferred to a polyvinylidene difluoride (PVDF) membrane. Immunoblots were subjected to incubation with primary antibody (sc-524; Santa Cruz Biotechnology) (1:500 dilution) targeting the amino-terminus of MEK-2 or primary antibody against Raf-1 (sc-7198; Santa Cruz Biotech) (1:1000 dilution). Primary antibodies were detected with the corresponding horseradish peroxidase-linked secondary antibodies. Blots were developed with the ECL chemiluminescence detection system according to the manufacturer's protocol (Amersham Pharmacia Biotech).

Zebrafish maintenance and LeTx treatment

Zebrafish (*Danio rerio*) were obtained from a local retailer and maintained at 28.5°C on a 14 h light/10 h dark cycle. Embryos were collected and maintained at 28.5°C and the stage of development was determined by morphology and reported as hours post fertilization (hpf). Fish embryos were placed in a 24-well plate with ten fish/ml/well and incubated with 20 μg/ml LF and 20 μg/ml PA and were observed for seven days post-treatment for morphological changes.

Inactivation of GSK-3β

To implicate GSK-3β signaling in LeTx-mediated cytotoxicity, RAW and IC-21 macrophages were treated with 20 mM LiCl for 2 h. Cells were subsequently treated with a range of LF (1.0 μg/ml to 0.005 ng/ml) plus PA (2.0 μg/ml) or 20 mM Tris as a buffer control. Cell viability, as determined by CCK-8 assay, was compared between LeTx-treated cells in the presence and absence of LiCl verses the corresponding Tris buffer controls.

Analysis of GSK-3β

As described previously for MEK-2 and Raf-1 analysis, protein extracts were taken from the phenolic phase of a Trizol extraction (Invitrogen Life Technologies) of RAW 264.7 cells treated with LeTx or control. Samples were taken from NIH-3T3 and IC-21 cells 1.5 h, 3 h, 6 h, 12 h, and 24 h following treatment with LeTx or control as indicated for RAW 264.7 cells. Additionally, samples were collected from "TIR" and "non-TIR" RAW 264.7 cells following

treatment with low dose LeTx or treatment with both low and high dose LeTx 1 h, 3 h, 6 h, 10 h, 24 h, and 48 h. The protein samples were solubilized with 1% SDS and the relative concentrations were determined by the Bronsted-Lowry method (Lowry, Rosebrough et al. 1951). For each sample subjected to analysis of GSK-3β, 10 μg of protein was separated by SDS-PAGE and then electro-transferred to a polyvinylidene difluoride (PVDF) membrane. Immunoblots were subjected to incubation with primary antibody specific for the carboxy-terminus of GSK-3β (#361528; Calbiochem) (1:1000 dilution). Primary antibody was detected with the corresponding horseradish peroxidase-linked secondary antibody. Blots were developed with the ECL chemiluminescence detection system according to the manufacturer's protocol (Amersham Pharmacia Biotech). Relative levels of GSK-3β were determined by densitometry using NIH Image V. 1.62 software.

In vitro stability of GSK-3β

The ability of LF to mediate direct cleavage of GSK-3β was analyzed using purified purified GSK-3β (Upstate) in combination with LF. Cleavage of GSK-3β was examined by incubating 2.5 μg of GSK-3β with 1μg of LF or Tris buffer control at 37 °C for 2 h and resolving with SDS-PAGE.

Comparative analysis of LF-mediated cytotoxicity and loss of GSK-3β

RAW 264.7 macrophages were grown in 25 cm^2 tissue culture-treated flasks as a confluent monolayer and treated with 2 μg/ml LF and PA, in the presence and absence of lactacystin, in a volume of 5 ml. Samples subjected to pretreatment

with 10 μM lactacystin (Calbiochem) were incubated for 30 min at 37°C prior to treatment with LeTx. Supernatant was collected from each flask (100 μl per flask) at designated time-points and used in a standard CytoTox One assay (Promega), performed according to manufacturer's instructions. After collecting supernatant to use in the cytotoxicity assay, cell extracts were collected from each flask in order to determine the relative level of GSK-3β at the designated time-points. To do so, the existing media was removed and cells were rinsed briefly with PBS. Lysis buffer (1% SDS, 50 mM Tris-Cl, 5 mM EDTA, pH 7.4) was added (2 ml per flask) and the flasks were then incubated on ice for 10 min. The lysates were then passed through a syringe 5 times prior to centrifugation at 10,000 x g for 10 min in order to pellet cell debris. The resultant supernatant was collected from each sample and relative amounts of protein were determined with the *DC* Protein Assay (Bio Rad), performed according to manufacturer's instructions.

Relative levels of GSK-3β and GAPDH were analyzed throughout the time-course. For each time-point, 10 μg of protein was separated by SDS-PAGE and then electro-transferred to a polyvinylidene difluoride (PVDF) membrane. Immunoblots were subjected to incubation with primary antibody (#9332; Cell Signaling) (1:50 dilution) that detects total levels of endogenous GSK-3β or with primary antibody against GAPDH (Advanced ImmunoChemical) (1:400 dilution). Primary antibodies were detected with the corresponding horseradish peroxidase-linked secondary antibodies. Blots were developed with the ECL chemiluminescence detection system according to the manufacturer's protocol

(Amersham Pharmacia Biotech). Densitometry analysis was performed in order to assess the relative levels of GSK-3β and GAPDH using a FluorChem 8900 imager (Alpha Innotech) and the accompanying software. The program assigned an integrated density value (IDV) for each sample based on pixel intensity. The IDV values obtained from that analysis were then plotted against percent cytotoxicity for each time-point.

To determine whether or not the observed loss of GSK-3β was linked to LF's inhibition of MAPK signaling, RAW macrophages were treated with PD 98059, a chemical inhibitor of MEK, or LeTx. Specifically, cells were treated with 20 μM PD 98059 (Calbiochem) or 2 μg/ml LeTx in a volume of 5 ml. Cell extracts were collected 30 min, 60 min, 90 min, 120 min, and 150 min post-treatment according to the method described above. Protein concentration was determined for each extract using the *DC* assay described previously and relative levels of GSK-3β were then examined in response to each treatment. Immunoblot analysis was performed as described above, probing each extract with a primary antibody for GSK-3β (# 361528; Calbiochem) (1:1000 dilution) in the presence and absence of GSK-3β blocking peptide (# 361529; Calbiochem).

Immunostaining for GSK-3β and β-catenin

RAW 264.7 macrophages were grown on coverslips in 12-well plates. Cells were treated with 2 μg/ml LeTx in the presence and absence of lactacystin. Controls were subjected to a 30 min pretreatment with 10 μM lactacystin (Sigma) at 37 °C

and existing media was replaced with fresh RP-10 prior to LeTx treatment. Cells were then fixed 0.5 h, 1 h, 1.5 h, 2 h, and 2.5 h following treatment with LeTx according to the company protocol supplied with the primary antibodies. Briefly, existing media was aspirated from each well and cells were fixed with 4% paraformaldehyde by incubating 15 min at room temperature. Cells were then wash three times with PBS and incubated for 5 min at room temperature per wash. Cells were then incubated at –20 °C in 100% methanol for 10 min. Cells were subjected to a final series of washes with PBS and incubated three times for 5 min at room temperature. The cells were then subjected to a blocking step prior to immunolabeling in which the cells were incubated at 37 °C for 2.5 h in blocking buffer containing 1% BSA, 1X TBS, 5% goat serum. Cells were then washed three times and incubated for 5 min at room temperature in blocking buffer. Primary antibody corresponding to GSK-3β (Cell Signaling) or phospho-β-catenin (Cell Signaling) was added at a 1:25 dilution in blocking buffer and cells were incubated overnight at 4 °C. Cells were then washed with blocking buffer for a total of three times as described above. Cells were then incubated at 37 °C for 2.5 h with corresponding rhodamine-conjugated secondary antibodies (2 μg/ml) (Molecular Probes) prepared in blocking buffer. Cells were washed with blocking buffer as described previously and subjected to an additional wash with PBS. Cells were then stained with 300 nM 4’6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes) for 5 min at room temperature. Cells underwent a final series of washes with PBS before mounting the coverslips with ProLong Anti-fade medium (Molecular Probes). IC-21 macrophages were subjected to the same staining protocol to serve as untreated controls. Samples

were then viewed with an Olympus BX61 epifluorescence microscope using an UPLAN-APO 60X oil immersion lens and analyzed using Spot RT software (version 3.5).

Semi-Quantitative Real-Time PCR

Real-time PCR was performed on total RNA extracted from LeTx-treated and mock-treated macrophages. Three primer sets were designed for Kif1C using Primer 3 software (Rozen and Skaletsky 2000). All primers were 21 bases in length. Pilot experiments showed optimal reaction with the primer set covering a region starting at nucleotide 732 and ending at nucleotide 832 in the open reading frame of *kif1C* and this pair was used throughout the real-time PCR analysis. A similar approach was used for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference sample as well and optimal primers were used covering a region starting at nucleotide 764 and ending at nucleotide 872. In each reaction, 500 ng of total RNA was reverse transcribed using the TaqMan reverse transcription kit (Applied Biosystems) according to manufacturer's instructions. Product from the reverse transcription was used as template in a subsequent real-time PCR. Each 25 µl reaction contained 400 nM primer, 0.5 ng of template, and 12.5 µl 2 X SYBR-green PCR master mix (Applied Biosystems) which includes dNTPs and AmpliTaq gold polymerase. The cycle protocol for real-time PCR was as follows: 1 cycle at 95° C for 10 min; 40 cycles of 95° C for 15 s, 48°C for 30 s, 60° C for 30 s, and a final cycle of 60° C for 10 min. These real-time PCR reactions and detection of accumulated product were performed in a Cepheid Smart-Cycler (Cepheid) and data was analyzed using the accompanying software.

Relative levels of expression were determined based on calculations involving the critical threshold (C_T) value of each sample, which was considered to be the point of greatest change in SYBR-green fluorescence along the curve. Calculations of fold changes in mRNA levels were made using the C_T value in a standard curve approach according to manufacturer's instructions (Applied Biosystems). All samples and reactions were performed in quadruplicate.

Analysis of tubulin

Refer to previous sections regarding the analysis of MEK-2, Raf-1, and GSK-3β for method of cell treatment and protein extraction. The protein samples were solubilized with 1% SDS and the relative concentrations were determined by the Bronsted-Lowry method (Lowry, Rosebrough et al. 1951). For each sample subjected to analysis of tubulin, 10 μg of protein was separated by SDS-PAGE and then electro-transferred to a polyvinylidene difluoride (PVDF) membrane. Immunoblots were subjected to incubation with primary antibody against alpha-tubulin (CP06; Oncogene research product) (1:10,000 dilution). Primary antibody was detected with the corresponding horseradish peroxidase-linked secondary antibody. The blot was developed with the ECL chemiluminescence detection system according to the manufacturer's protocol (Amersham Pharmacia Biotech). Relative levels of tubulin were determined by densitometry using NIH Image V. 1.62 software.

In vitro stability of tubulin

The ability of LF to mediate direct cleavage of tubulin was analyzed using purified tubulin from bovine brain (Molecular Probes) in combination with LF. Cleavage of tubulin by LF was assayed by incubating 5 μg of tubulin with 1 μg of LF or Tris buffer control at 37 °C for 2.5 h and resolving with SDS-PAGE.

Rate of PA-mediated toxin entry

The rate of cell entry of PA-mediated toxins was examined in RAW 264.6 macrophages, NIH-3T3 fibroblasts, HeLa endothelial cells, and IC-21 macrophages. Cells from each line were grown in a 96-well plate and treated with 2 μg/ml PA and 2 μg/ml LfnTcsL or LfnTcdB per well. Following toxin treatment, 50 nM bafilomycin A (Sigma) was added to each test well every 10 min for 2 h. The plates were incubated at 37 °C during the course of treatment and viability was then determined 24 h following toxin treatment. Viability was determined with CCK-8, as described previously, or according to cell rounding.

RESULTS

Immunoblot analysis of LF-mediated cleavage of MEK-2 in RAW 264.7 macrophages

The time-frame for LF-mediated cleavage of MEK in LeTx-sensitive macrophages was established with immunoblot analysis in which an antibody specific for the N-terminus of MEK-2 was used. MEK-2 is one of six MAPKKs cleaved by LF; the cleavage site for each is located at the N-terminus. Thus, the use of an antibody specific for the N-terminus allowed us to pinpoint the cleavage event during the course of intoxication in sensitive macrophages. This, in turn, established a point of reference that would allow us to examine specific cellular events that occurred prior to and subsequent to MEK cleavage after treatment with LeTx.

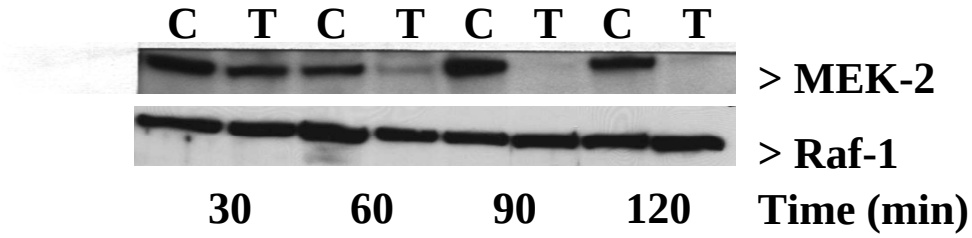


Fig. 6. **Immunoblot analysis of proteolytic cleavage of MEK-2 in LeTx-treated RAW 264.7 macrophages** To determine the time-course of LF-mediated cleavage of MEK-2, RAW 264.7 cells were treated with LeTx and extracts were collected at 30 min, 60 min, 90 min and 120 min time-points following treatment. Ten micrograms of extract was resolved by SDS-PAGE, transferred to PVDF membrane, and immunoblotted using primary antibody reactive to the amino-terminus of MEK-2. Time-points and samples are labeled within the figure. C= control, mock-treated samples; T= LeTx-treated samples. Immunoblot detection of Raf-1 was included to normalize for gel loading and variations in protein abudance.

mRNA profiles in LeTx-treated RAW 264.7 macrophages

DNA array profiling can be used to gain insight about cellular activity as it relates to changes in gene expression. In the case of analyzing toxin-treated cells, such a profile may reveal disregulation of genes controlled by a particular signaling pathway, thus providing clues to toxin activity for further investigation. For these reasons, we analyzed mRNA changes in RAW 264.7 macrophages following intoxication by LeTx for 30 min or 90 min. As shown in Fig. 6, these are time-points at either 30 min prior to or 30 min following cleavage of MEK-2. Approximately 4 h following treatment with LeTx, these cells begin to lose viability and show signs of necrotic cell death (data not shown).

The Mouse Atlas™ 1.2 membrane array system, which contains 1,185 gene targets that can be grouped into 157 functional categories, was selected for the mRNA analysis. For each time-point, four independent experimental sets were generated for both toxin-treated and mock-treated samples (a total of 16 membranes). Total RNA was extracted from the treated cells, then reverse transcribed, and radiolabeled with [^{33}P]dCTP. Label incorporation routinely exceeded 90% efficiency. Following the labeling procedure, an equal amount (based on nucleic acid concentration) of probe was hybridized to the membrane arrays. Following this standard treatment, images were captured and spot intensity was quantified.

To identify differentially expressed genes, results from a standard paired T-test were compared to those from an associative analysis. A threshold of $p<0.005$ was

used to deem significance for both statistical analyses. Genes identified by both the standard paired T-test and associative analysis are likely real positives, those selected by the standard T-test only may contain false positives, and those selected by the associative analysis only are potentially true positives. Candidate genes expressed under only one condition, experimental or control, were not subjected to ratio analysis and were classified as increased or decreased expression. Of the 1185 genes, detectable changes in mRNA levels were found in 108 genes 30 min following toxin treatment. By 90 min, there were 83 differentially expressed genes in the intoxicated macrophages.

Putative signaling pathways targeted by LeTx

Interestingly, analysis of the DNA array data did not reveal a notable change in expression of genes regulated by ERK 1,2 signaling. Of the differentially expressed genes, approximately 15% could be linked to known regulation by ERK 1,2. A similar level of ERK 1,2-regulated genes was detected if a set of genes were selected at random from the array, indicating that toxin treatment did not dramatically change the expression of ERK 1,2-regulated genes. Furthermore, we found that treatment with PD 98059, an inhibitor of Raf /MEK interaction and ERK1,2 activation, was not toxic to either LeTx-sensitive or LeTx-resistant cells (data not shown). Finally, p38 and JNK signaling pathways were not active in either control or experimental conditions, as determined by phosphorylation profiles, indicating these LF-targeted pathways did not impact our analyses (data not shown).

These results suggested the inactivation of MAPKKs might not be the sole contributing factor to the changes in physiology of LeTx-treated cells under these experimental conditions. For this reason, the differentially expressed genes were further analyzed for other pathways possibly disrupted by LeTx. As summarized in Table 1, several components of the Wnt signaling pathway appeared to be impacted when RAW 264.7 cells were treated with LeTx. Along with alterations in components of the Wnt pathway and genes regulated by Wnt, we also detected changes in genes regulated by GSK-3β. GSK-3β controls levels of β-catenin by phosphorylation which targets β-catenin for ubiquitination and proteasome-mediated destruction. Activation of the Wnt pathway results in GSK-3β inactivation, via dishevelled protein, and subsequent accumulation of β–catenin in the nucleus where transcription is regulated. Thus, it seemed reasonable that the changes in Wnt signaling could be part of a compensatory response resulting from inactivation of GSK-3β.

TABLE 1. Genes differentially expressed in LeTx -treated RAW cells associated with Wnt signaling and GSK-3β.

Genes names Timepoint	Accession #	p Value[a]	p' Value[a]	Fold[b]

-Dishevelled, dsh homolog (Drosophila)^ 30 min	U10115		8.5065E-05	1.85
-Low density lipoprotein receptor^ 30 min	Z19521		0.00127	1.65
-Discs, large homolog 4 (Drosophila) ^ 30 min	D50621		0.00153	-1.64
-Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain^ 30 min	U65091	0.03887	4.75066E-18	+
-Matrix metalloproteinase 9 ◊ 30 min	X72795	0.02438	6.23703E-54	+
-Matrix metalloproteinase 14 ◊ 30 min	X83536	0.00977	2.40232E-36	+
-Fibroblast growth factor receptor 4 ◊ 30min	X59927	0.03683	2.61219E-18	+
-Dickkopf homolog 1 (Xenopus laevis)^ 30 min	AF030433		1.65271E-12	+
-Frizzled homolog 7 (Drosophila)^ 30 min	U43320		1.53642E-11	+
-Frizzled homolog 6 (Drosophila)^ 30 min	U43319		7.13486E-77	+
-cAMP responsive element binding protein ⟨ 30 min	M95106		2.109E-38	-
-Dishevelled 2, dsh homolog (Drosophila)^ 90 min	U2416		1.44327E-13	3.97
-Catenin (cadherin-associated protein), delta 2^ (neural plakophilin-related arm-repeat protein) 90 min	U90331		0.00011	2.04
-Eph receptor B4 ◊ 90 min	Z49085	0.00951	1.60623E-10	+
-Discs, large homolog 4 (Drosophila) ^ 90 min	D50621		0.00150	+
-Frizzled homolog 7 (Drosophila)^ 90 min	U43320		0.00179	+
-Matrix metalloproteinase 2 ◊ 90 min	M84324	0.00094	2.17779E-07	+
-CCAAT/enhancer binding protein (C/EBP), delta ⟨ 90 min	X61800	0.01733	3.10031E-16	-
-Dishevelled 3, dsh homolog (Drosophila)^ 90 min	U41285		1.12474E-07	-

[a] P and P' values represent levels of significance in the standard paired analysis and the associative analysis respectively.
[b] Fold refers to the difference in gene expression between control and experimental samples for genes expressed in both conditions (normalized value > 0.48, cf. Methods). Genes expressed only in one condition are represented by +(experimental) or -(control). Genes involved with Wnt signaling pathway are represented by ^. Genes characterized as direct targets of Wnt signaling are represented by ◊. Genes that are considered to be downstream targets of GSK-3β are represented by ⟨.

Effect of LeTx on zebrafish development

The role of GSK-3β signaling in macrophage physiology is poorly defined, and to our knowledge there are no reports of Wnt or GKS-3β pathways modulating macrophage activity. GSK-3β activity, via Wnt signaling, has been largely defined in embryonic development. To further address our hypothesis that LeTx is capable of modulating GSK-3β regulated activities, zebrafish embryos were treated with the toxin and analyzed for defects in development. Embryos were treated with the toxin at the very early four-cell stage of development. Following treatment, the embryos were examined for defects in embryo development and subsequent growth of the hatched fish. Control embryos were treated with PA alone, LF alone, and PA plus an enzymatically inactive mutant of LF (LF_{H686C}). Embryos were treated in triplicate in a 24-well plate (10 embryos/well), for a total of 30 embryos per sample. A dose range of LeTx consisting of 10 μg - 40 μg LF/well and a constant amount of PA at 40 μg/well was tested. Amounts of LF in excess of 20 μg were lethal to the embryos (data not shown). Yet, when embryos were treated with functional LeTx at the non-lethal dose, gross defects in development were identified. In contrast, control-treated fish developed with normal phenotypes. A representative embryo elaborating defects following toxin treatment is shown in Figure 7. Notably, embryos are slowed in development, demonstrate a delay in pigmentation, and show signs of cardiac hypertrophy when treated with LeTx. As can be seen in Fig. 7J, fish show a major malformation of the anterior ventral region. As will be discussed, each of these defects have been linked to Wnt activity in a variety of developmental models. Taken together these data further indicate, in a well-established model, that LeTx is capable of

interfering with Wnt related activities, which would occur as a result of the loss of GSK-3β.

QuickTime™ and a
TIFF (LZW) decompressor

Fig. 7. **LeTx treatment of embryonic zebrafish** Zebrafish embryos were treated with LeTx (20 μg/ml LF + 20 μg/ml PA) between the 4-16 cell stage of development. Control zebrafish were treated with an excess of PA (40 μg/ml). Lanes A-E, Control zebrafish; Lanes F-J, LeTx-treated zebrafish. Arrows indicate points of decreased pigmentation and cardiac hypertrophy following treatment with LeTx.

GSK-3β Signaling Pathway

We further analyzed the role of the GSK-3β signaling pathway by inactivating this protein with LiCl, as previously described by others (Stambolic, Ruel et al. 1996; Hedgepeth, Conrad et al. 1997), prior to treatment with LeTx.

Interestingly, as shown in Figure 8, pretreatment of cells with LiCl increases sensitivity to LeTx. RAW 264.7 macrophages pretreated with LiCl exhibited a 10-fold increase in sensitivity to LeTx. In light of these observations, we also determined if LiCl could convert LeTx-resistant cells to a sensitive phenotype. IC-21 macrophages, which are reported to be resistant to LeTx, were pretreated with LiCl and tested for sensitivity to the toxin. As shown in Figure 8, these cells became highly sensitive to LeTx following pretreatment with LiCl, further indicating that active GSK-3β promotes cell survival following treatment with LeTx. Finally, treatment with LiCl alone results in minimal cytotoxicity, further suggesting a synergy between loss of GSK-3β activity and other LeTx-related activities.

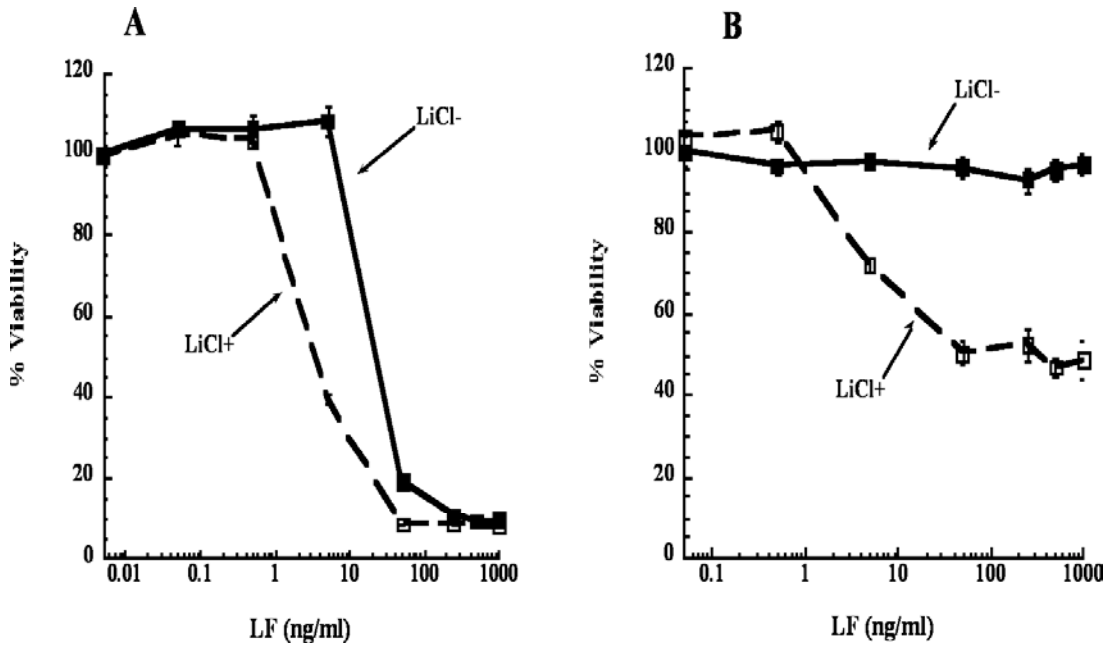
To determine if the enhanced sensitivity observed with LiCl was directly linked to toxin activity, we compared cell viability in response to treatment with PA, PA-LFn^{1-556} (a modified form of LF capable of entering cells but lacking enzymatic activity), and LeTx after pretreatment with LiCl. These conditions allowed us to determine whether or not enhanced cytotoxicity resulted from toxin binding or entry alone. No change in viability was detected after treatment with PA or PA-LFn^{1-556} in the presence or absence of LiCl (data not shown). Thus, the enhanced sensitivity can be attributed to the combined intracellular effects of LiCl and LeTx.

Finally, we examined whether the impact of LiCl on LeTx-sensitivity was cell-type specific. To do so, NIH-3T3 fibroblasts were subjected to the same

treatment conditions as the IC-21, resistant, macrophages. Unlike the IC-21 macrophages, pretreatment with LiCl did not render these cells susceptible to subsequent challenge with LeTx. No change in cell viability was detected in LeTx-treated NIH-3T3 cells in the presence or absence of LiCl. This data suggests that the impact of LiCl on LeTx-sensitivity is specific for macrophages.

Fig. 8. **Relative LeTx sensitivity in macrophages following treatment with LiCl** RAW 264.7 and IC-21 macrophages were pretreated with LiCl and subsequently challenged with LF ranging from 1.0 μg/ml to 0.005 ng/ml and a fixed amount of PA (2 μg/ml). Following a 15 h incubation with LeTx, cells were assayed for viability by CCK-8 staining. Panel A) Effects of pretreatment with LiCl on LeTx cytotoxicity in RAW macrophages; Panel B) Effects of pretreatment with LiCl on LeTx cytotoxicity in IC-21 macrophages. Solid line = pretreatment with buffer control; dashed line = pretreatment with 20 mM LiCl. Each assay was performed in triplicate and the error bar represents the standard deviation from the mean.

Fig. 8


A
B
% Viability
LF (ng/ml)
LiCl-
LiCl+
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000

In vivo and in vitro analysis of GSK-3β following treatment with LeTx

It was unclear as to whether LiCl pretreatment was potentiating a cytotoxic activity of LeTx or attenuating a protective response. Furthermore, array data suggested that genes regulated by GSK-3β activity, and unrelated to Wnt signaling, were also modulated in LeTx-treated cells (see Table 1). Additionally, immunoblot analysis of supernatant from LeTx-treated cells did not reveal any detectable Wnt-3A protein (data not shown), suggesting inactivation of GSK-3β was not due to increased Wnt expression, and may represent a more global change in GSK-3β. To address this possibility, lysates from LeTx-treated RAW 264.7 macrophages were immunoblotted with GSK-3β anti-serum. As shown in Figure 9A, cells treated with a cytotoxic dose of LeTx showed a marked reduction in the level of detectable GSK-3β. Furthermore, cells treated with a non-toxic dose of LeTx (5 ng/ml LF, plus 1 μg/ml PA) show an approximately 60% decrease in detectable GSK-3β. A modified form of GSK-3β was not detected in the lysates of toxin-treated cells, suggesting a near complete degradation of the protein rather than limited-site cleavage. This possibility was further confirmed by testing the ability of LF to directly cleave GSK-3β in an *in vitro* reaction. As shown in Figure 9B, co-incubation of LF and GSK-3β did not result in any detectable degradation of GSK-3β. Addition of cell lysates to this reaction did not trigger degradation of GSK-3β (data not shown). Finally, there was no detectable change in the levels of GSK-3β in LeTx-resistant NIH-3T3 fibroblasts, IC-21

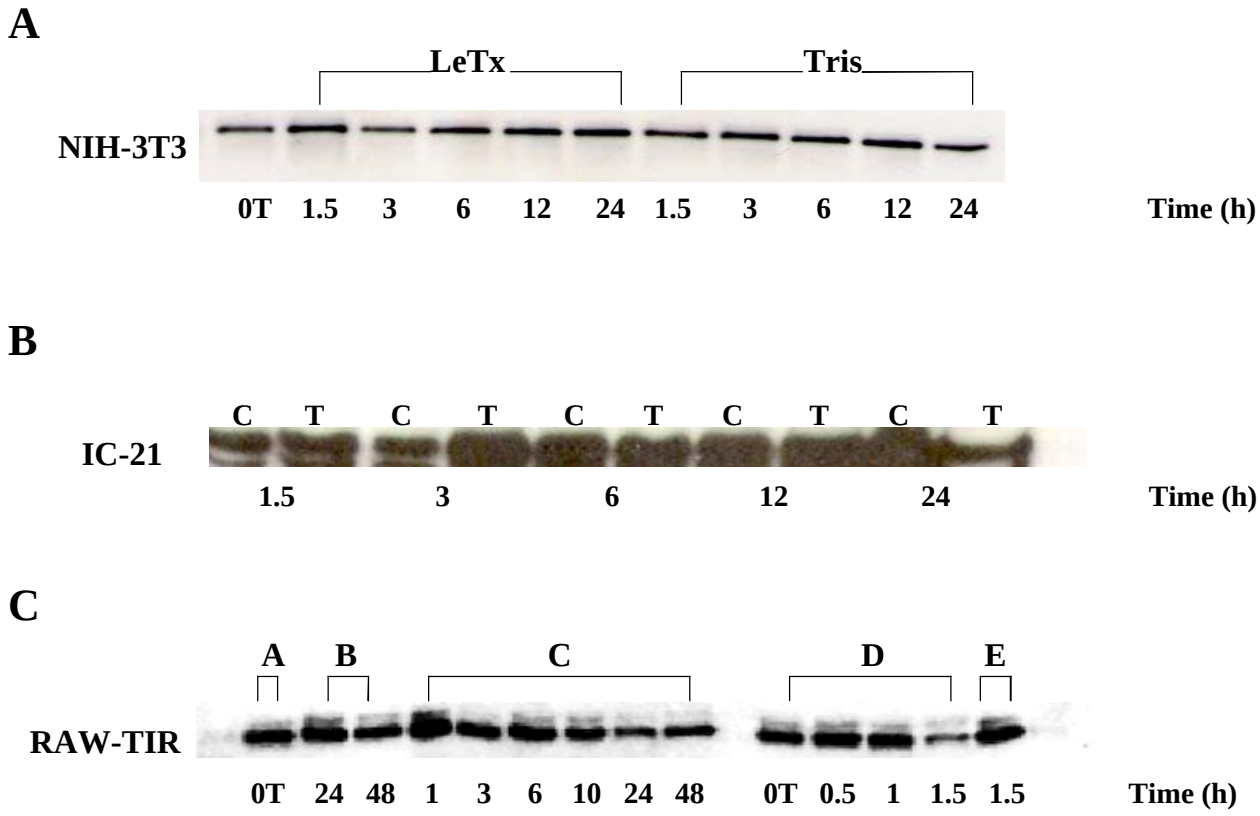
macrophages, or in RAW-TIR macrophages (cells pretreated with low-dose LeTx to confer resistance to subsequent challenge with a cytotoxic dose of LeTx). These data, shown in Figure 10, further suggest a correlation between decreases in GSK-3β and LeTx sensitivity.



Fig. 9. ***In vivo* and *in vitro* analysis of GSK-3β after treatment with LeTx** Panel A) Immunoblot of GSK-3β from cells treated with LeTx. Lysates were collected 2 h following treatment with LeTx, resolved by SDS-PAGE, and immunoblotted using GSK-3β-specific antibody. Lane 1, Buffer Control; Lane 2, 2 μg/ml LF; Lane 3, 5 ng/ml LF. PA was included at a constant amount of 2 μg/ml. The corresponding blots and conditions are shown within the figure. Panel B) *In vitro* stability of GSK-3β in the presence of LF. Purified GSK-3β (2.5 μg) was incubated with 1 μg of LF for 2 h at 37° C, resolved by SDS-PAGE, and stained with coomassie blue. C=GSK-3β; T=GSK-3β plus LF. Immunoblot detection of Raf-1 was included to normalize for gel loading and variations in protein abundance.

Fig. 10. **Immunoblot analysis of GSK-3β in LeTx-resistant cell lines** Panel A) Immunoblot of GSK-3β•from NIH-3T3 fibroblast cells treated with LeTx. Lysates were collected 1.5 h, 3 h, 6 h, 12 h, and 24 h following treatment with LeTx, resolved by SDS-PAGE, and immunoblotted using GSK-3β-specific antibody. The corresponding conditions are shown within the figure. Panel B) Immunoblot analysis of GSK-3β from IC-21 macrophages treated with LeTx. Lysates were collected and probed as indicated for the NIH-3T3 samples. Panel C) Immunoblot analysis of GSK-3β in RAW "TIR" macrophages versus "non-TIR" macrophages. "A" corresponds to untreated control; "B" corresponds to cells treated with low, TIR-inducing, dose LeTx; "C" corresponds to TIR samples that were first subjected to low dose treatment with LeTx followed by subsequent high dose LeTx; "D" corresponds to non-TIR samples; "E" corresponds to untreated control for non-TIR samples; the designated time-points refer to time (h) post-treatment with high dose LeTx.

Fig. 10


A
LeTx
Tris
NIH-3T3
0T 1.5 3 6 12 24 1.5 3 6 12 24
Time (h)
B
C T C T C T C T C T
IC-21
1.5 3 6 12 24
Time (h)
C
A B C D E
RAW-TIR
0T 24 48 1 3 6 10 24 48 0T 0.5 1 1.5 1.5
Time (h)

Loss of GSK-3β in LeTx-treated RAW 264.7 macrophages

To dismiss the possibility that the observed loss of GSK-3β in RAWs was merely due to the fact that cells were dying in response to the toxin, cytotoxicity was compared with levels of this protein throughout a time-course. Cells were treated with LeTx in the presence and absence of proteasome inhibitor, lactacystin. Pretreatment with lactacystin served to protect the cells from high dose treatment with LeTx and to prevent the subsequent loss of GSK-3β via proteasome-mediated destruction, thereby serving as a control for LeTx-treated RAWs. At designated time-points, cytotoxicity and GSK-3β levels were determined following treatment with LeTx in the presence and absence of lactacystin. Cytotoxicity was determined using the CytoTox One Kit which measures the level of lactate dehydrogenase released from damaged cells. As shown in Figure 11, a decline in the level of GSK-3β was first observed between 90 and 105 min post-treatment with LeTx. However, a significant increase in cytotoxicity was not observed until 120 min following treatment with LeTx; refer to Figure 11. Together, these findings suggest that the observed loss of GSK-3β is directly related to LF's activity within RAW macrophages and is not part of a general loss of protein that occurs as cells succumb to the toxin.

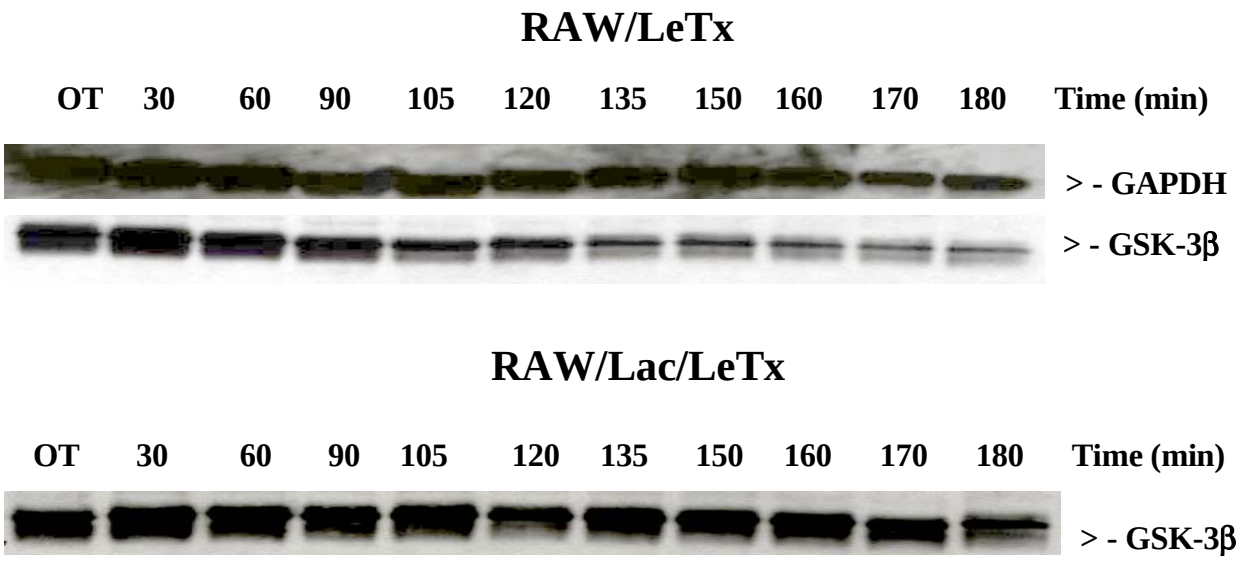
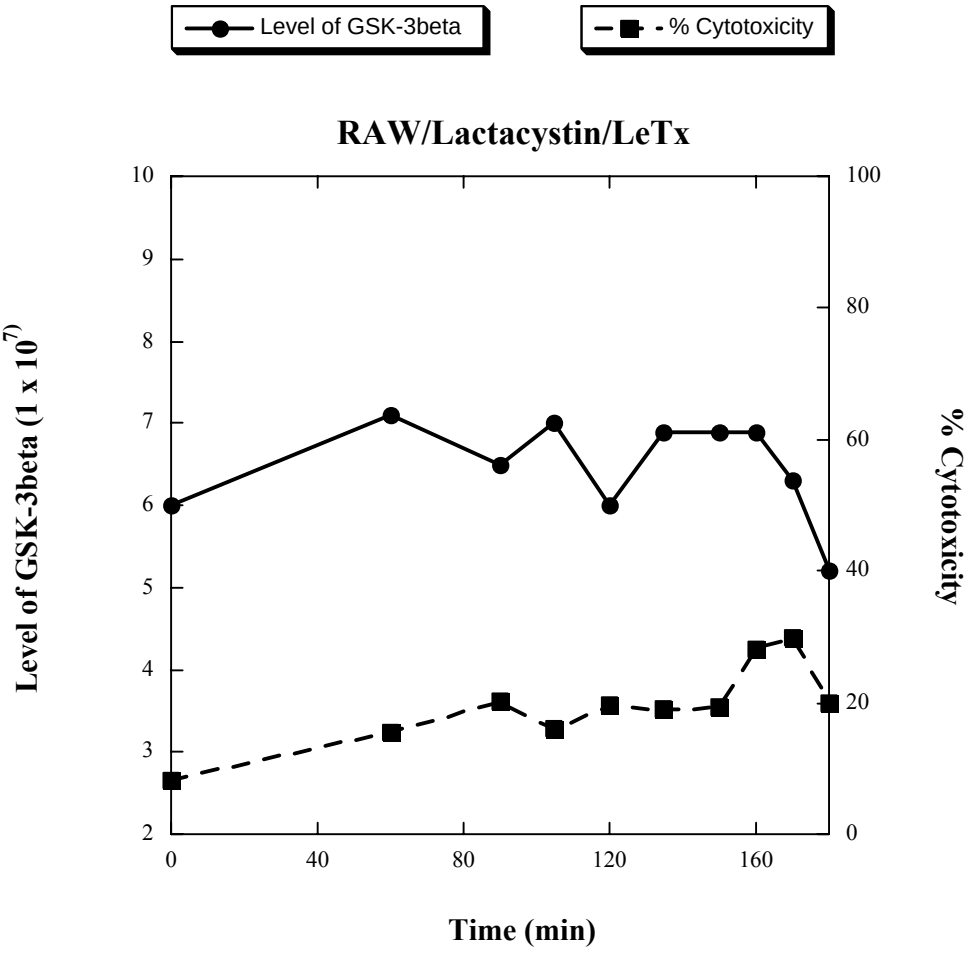


Fig. 11. **Immunoblot analysis of GSK-3β in LeTx-treated RAW 264.7 macrophages in the presence and absence of proteasome inhibitor** The level of GSK-3β was examined in LeTx-treated macrophages in the presence and absence of the proteasome inhibitor, lactacystin. Cells pretreated with proteasome inhibitor were incubated with 10 μM lactacystin for 30 min at 37°C prior to treatment with LeTx (2 μg/ml). Extracts were collected at 30 min, 60 min, 90 min, 105 min, 120 min, 135 min, 150 min, 160 min, 170min, and 180 min time-points following treatment with LeTx. Ten micrograms of extract was resolved by SDS-PAGE, transferred to PVDF membrane, and immunoblotted using primary antibody reactive to GSK-3β. Time-points and samples are labeled within the figure. Immunoblot detection of GAPDH was included to normalize for gel loading and variations in protein abudance.

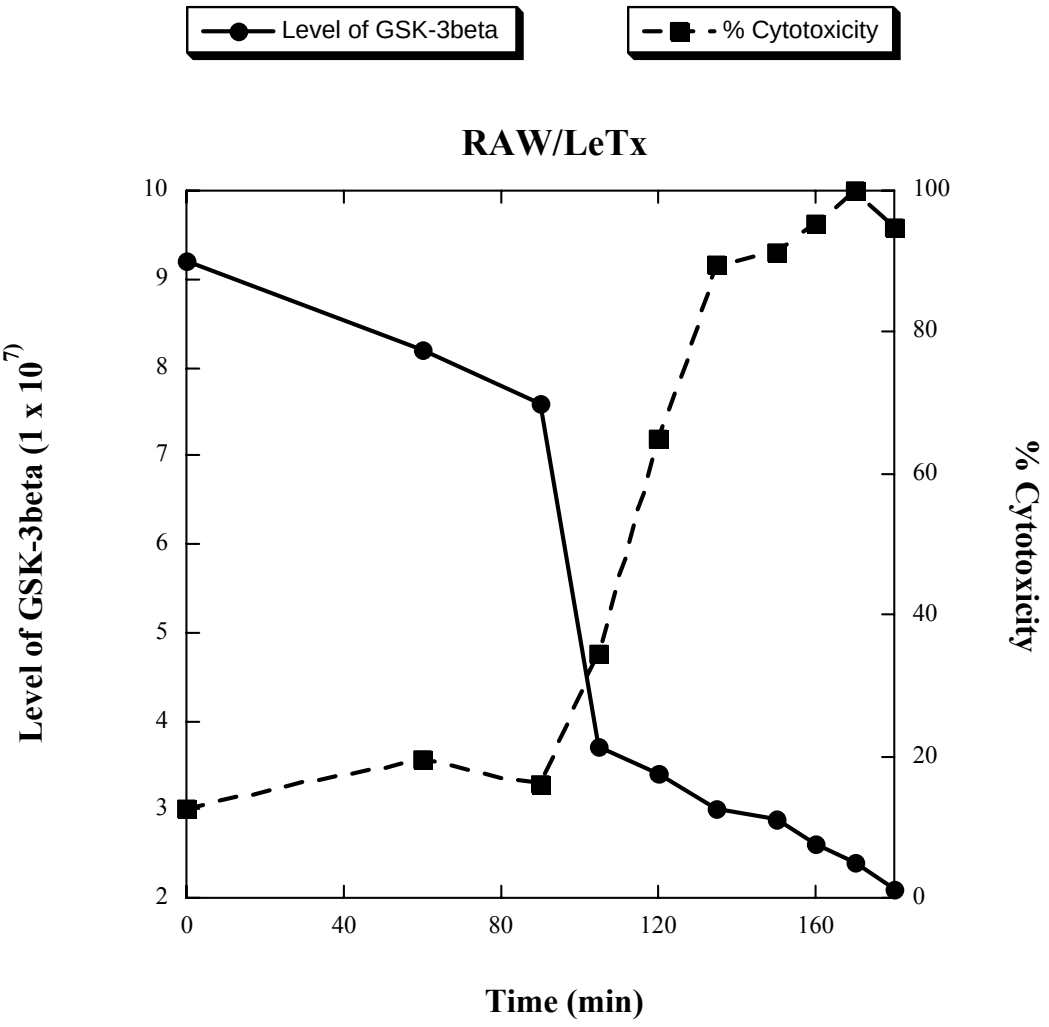
Fig. 12. **Levels of GSK-3β versus % cytotoxicity in LeTx-treated RAW 264.7 macrophages** A time-course was performed in which RAW cells were treated with 2 μg/ml LeTx for a total of 3 h. Within that time-frame, levels of GSK-3β and % cytotoxicity were determined at designated time-points. For each time-point, supernatant was collected and used to perform a CytoTox One assay to determine % cytotoxicity while cell extracts were collected to perform immunoblot analysis, shown in Figure 11. Relative band intensities were determined using FluorChem 8900 software which assigns a value for each sample based on pixel intensity. These values were then plotted against % cytotoxicity for each time-point in order to compare levels of GSK-3β and % cytotoxicity throughout the time-course. Panel A) Relative level of GSK-3β versus % cytotoxicity in LeTx-treated RAW 264.7 cells, pretreated with lactacystin. Panel B) Relative level of GSK-3β versus % cytotoxicity in LeTx-treated RAW 264.7 cells, in the absence of lactacystin. Panel C) Relative level of GAPDH versus % cytotoxicity in LeTx-treated RAW 264.7 cells. GAPDH was used as a control to show that levels of this protein remained constant in comparison to those of GSK-3β.

Fig. 12

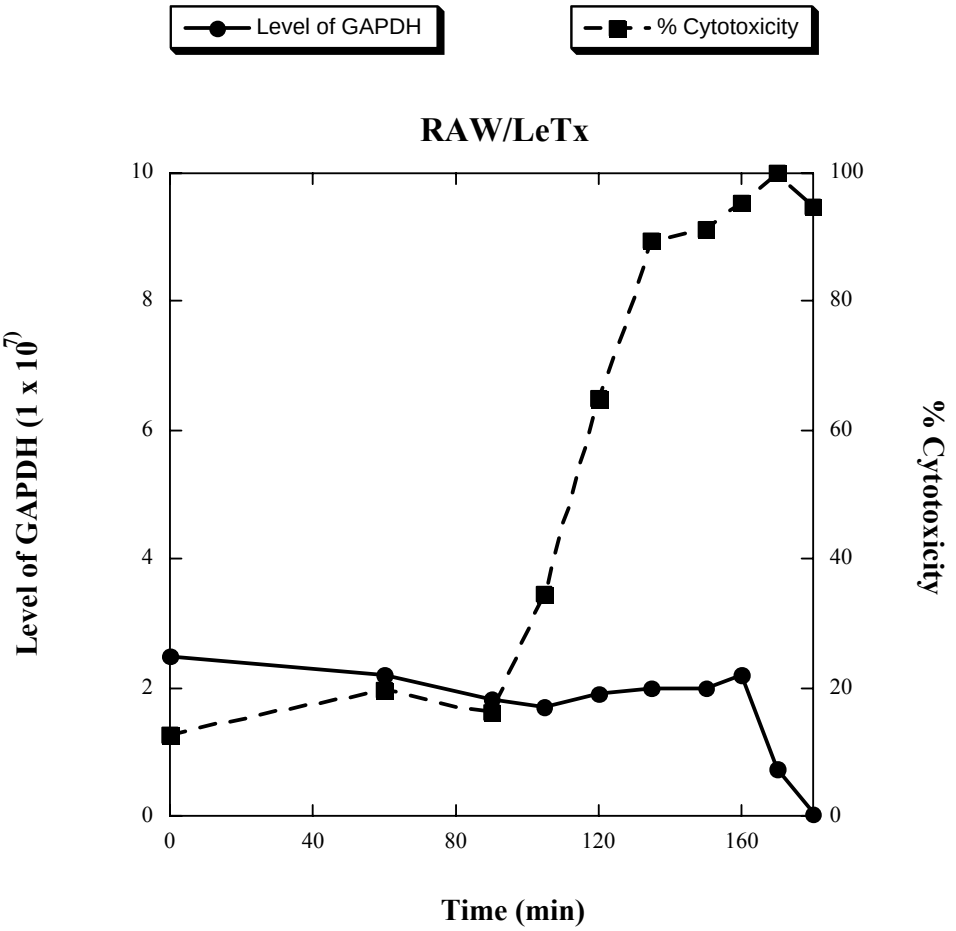
A


Level of GSK-3beta
% Cytotoxicity
RAW/Lactacystin/LeTx
Level of GSK-3beta (1 x 10^7)
% Cytotoxicity
Time (min)
10
9
8
7
6
5
4
3
2
100
80
60
40
20
0
0
40
80
120
160

B


Level of GSK-3beta
% Cytotoxicity
RAW/LeTx
Level of GSK-3beta (1 x 10^7)
% Cytotoxicity
Time (min)
10
9
8
7
6
5
4
3
2
100
80
60
40
20
0
0
40
80
120
160

C


Level of GAPDH
% Cytotoxicity
RAW/LeTx
Level of GAPDH (1 x 10^7)
% Cytotoxicity
Time (min)
0
2
4
6
8
10
0
20
40
60
80
100
0
40
80
120
160

The correlation between toxin activity and loss of GSK-3β caused us to question whether or not this was linked to inhibition of MAPK signaling or if it resulted from a separate activity of LF. In order to determine this, levels of GSK-3β were examined in RAW macrophages treated with PD 98059, a chemical inhibitor of MAPK signaling. PD 98059 disrupts MAPK signaling by preventing the phosphorylation of MEK, which, in turn prevents the activation of the downstream kinase, ERK. In doing so, this inhibitor mimics LF's cleavage of MEK and subsequent disruption of this pathway. Hence, we compared the level of GSK-3β in RAW macrophages treated with PD 98059 or LeTx. As shown in Figure 13, there was no change in the level of GSK-3β following treatment with PD 98059 alone, thereby suggesting that the loss of GSK-3β is separate from LF's inhibition of MAPK signaling.

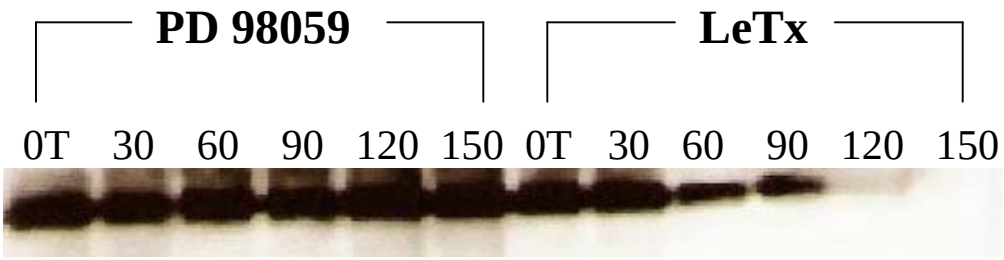


Fig. 13. **Immunoblot analysis of GSK-3β in response to MAPK inhibition versus LeTx in RAW 264.7 macrophages** In order to determine whether the loss of GSK-3β occurs as a result of MAPK inhibition alone or if it results from a separate activity of LF, RAW 264.7 cells were treated with PD 98059 or LeTx and extracts were collected at 30 min, 60 min, 90 min, 120 min, and 150 min time-points following treatment. Ten micrograms of extract was resolved by SDS-PAGE, transferred to PVDF membrane, and immunoblotted using primary antibody reactive to GSK-3β. Time-points and samples are labeled within the figure.

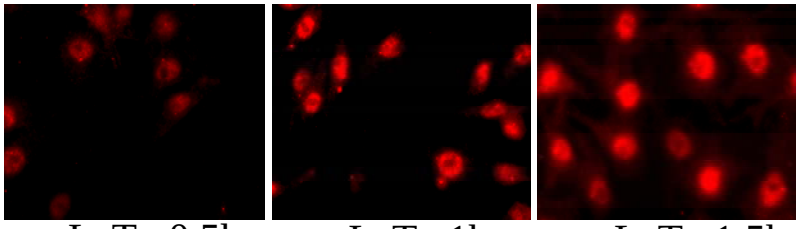
Cellular localization of GSK-3β and related proteins

The contribution of GSK-3β in governing susceptibility to LeTx, as shown with the LiCl assays, combined with the observed changes in the level of this protein in LeTx-sensitive cells caused us to examine its cellular localization patterns. Additionally, we investigated the localization of β-catenin due to its central role in GSK-3β-regulated activities, particularly those involved in the Wnt signaling which could account for the defects observed in the LeTx-treated zebrafish. Immunostaining was performed using primary antibodies specific for GSK-3β and β-catenin to probe LeTx-treated RAW cells throughout a time-course. We used RAW 264.7 cells pretreated with lactacystin as a control since these cells are protected from subsequent challenge with LeTx and, as shown previously, do not experience a change in the level of GSK-3β. Comparisons were then made in the localization patterns of GSK-3β and β-catenin in the presence and absence of lactacystin. There was a noticeable decline in GSK-3β at later time-points, comparable to what had been observed with immunoblot analysis. However, the staining patterns were as expected up until those times with GSK-3β concentrated in the cytoplasm (data not shown). Surprisingly, the most notable staining patterns were observed for phospho-β-catenin. As shown in Figure 14, all samples revealed nuclear localization of this protein. Additionally, the most noticeable change that occurred in response to LeTx was hyper-phosphorylation of β-catenin 1 h and 1.5 h post-treatment. This trend was not observed in corresponding cells that had been pretreated with lactacystin or in IC-21 macrophages that served as an untreated control.

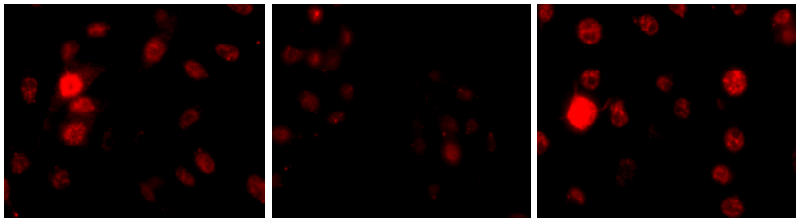
Fig. 14. **Cellular localization of phospho-β-catenin in LeTx-treated RAW 264.7 macrophages** Panel A) Cells were treated with LeTx or Tris buffer control for 2.5 h. Cells were fixed and stained for phospho-β-catenin at designated time-points. Panel B) Cells pretreated with lactacystin followed by treatment with LeTx or Tris buffer control and analyzed as described for Panel A. Samples and time-points are designated within the figure.

A

RAW/LeTx



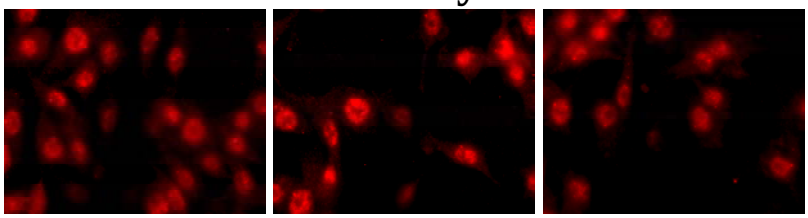
LeTx 0.5h | LeTx 1h | LeTx 1.5h



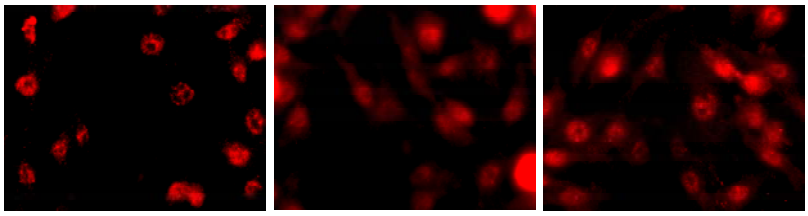
LeTx 2h | LeTx 2.5h | Tris 2.5h

B

RAW/Lactacystin/LeTx



LeTx 0.5h | LeTx 1h | LeTx 1.5h



LeTx 2h | LeTx 2.5h | Tris 2.5h

Differential expression of kinesin motor proteins

Reports have implicated a kinesin motor protein, Kif1C, as a factor that governs macrophage resistance to LeTx activity (Roberts, Watters et al. 1998; Watters, Dewar et al. 2001). In our analysis, two kinesins, kinesin family member Kif5C and kinesin family member Kif3C, were up-regulated, while kinesin family member Kif3B was down-regulated in toxin treated cells (see Table 2). It has recently been reported that APC and Kif3B proteins co-localize at microtubules along with β-catenin following phosphorylation by GSK-3β (Jimbo, Kawasaki et al. 2002). This observation, along with the report of Watters et al. (Watters, Dewar et al. 2001), suggest that kinesins not only confer resistance to LeTx, but also respond to LeTx activity by altering transcript levels. If such a hypothesis is true, then we reasoned that resistant versus sensitive cells might show differential expression of Kif1C. Therefore, the level of mRNA encoding for Kif1C was also examined by real-time PCR in IC-21 and RAW 264.7 macrophages following treatment with LeTx. As shown in Table 3, unlike the results from LeTx-treated RAW 264.7 cells, in IC-21 cells there is a decrease in the level of mRNA encoding Kif1C following treatment with LeTx. Kif1C mRNA expression decreased 8.7 fold in IC-21 macrophages, while LeTx-sensitive RAW 264.7 macrophages showed a 2.3 fold increase in expression. These data suggest that in addition to single nucleotide polymorphisms in Kif1C between LeTx-resistant and sensitive macrophages, the levels of expression in response to the toxin may be a determining factor in cell survival during exposure to the toxin.

TABLE 2. Genes differentially expressed in LeTx -treated RAW cells related to kinesin motor proteins.

Genes names	Accession #	p Value[a]	p' Value[a]	Fold[b]	Timepoint
-Kinesin family member 5C	X61435	0.00615	9.51463E-07	2.47	90 min
-Kinesin family member 3C	AF013116	0.00074		1.86	90 min
-Kinesin family member 3B	D26077	0.02960	9.98407E-06	-2.26	90 min

a P and P' values represent levels of significance in the standard paired analysis and the associative analysis respectively.
b Fold refers to the difference in gene expression between control and experimental samples for genes expressed in both conditions (normalized value > 0.48, cf. Methods).

Table 3. Real-time PCR Analysis of Kif1C in LeTx-Resistant and LeTx-Sensitive Macrophages

		IC-21		RAW
GAPDH	[a]C_T	Fold Change	[a]C_T	Fold Change
Tris	21.4		18.4	
LeTx	20.6		19.8	
Kif1C				
Tris	25.9		26.9	
LeTx	28.4	**8.7 decrease**	27.1	**2.3 increase**

[a]Mean average of four independent real-time PCR analyses. Fold change was determined by comparison with the standard curve generated for each target gene.

Decreased levels of tubulin in LeTx-treated RAW 264.7 macrophages

Microtubules are a major intersecting point for the processes mentioned above. GSK-3β•activity and motor protein activity each involve a functional microtubule network (Flaherty, Soria et al. 2000; Cui, Dong et al. 2002). Furthermore, GSK-3β is reportedly involved in regulating microtubule stability (Sang, Lu et al. 2001), and the array data indicated changes in microtubule-interacting proteins. As summarized in Table 4, levels of tubulin, beta 4 are reduced 30 min after treatment with LeTx, microtubule-associated protein EB family member 1 (EB1) is reduced by at least 11 fold 90 min following treatment with LeTx, and microtubule-associated protein 4 is also altered in expression following toxin treatment, making the overall stability of tubulin following treatment with LeTx of interest. As shown in Figure 15A, following treatment with LeTx, there is a notable decrease in the level of cellular tubulin in RAW 264.7 cells. Densitometry analysis indicated a 60% reduction in total intact tubulin following treatment of the cells with LeTx. This decrease occurs before cell death and cells maintain normal levels of Raf (control) at the time-point of decreased tubulin. Similar to the results with GSK-3β, LF does not appear to directly cleave tubulin. As shown in Figure 15B, there was no detectable loss of tubulin when the protein was incubated with LF.

TABLE 4. Genes differentially expressed in LeTx -treated RAW cells linked to microtubule stability.

Genes names Timepoint	Accession #	p Value[a]	p' Value[a]	Fold[b]
-Microtubule-associated protein 4 30 min	M72414		8.65563E-11	2.82
-Tubulin, beta 4 30 min	M28730	0.00272	2.19067E-11	-
-Microtubule-associated protein, RP/EB family, member 1 90min	U51196	0.00225	4.72168E-38	-11.35

[a] P and P' values represent levels of significance in the standard paired analysis and the associative analysis respectively.
[b] Fold refers to the difference in gene expression between control and experimental samples for genes expressed in both conditions (normalized value > 0.48, cf. Methods). Genes expressed only in control sample is indicated by(-).

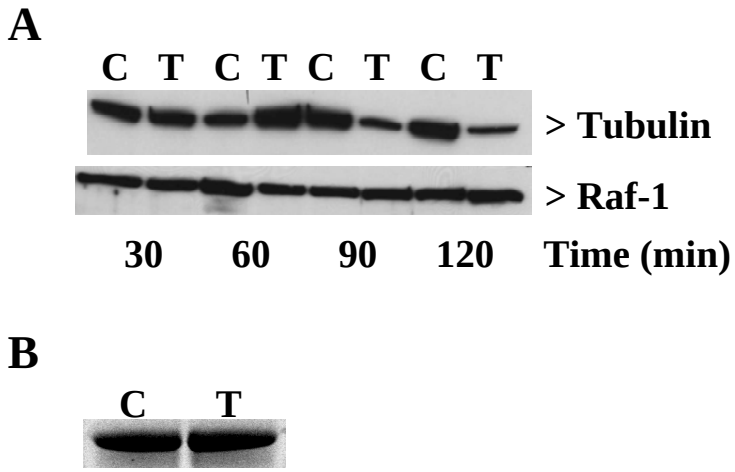


Fig. 15. **Changes in cellular tubulin levels following treatment with LeTx** Panel A) Cell lysates were collected at the indicated time-points from LeTx-treated and mock-treated controls RAW 264.7 macrophages. Extracts were then examined by immunoblot analysis for decreases in total tubulin, in correspondence with cleavage of MEK-2 as shown in Fig. 6. The corresponding conditions are shown within the figure. Panel B) Stability of alpha-tubulin in the presence of LF. Purified tubulin was incubated with LF for 2.5 h at 37°C, resolved by SDS-PAGE, and stained with coomassie blue. C= control; mock-treated and T=LeTx-treated samples. Immunoblot detection of Raf-1 was included to normalize for gel loading and variations in protein abudance.

Difference in PA-mediated entry among LeTx-sensitive and resistant cell types

Preliminary data obtained from immunoblot analysis of MEK-1 in LeTx-resistant and sensitive cells revealed differences in the time-frame for this cleavage event. As shown in Figure 16, MEK cleavage occurred within 90 min following LeTx treatment in RAW macrophages, at which point no MEK was detected with the N-terminal antibody, thus indicating complete cleavage of this protein. In contrast, cleavage occurred much later in IC-21 macrophages. MEK became non-reactive with the antibody used 6 h after toxin treatment, thus indicating complete cleavage from that point on. Additionally, NIH-3T3 fibroblasts exhibited a delay in MEK cleavage compared to LeTx-sensitive RAW cells, with a profile similar to that obtained for IC-21 cells (data not shown). Together, these data suggested that perhaps differences in rate of entry contributed, at least in part, to susceptibility to LeTx. Therefore, we compared the rate of entry of PA-mediated toxins among LeTx-sensitive and LeTx-resistant cell lines based on changes in cell viability.

We examined changes in cell viability among macrophage and non-macrophage cell lines in response to treatment with different PA-mediated toxins. Specifically, we treated RAW, NIH-3T3, HeLa, and IC-21 cells with fusions of LF and *C. sordellii* lethal toxin (TcsL) or LF and *C. difficile* toxin B (TcdB). These fusions consisted of a portion of LF, which contained the PA-binding site but lacked the enzymatic moiety, fused to the enzymatic domain of TcsL or TcdB. We chose to use these particular fusions because all cell lines in our assay experience a significant cytopathic effect in response to these modified toxins

unlike LeTx, although both the fusions and LeTx rely on PA-mediated cell entry. Following treatment with the fusions, bafilomycin was added at 10 min intervals for 2 h and cell viability was examined throughout the time-course. Bafilomycin blocks acidification of the toxin-containing endosome, thereby inhibiting the release of pH-dependent toxins into the cytosol. In this case, the use of bafilomycin allowed us to determine the time-frame for release of PA-mediated toxins into the cytosol of different cell lines.

The bafilomycin assays revealed significant differences between macrophage and non-macrophage cell lines in response to treatment with the toxin fusions. Most pronounced was the difference in the viability of NIH-3T3, HeLa, and RAW cells in response to treatment with PA, $LFnTcsL^{1-556}$ followed by treatment with bafilomycin. As shown in Figure 17, bafilomycin conferred significant protection in NIH-3T3 cells against the fusion for the duration of the 2 h time-course whereas the RAW cells experienced no protective effect. Likewise, HeLa cells revealed a significant protective effect from bafilomycin throughout the same time-course. IC-21 cells exhibited a minimal protective effect through the 50 min time-point but showed no significant protection when bafilomycin was added beyond that point (data not shown). Data derived from assays in which PA, $LFnTcdB^{1-556}$ was used correlated with data from the PA, $LFnTcsL^{1-556}$ assays with respect to each cell type. Taken together, these findings indicate that there is a difference in the rate of entry of PA-mediated toxins in different cell lines. In particular, it seems that PA-mediated toxin entry occurs much quicker in macrophages compared to the other cell lines tested. This, in turn, may contribute

to differences in susceptibility to LeTx. However, it should be noted that the difference in LeTx sensitivity among RAW and IC-21 macrophages suggests that additional factors, such as those described previously, govern macrophage sensitivity.

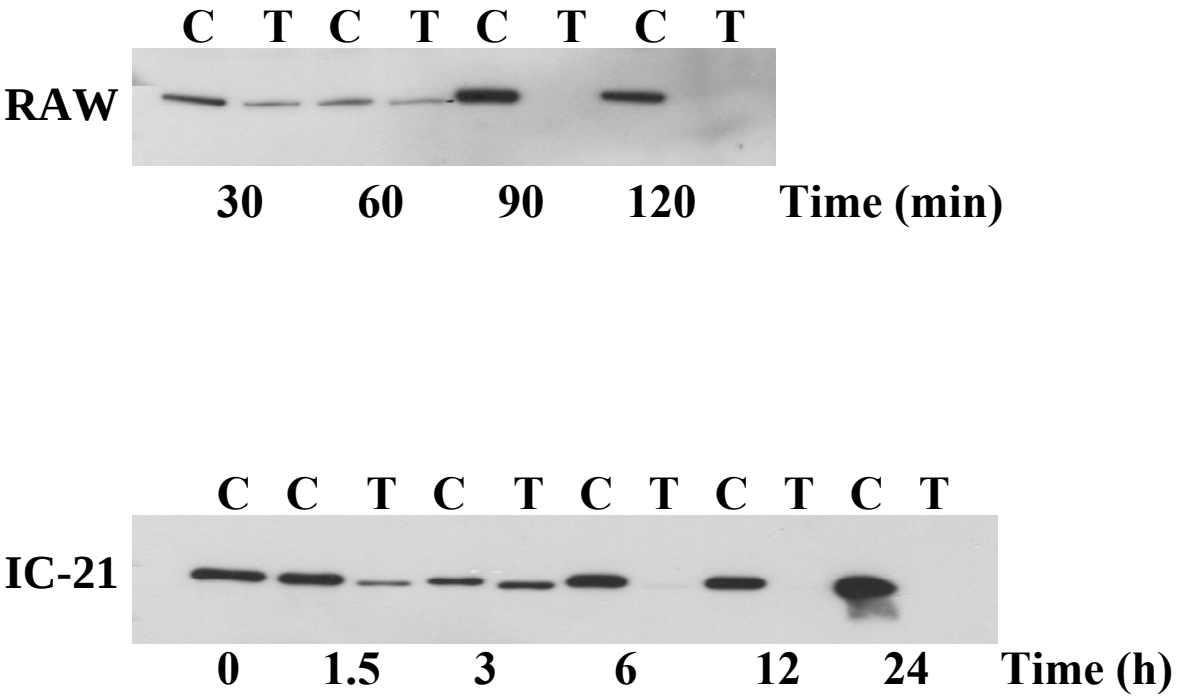


Fig. 16. **Immunoblot analysis of MEK cleavage in LeTx-sensitive and resistant cells** In order to determine the time-course of LF-mediated cleavage of MEK-1, RAW 264.7 and IC-21 cells were treated with LeTx and extracts were collected at designated time-points following treatment. Ten micrograms of extract was resolved by SDS-PAGE, transferred to PVDF membrane, and immunoblotted using primary antibody reactive to the amino-terminus of MEK-1. Time-points and samples are labeled within the figure. C= control, mock-treated samples; T= LeTx-treated samples.

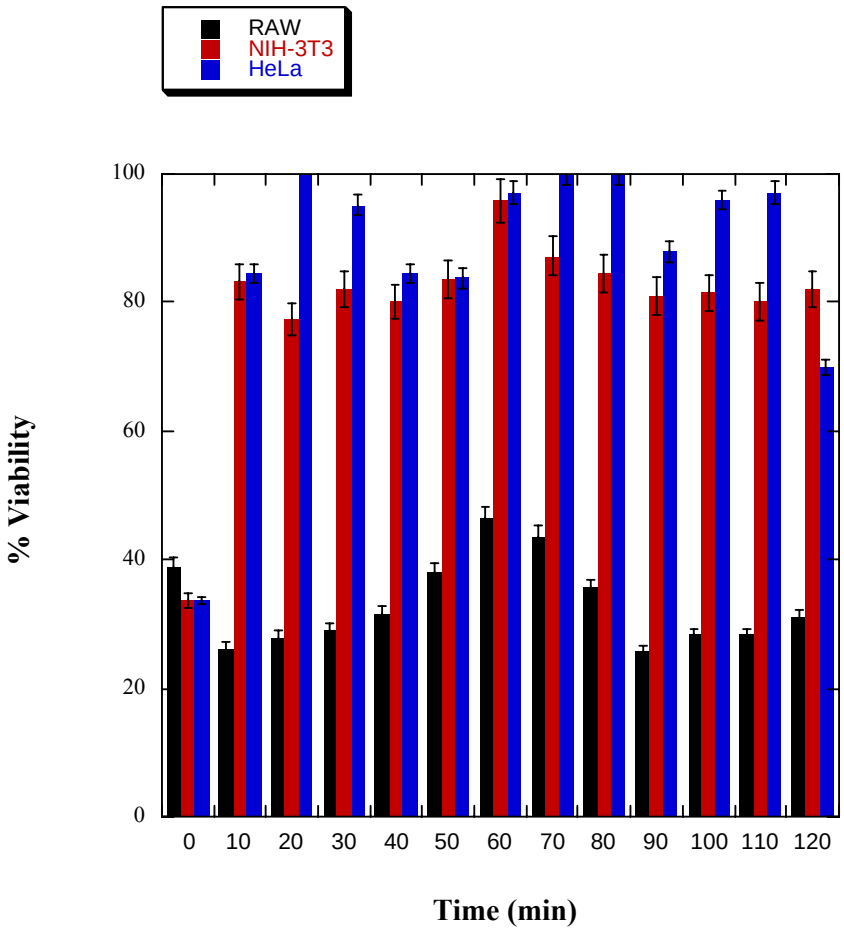


Fig. 17. **Difference in the rate of PA-mediated entry among LeTx-sensitive and resistant cell lines** RAW 264.7 macrophages, NIH-3T3 fibroblasts, and HeLa endothelial cells were treated with 2 μg/ml PA and LFnTcsL$^{1-556}$. Bafilomycin A was then added every 10 min for 2 h and cell viability was determined for each of the designated time-points 24 h following toxin treatment. Samples and time-points are designated within the figure.

DISCUSSION

The DNA array studies allowed us to characterize previously unidentified changes in macrophage physiology that occur following intoxication by LeTx. Reportedly, LeTx is cytotoxic to a very limited number of cell lineages although MAPKKs undergo cleavage by LF in a variety of different cell types. Consequently, yet undefined events outside of the MAPK signaling pathways may also contribute to LeTx's cytotoxic activity. As a broader approach to analysis of LeTx-intoxicated macrophages, we used a DNA membrane array to evaluate expression of over 1,000 categorized genes. The DNA array data was scrutinized to determine whether or not transcriptional changes are the result of disruption of an upstream regulatory pathway or part of a compensatory response by the cell. Thus, this data was used as a guide to find LeTx-induced changes at the protein level. We believe our data reflects both pathway disruption (e.g. GSK-3β) and compensatory responses (e.g. kinesin motor proteins). Using this perspective, analysis of the expression data suggested that the Wnt signaling pathway could be disrupted in LeTx-treated macrophages. Additionally, our DNA array analysis indicated that kinesin motor-protein expression and tubulin stability may be altered in these cells in response to treatment with LeTx.

Wnt signaling, which is regulated by GSK-3β, plays a major role in various stages of embryonic development. Thus, we used a developing zebrafish model to further confirm LeTx's impact on this pathway. Phenotypic changes, indicative of defects in Wnt signaling, were revealed in toxin-treated embryos. Temporal

loss of pigmentation and cardiac hypertrophy were the two prominent phenotypes of LeTx-treated embryos. Both of these developmental processes have been shown to involve regulation by Wnt. Specifically, Dorsky and colleagues reported on the requirement of Wnt in regulating pigment cell formation in zebrafish (Dorsky, Raible et al. 2000). Furthermore, Wnt signaling is known to be involved with cardiogenesis and modulation of cardiac hypertrophy via GSK-3β-related activities (Hardt and Sadoshima 2002). Thus, the resulting phenotypes from LeTx-treated embryos correspond with the developmental defects expected following Wnt inactivation.

Although disruption of GSK-3β was first indicated by the observed transcriptional changes associated with Wnt signaling, we also detected changes in a group of genes regulated by GSK-3β outside of the Wnt pathway. This observation suggested these changes might be due to an overall loss of GSK-3β activity after treating cells with LeTx. In line with this, results from the LiCl inhibitor studies suggest that GSK-3β is necessary for survival of LeTx-treated cells. Inhibition of this protein and its related activities via LiCl not only enhanced cytotoxicity in LeTx-sensitive macrophages, but converted LeTx-resistant macrophages to a sensitive phenotype. In addition to relating GSK-3β-regulated activities to LeTx sensitivity in macrophages, we also correlated a direct loss of this protein to cell sensitivity. Specifically, loss of GSK-3β was only observed in LeTx-sensitive cells; i.e. RAW 264.7 macrophages or IC-21 macrophages that had been pretreated with LiCl. Additionally, loss of GSK-3β occurred well before changes

in cell viability were detected, thereby indicating that this effect is specifically related to the cellular activities of LF. Furthermore, the observed loss of GSK-3β was not solely attributed to LF's inhibition of MAPK signaling, as levels of this protein remained unchanged in response to treatment with PD 98059, a chemical inhibitor of MEK. Together, these findings suggest that the loss of GSK-3β is not an inconsequential downstream event in intoxication. Instead, the observed changes regarding GSK-3β indicate a specific effect of LF which, in turn, relates to the susceptibility of different cell lineages to LeTx.

A plausible explanation relating the observed effects to cytotoxicity in LeTx-treated macrophages would link LiCl inactivation of GSK-3β to inhibition of NF-κB–regulated expression of factors that are important for cell survival. A report by Hoeflich and colleagues found that NF-κB is regulated by GSK-3β within transcriptional complexes (Hoeflich, Luo et al. 2000). Thus, inactivation of GSK-3β by LiCl could block NF-κB transcriptional regulation. Park and colleagues (Park, Greten et al. 2002) have recently shown that NF-κB synergizes with p38 to regulate survival genes in the presence of inflammatory stimuli and that LeTx, by inactivating the p38 pathway, promotes cell death. Thus, blocking GSK-3β function by LiCl treatment may heighten sensitivity to LeTx by preventing the expression of factors that promote survival. It must be noted, however, that LiCl treatment may be having a broad effect on cell signaling; thereby impacting targets outside of the GSK-3β pathway. For this reason, the LiCl sensitization data should be considered predominantly in light of results from the zebrafish,

immunoblot, and DNA array experiments. In fact, detailed analysis of the DNA array data does further support the putative loss of GSK-3β in LeTx-treated cells. β-catenin, AP-1, cyclic AMP-response element binding protein, NFκB, Myc, heat shock factor-1, nuclear factor of activated T-cells, and CCAAT/enhancer-binding proteins are known targets of GSK-3β regulation and are represented on the membrane arrays (Wang, Fiol et al. 1994; Welsh, Wilson et al. 1996; Hoeflich, Luo et al. 2000). Of these, β-catenin, cyclic AMP-response element binding protein, and CCAAT/enhancer-binding protein show changes in expression following treatment of macrophages with LeTx.

It seems likely that GSK-3β signaling is most important at the low, apoptosis-inducing, levels of LeTx treatment. LiCl treatment did not alter the high-dose effects of LeTx but enhanced cytotoxicity at low-doses of the toxin. Furthermore, cell death in IC-21 macrophages occurred at a relatively slow rate, indicative of apoptosis. This would be in agreement with a study that reported apoptotic cell death in RAW 264.7 macrophages in response to treatment with sublytic amounts of LeTx (Popov, Villasmil et al. 2002). Collectively, these data indicate that necrotic cell death occurs even in the absence of inhibition of GSK-3β, but the low-dose effects of the toxin are enhanced in cells in which GSK-3β has been attenuated.

Based on the link between GSK-3β and LeTx sensitivity, we examined the staining and localization patterns of GSK-3β and related proteins in LeTx-treated

RAW cells. In doing so, we found that β-catenin is hyper-phosphorylated in response to treatment with LeTx. Phosphorylation of this protein is normally regulated by GSK-3β, targeting it for proteasome-mediated degradation. In this regard, GSK-3β actively inhibits β-catenin-mediated transcription associated with Wnt signaling. However, since we observed a loss of GSK-3β and its associated activities, we were surprised to observe the hyper-phosphorylation of a protein normally regulated by GSK-3β. Additionally, our data revealed nuclear localization of phospho-β-catenin in LeTx-treated RAW cells. We had expected that LeTx's disruption of GSK-3β would have resulted in the accumulation of unphosphorlyated β-catenin in the cytoplasm prior to its translocation to the nucleus. However, reports indicate that disruption of signaling involving Wnt or cell-cell adhesion, in which β-catenin is a central mediator, can result in accumulation of β-catenin in cytoplasm or nucleus (Roose, Huls et al. 1999; Behrens 2000). Interestingly, hyper-phosphorylation of β-catenin was not observed in LeTx-treated RAW cells that had been pretreated with proteasome inhibitor. In this case, pretreatment with proteasome inhibitor confers protection from LeTx for the duration of the time-course. Interestingly, the staining pattern of β-catenin in these cells was similar to that of IC-21 macrophages. In total, these results suggest that localization and hyper-phosphoylation of β-catenin may contribute to LeTx sensitivity.

Separate from the findings regarding GSK-3β and its related activities, the array data also revealed changes in the expression of kinesin motor proteins. These

changes were of immediate interest because of the findings of Watters and colleagues which linked a kinesin-like motor protein, Kif1C, to cellular resistance to LeTx (Watters, Dewar et al. 2001). Our experiments examined the role of motor proteins from a perspective different from Watters et al., which identified the resistance loci based on single nucleotide polymorphisms in the *kif1C* gene. Yet, there are correlations between these two studies. In addition to the array data which showed changes in the expression of various kinesins in response to LeTx, we also demonstrated differential expression of Kif1C among sensitive and resistant macrophages. Specifically, we observed a difference in the level of Kif1C in LeTx-treated RAW and IC-21 cells. Watters and colleagues also found that destabilizing the cellular localization of Kif1C with brefeldin-A converts resistant IC-21 cells to a LeTx-sensitive phenotype. Thus, alterations in Kif1C's ability to function correctly may be linked to LeTx-sensitivity. In the absence of the ability to make these adjustments, cells would be rendered more susceptible to the toxin, as is the case with RAW 264.7 macrophages.

Taken together, the findings from the array studies allow for an encompassing model that could account for the observed effects, centering around the loss of GSK-3β. In such a case, loss of GSK-3β and subsequent alterations in synergy with NF-κB may render the cell incapable of promoting survival. Within the same model, loss of GSK-3β could lead to alterations in microtubule stability and corresponding compensatory responses involving kinesin protein expression. Defining specific details of such a model will require continued investigation. Furthermore, such a model should not dismiss a role for inactivation of MAPKKs

as a contributing factor. The instigating event leading to loss of GSK-3β is not clear and could be a downstream result of MAPKKs inactivation. However, this cannot be the full explanation for cell death since other cells with inactivated MAPK signaling do not necessarily succumb to the toxin.

Our findings provide additional evidence to support the proposed role of the proteasome in mediating sensitivity to LeTx (Tang and Leppla 1999). First, in examining the loss of GSK-3β versus cytotoxicity in LeTx-treated RAW cells, pretreatment with proteasome inhibitor, lactacystin, protected the cells for the duration of the time-course. In addition, cells pretreated with lactacystin showed no change in the level of GSK-3β in response to LeTx. Lastly, the hyper-phosphorylation of β-catenin, observed with immunostaining, occurred in the absence of lactacystyin whereas cells pretreated with lactacystin did not show this alteration. Together, our findings further emphasize the role of the proteasome in mediating sensitivity to LeTx.

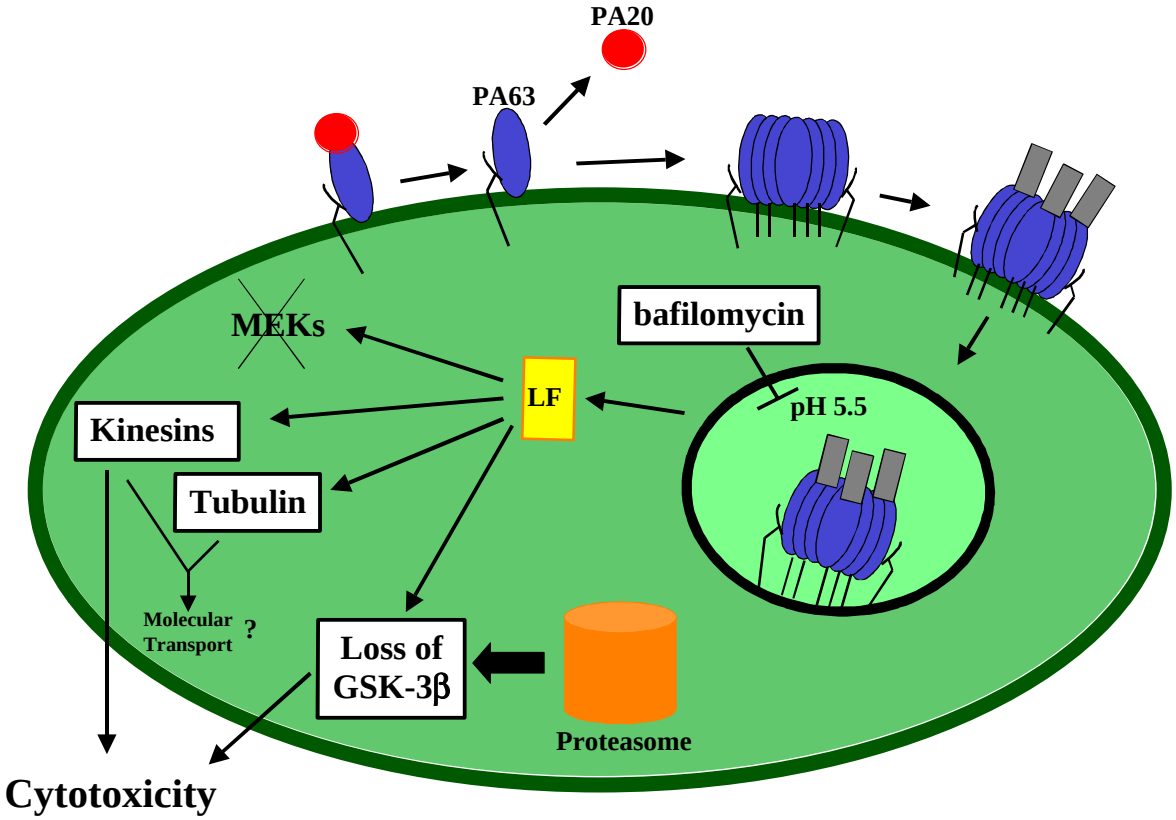
The bafilomycin assays suggest that the rate of PA-mediated toxin entry may play a role in governing sensitivity to LeTx among different cell types. The data revealed that significant protection could be achieved for the non-macrophage cell lines, NIH-3T3 and HeLa, when treated with bafilomycin up to two hours following treatment with PA-mediated toxins. In contrast, bafilomycin offered no protection to RAW macrophages and limited protection to IC-21 macrophages within that same time-frame, suggesting that entry occurs more rapidly within this cell type. It has been shown that anthrax toxin entry is governed by the

association of its cell surface receptor with lipid rafts (Abrami, Liu et al. 2003). Although the reported receptor of anthrax toxin (ATR) is widely expressed, it is possible that there is a different mechanism of cell entry, other than a lipid raft-mediated process, or a difference in the rate of entry among different cell types. These possibilities could account for differences in LeTx sensitivity observed *in vitro*.

Summarized in Figure 18, the findings presented in this thesis provide new insight regarding LeTx's mechanism of action *in vitro* and identify cellular factors involved in mediating susceptibility to this toxin. These data identify a potential activity of LF, separate from its inhibition of MAPKKs, which contributes to LeTx sensitivity. Specifically, toxin treatment causes alterations in GSK-3β in LeTx-sensitive cells. Additionally, chemical inhibition of GSK-3β with LiCl converts resistant cells to a sensitive phenotype. Taken together, these results suggest that GSK-3β activity is required to promote cell survival in response to LeTx and that the observed cytotoxicity in RAW macrophages results from disruption of GSK-3β and its related activities. In addition to impacting GSK-3β, LeTx was shown to alter the expression of kinesin motor proteins as well as microtubule-associated proteins. The kinesin motor protein Kif1C was previously linked to LeTx sensitivity based on a single nucleotide polymorphism in this gene in resistant versus sensitive macrophages. The data presented in this thesis not only demonstrate differential expression of kinesin motor proteins in LeTx-sensitive macrophages but also reveal differential expression of Kif1C in sensitive versus resistant macrophages. In regards to the impact of LeTx on microtubule

stability, my studies revealed differential expression of microtubule-associated proteins and tubulin in LeTx-sensitive cells. Furthermore, these cells exhibit a dramatic reduction in the level of tubulin in response to LeTx. Finally, results from the bafilomycin assays implicate the rate or mechanism of entry as an additional factor in governing sensitivity to LeTx. In summary, results from these studies portray LeTx as a virulence factor with multi-faceted activities and should encourage the continued investigation of this toxin's ability to impact multiple cellular events.

Fig. 18



Previous Findings	**Significant Findings From My Work**
Kif1C linked to LeTx—resistance in macrophages	**Kif5C, Kif3C, Kif3B** motor proteins are differentially expressed in LeTx-treated RAW 264.7 cells
	Kif1C is differentially expressed between LeTx-resistant and sensitive macrophages
	Tubulin & microtubule-associated proteins are differentially expressed in RAW 264.7 cells
	Tubulin levels decline in LeTx-treated RAW 264.7 cells
	LeTx impacts **Wnt signaling**
	GSK-3β - regulated genes (Wnt and non-Wnt related) are differentially expressed in RAW 264.7 cells
	Inhibition of GSK-3β converts resistant macrophages to a sensitive phenotype
	Levels of GSK-3β decline in LeTx-sensitive macrophages
Proteasome activity mediates LeTx-sensitivity in macrophages	**Inhibition of the proteasome** prevents changes in the level of GSK-3β in LeTx-treated RAW 264.7 cells
	Differences in the **rate of entry** of LeTx may contribute to differences in sensitivity among various cell types

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