ANTHRAX LETHAL AND EDEMA TOXINS EFFECTS ON HUMAN PERIPHERAL CELLS

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To all who have provided their support, knowledge, challenges and guidance along my journey

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ATP	Adenosine triphosphate: A nucleotide which performs many essential functions within the cell. It is also the substrate for cAMP
cAMP	Cyclic adenosine monophosphate: A second messenger chemical produced by adenylyl cyclase from ATP
EF	Edema factor: An exotoxin produced by *B. anthracis* and a known adenylyl cyclase which increases intracellular cAMP
ERK	Extracellular signal-regulated kinases: Members of the MAPK family which are phosphorylated by MAPKK 1 and MAPKK 2
ET	Edema toxin: The combination of EF and PA
Fsk	Forskolin: A chemical produced in plants which increases intracellular cAMP levels
JNK	c-Jun N-terminal kinases: Members of the MAPK family which are phosphorylated by MAPKK 4 and MAPKK 7
LF	Lethal factor: An exotoxin produced by *B. anthracis* and a zinc dependent metalloprotease which cleaves the N-terminus of MAPKKs
LT	Lethal toxin: The combination of LF and PA
MAPKK/MEK	Mitogen activated protein kinase kinase: Kinases which phosphorylate downstream substrates typically at serine and threonine
p38	p38 mitogen-activated protein kinases: Members of the MAPK family which are phosphorylated by MAPKK 3 and MAPKK 6
PA	Protective antigen: An exotoxin produced by *B. anthracis* which binds to host cell receptors facilitating entry of EF and/or LF

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Bacillus anthracis is a gram-positive bacterium known to produce two toxins: edema toxin (ET) and lethal toxin (LT). ET is an adenylyl cyclase that increases intracellular cAMP levels, while LT cleaves and inactivates members of the mitogen-activated protein kinase kinase family (MAPKK or MEK). The effects of these toxins are typically studied independently of one another. We have now investigated the effects of dual toxin treatment on multiple cell types. We found that treatment of cells with both ET and LT diminished production of cAMP in a dose dependent manner, and also showed forskolin, a stimulant of endogenous mammalian adenylyl cyclases was inhibited by anthrax LT.

We sought to further elucidate how inhibition of the MAPKK signaling cascade attenuated cAMP. We found that the proteolytic activity of LT was required for inactivation of adenylyl cyclase activity. The inhibition could not be explained by secretion of either cAMP itself or its substrate ATP, as extracellular levels of cAMP and ATP were not increased after dual toxin treatment. Because LT inhibits the MAPKK pathway, we utilized specific MAPKK pathway inhibitors to further characterize which specific pathway or pathways (p38, JNK, or ERK) regulated cAMP production. We found that all MAPKK pathways must remain functional in order for ET and endogenous adenylyl cyclases to generate significant increases in intracellular

cAMP concentrations. We also found that inhibition of cAMP production by LT required intact cells, as the production of cAMP was not inhibited in cell extracts treated with dual toxins. These findings suggested LT may be acting via a post-translational signaling mechanism downstream of the MAPKK pathways. To further characterize potential targets of cAMP inhibition by LT, 2DIGE was utilized to compare ET versus ET plus LT treated cells. Twenty eight candidate proteins were found to be differentially modified including actin regulatory, stress response, glycolytic and heat shock proteins. We conclude the production of cAMP by adenylyl cyclases requires the activation of all three MAPKK pathways, and we have identified a number of potential downstream protein candidates that may serve to regulate adenylyl cyclase activity.

We have also measured the transcriptional response of human peripheral monocytes after treatment with anthrax LT and have found multiple changes in the signaling pathways, outside of the well-known MAPKK pathways. We confirmed monocyte susceptibility to anthrax LT and these results will help to further elucidate the role of LT on immune cell inhibition during an anthrax infection. Additionally, treatment of human platelets with either LT or ET has no effect on platelets or their function. These findings can be explained by a failure of toxin to bind to the platelet surface due to low levels of the anthrax receptors on human platelets. This is the first finding indicating anthrax toxins do not directly impair human platelet function, thus, the internal hemorrhage often observed in persons who succumb to anthrax is attributed to other defects in hemostasis.

CHAPTER 1
INTRODUCTION

Bacillus Anthracis History

B. anthracis, the causative agent of anthrax, has laid the foundation for some of the most valuable scientific breakthroughs, along with some of the greatest human and animal threats. Anthrax is a zoonotic disease, in which sheep, horses, goats and cattle are the most susceptible animals [1-3]. Anthrax infections have been documented as far back as 1491 B.C. in Egypt, in which the Old Testament references two plagues, the 5th (the plague of cattle and livestock) and 6th plagues (boils), believed to be caused by anthrax. Other historic references of anthrax describe cattle diseases in 500 B.C. in India, along with descriptions afflicting both livestock and people in ancient Greece from 1000 B.C. to 200 A.D. [4]. Virgil, a Roman poet and scientist, wrote descriptive accounts of anthrax occurring in the Roman region of Noricum between 28 and 29 B.C. He termed the outbreak "the murrain of Noricum" and addressed detailed occurrences: "If anyone wore a garment made from the tainted wool, his limb was soon attacked by inflamed papules and a foul exudates, and if he delayed too long to remove the material a violent inflammation covered the parts it had touched" [5].

Anthrax was utilized in the late 1800s by scientists to help test and establish critical concepts in both microbiology and immunology. Anthrax has aided in the formulation of Koch's postulates in the late 1870s after Robert Koch identified the bacillus in blood smears, grew the bacterium in culture, and developed an animal model for infection [6-8]. Anthrax also played a critical role in immunology in which the first live bacterial vaccine was developed in cattle using an attenuated strain by Louis Pasteur in 1881 [9].

During the 19th century in Europe, many sheep workers were exposed to anthrax. Workers would be exposed to anthrax spores during the separation of wool, which led to anthrax being

labeled as “Woolsorters disease”. Several hundred cases of “Woolsorters disease” during this era resulted in the establishment of disinfection stations and occupational regulations for anthrax [10]. More recent accounts of anthrax have occurred in 1979 in the former Soviet Union when anthrax was released by a military facility causing over 100 fatalities, and in 1957 in the United States, in which workers at a goat-hair processing facility contracted inhalation anthrax, killing 4 individuals [11, 12].

Anthrax has not been documented to be transmitted from person-to-person. Human cases of anthrax are typically associated with contact of infected animal by-products such as wool, goat hair or hide. In the United States, anthrax is known to be endemic in Texas, Oklahoma, and Mississippi [13]. Currently, the biggest threat of anthrax is its use as a bioterrorist agent. Because inhalational anthrax is highly fatal and easily disseminated, its use in biological warfare is highly feared. In 2001, anthrax was released in the U.S. postal system and resulted in 11 cases of inhalation anthrax, 11 cases of cutaneous anthrax, and 5 fatalities [14].

Bacillus anthracis Microbiology

B. anthracis is a gram-positive, non-motile, rod-shaped, bacillus that is non-hemolytic when grown on sheep blood agar. The bacterium can be identified with staining using McFadden’s methylene blue. Other identification markers for anthrax include the presence of the protective antigen toxin, lysis by gamma phage, and capsule detection using fluorescent antibodies [15, 16]. The optimal growth conditions for *B. anthracis* is 35°C, and temperatures higher than 45°C lead to the loss of the capsule component yielding avirulent or attenuated strains [17]. *B. anthracis* is also capable of undergoing sporulation under non-optimal growth conditions, specifically when infected host tissue is exposed to oxygen. Although the bacterium can establish an infection if ingested, it is the spore form of *B. anthracis* that is typically associated with disease in both humans and animals.

The spore structure of anthrax is metabolically inactive and is composed of multiple layers: an outer exosporium, followed by a spore coat, cortex, and finally the core, where the bacteria's nucleic acid can be found. The route of spore contact with a susceptible host dictates the form (cutaneous, inhalation, or indigestion) of disease, and is generally a good predictor of severity and outcome of infection. The spore form of anthrax is highly resistant to drying and most disinfectants, allowing it to survive for many decades or even centuries in harmful environmental conditions. The recommended method for decontamination of the spores is exposure to temperatures of at least 120°C for a minimum of 15 minutes [18]. Typical human contact with anthrax spores is through animal carcasses or by-products from animals that have succumbed to the disease. Interestingly, it has been shown that animal carcasses which have not been opened will result in little environmental contamination of spores [19].

Anthrax Exposure

Anthrax spores will infect animals, including humans, through a cutaneous, inhalational, or gastrointestinal route. Cutaneous anthrax is the most prevalent form of anthrax with >95% of reported cases being cutaneous [20, 21]. Cutaneous anthrax is associated with the lowest mortality, and will usually remain localized to the dermis or subcutaneous tissue, although septicemia and toxemia may occur in severe cases. If cutaneous anthrax is left untreated, there is a 20% mortality rate associated with this route of exposure, while treated cases have <1% mortality rate [22, 23]. The initial site of infection is believed to form at a pre-existing lesion or abrasion in the skin, followed by a one to seven day incubation period, after which a papule appears at the site of infection. After five to seven days of the initial papule formation, the vesicle then ruptures, leaving a black, painless, ulcer-like eschar. After two to three weeks the eschar will subside, leaving a scar at the site of the lesion.

Gastrointestinal anthrax occurs after ingestion of contaminated meat. It is believed that these cases involve ingestion of poorly cooked meat containing vegetative bacilli [24]. This is the rarest form of anthrax, although under-reporting of infection is believed to be common. Two to five days after initial ingestion of infected meat products, symptoms appear and can include fever, nausea, vomiting, abdominal pain and sometimes respiratory distress. Because gastrointestinal anthrax has such a broad range of symptoms, it is very difficult to diagnose early, resulting in mortality as high as 50% [25]. The exact mechanism of how the bacilli penetrate the mucous membranes and enter the bloodstream remains unknown. Fatality from gastrointestinal anthrax occurs following toxemia, sepsis and shock, in which documented cases from the time of initial symptoms until death varies from two to five days [20, 26-28]. A few cases of oropharyngeal (middle throat) anthrax have also been documented [29-32].

The most feared form of anthrax is inhalational anthrax, due to its high mortality and hard to diagnose "flu-like symptoms". Inhalational anthrax occurs after *B. anthracis* spores are inhaled into the lungs. Exposure typically occurs during occupational contact with air-borne spores from infected animal by-products, but the intentional release of anthrax spores has been documented. The onset of symptoms typically occur four to six days after spore inhalation and symptoms can include fever, chills, sweats, fatigue, nonproductive cough, muscle aches, and nausea or vomiting. The mild, initial phase of inhalation anthrax is followed by a sudden onset of more severe symptoms including dyspnea, disorientation, cyanosis, followed by coma and death. Without treatment, death occurs in 90% of inhalational anthrax victims, while early intervention can lower fatality to 40% [24].

Pathogenesis

Death from anthrax is believed to be caused by toxin-induced vascular collapse leading to hypoxia. Virulent strains of *B. anthracis* harbor two plasmids: pXO1 and pXO2. The pXO1

plasmid is 182 kb in size and encodes the tripartite toxin components: EF, LF and PA. The pXO2 plasmid is 96kb and encodes the anthrax capsule. Expression of both plasmids is regulated by atxA which is encoded on the pXO1 plasmid and has a positive effect on the transcription of the pXO2 gene regulators acpA and acpB [33]. Plasmid expression is modulated by CO2 levels, which at high levels, can also directly activate acpA and acpB. Without either of these plasmids, *B. anthracis* shows extremely reduced virulence, and culture filtrates from avirulent, uncapsulated strain are used in current human vaccines [34-36].

Capsule

Virulent strains of *B. anthracis* express an anti-phagocytic capsule composed of poly-D-glutamic acid. The capsule is expressed only in the presence of CO2 together with bicarbonate or serum and not during *in vitro* aerobic culturing conditions. When the isolated capsule is injected into animals, it has been shown to be excreted in an intact form, suggesting that the capsule is not antigenic [37]. This non-immunogenic capsule allows *B. anthracis* to evade the host immune system. The role of the capsule is more crucial during the initial phases of infection, allowing the bacterium to establish an infection and disseminate from the initial site of infection, while the toxins are more important during the later stages of anthrax [38, 39].

Toxins

B. anthracis secretes three exotoxins: protective antigen, edema factor, and lethal factor. The anthrax toxins are defined as A-B toxins in which protective antigen serves as the B subunit and binds to host cell receptors, while edema factor and lethal factor serve as the catalytic subunits. Protective antigen (PA) is required for entry of the catalytic subunits and is termed edema toxin (ET) when administered in the presence of edema factor (EF), and is termed lethal toxin (LT) when the catalytic subunit lethal factor (LF) is combined with PA. Many animal models have been used to study the effects of the toxins, including studies on mice, guinea-pigs,

sheep, rabbits, and primates. Dogs, cats, pigs, and birds are considered more resistant to anthrax than other animals [40-43].

Protective antigen is an 83 kDa protein that binds to one of two host cellular receptors: tumor endothelial marker 8 (TEM8) and capillary morphogenesis gene 2 (CMG2) facilitating entry of EF and LF. Both anthrax receptors share a von Willebrand domain which is required for PA binding, although the von Willebrand domain is associated with binding to the extracellular matrix, the exact function of these receptors is unknown. After PA binds to the host cell receptors, a 20 kDa portion of PA is cleaved by furin [44]. After cleavage, the remaining 63 kDa PA forms a heptameric pre-pore, in which two to three subunits of EF and/or LF bind, followed by clathirin-mediated endocytosis and cellular processing of the toxins within the acidic compartments of cells [45-48]. Protonation of PA in the acidic endosomes leads to insertion of PA into the membrane, and formation of the pore required for translocation of EF/LF into the cytosol.

Edema factor is an 89 kDa calcium-dependent adenylate cyclase that increases intracellular cyclic adenosine monophosphate (cAMP) levels. EF is encoded by the cya locus and requires binding of calmodulin for its catalytic activity [33, 49-55]. The N-terminus of EF is highly homologous to LF and has been shown to be essential for binding to PA. The first accounts investigating edema toxin noted morphological changes in CHOK1A cells which were similar to cholera toxin, leading to the discovery that ET increases cAMP levels and posseses adenylate cyclase activity [54]. The effects of ET are reversible and it has been shown that EF has a half-life of less than two hours in the cytosol [54]. Because of its anti-phagocytic activities in both polymorphonuclear cells and macrophages, ET is predicted to play a more critical role in the early stages of infection [56, 57].

Lethal factor is a 90 kDa zinc-dependent metalloprotease that cleaves MEKs thereby preventing downstream activation of the p38, JNK and ERK pathways. LT has been shown to cleave the N-terminus of mitogen-activated protein kinase kinases (MAPKK) one through seven, with the exception of MAPKK five [58-61]. LF is encoded by the lef locus and mutations on its N-terminus prevent binding to PA, while the C-terminus contains its protease activity [62, 63]. Early studies with LT utilized mice, which showed varying levels of cytotoxicity *in vitro* and varying degrees of susceptibility to LT [46, 64-69]. It was later discovered that only certain strains of mice harboring the Nalp1b gene are susceptible to the cytotoxic effects of LT [70]. Investigations using human cells have shown LT blocks cytokine release in macrophages, increases apoptosis and cell permeability in endothelial cells, blocks neutrophil motility, and decreases proliferation and cytokine production of lymphocytes [64, 70-76].

cAMP Signaling

Cyclic adenosine monophosphate (cAMP) is a second messenger that regulates a diverse range of cell processes including cell metabolism, gene expression, cell division, exocytosis, and regulation of immune responses. cAMP is synthesized from ATP by adenylyl cyclases and is one of the most diverse and complex second messengers discovered. cAMP was discovered by Dr. Earl Sutherland during his investigations on the mechanisms of hormone action, and is known to be involved in numerous regulatory and metabolic processes in various organisms including amoebas, bacteria, plants, and humans [77, 78].

The initiation of mammalian cAMP production is through activation of a seven transmembrane-spanning G protein-coupled receptor (GPCR) upon binding to its cognate ligand which includes epinephrine, dopamine, prostaglandin, prostaglandin E2 (PGE2), adenosine, and glucagon, among others [79]. GPCR activation results in an exchange of GDP for GTP on a receptor coupled heterotrimeric G protein, causing the G protein to dissociate into a Gα and Gβɣ

subunit (Figure 1-3). This activation is transient as GTP hydrolysis will return the G protein into an inactivated state and reassociate with the Gβγ subunit. This hydrolysis is accomplished through a regulator of G protein Signaling (RGS) which acts as a GTPase-accelerating protein (GAP) [80].

The primary effector component of the heterotrimeric G protein is the 40-46 kDa Gα subunits, which can be further divided into two families: the stimulatory (Gαs) or inhibitory (Gαi) subunits [81]. Production of cAMP by adenylyl cyclases is accomplished through binding of the stimulatory Gαs subunit to adenylyl cyclases resulting in adenylyl cyclase activation and conversion of ATP to cAMP. GPCR can also lead to the release of a Gαi subunit which results in inhibition of adenylyl cyclases.

There are 10 known isoforms of mammalian adenylyl cyclases, nine of which encode a membrane-bound form, while one encodes a soluble isoform. These isoforms are further divided into five distinct families: the calcium-calmodulin sensitive forms (AC1, AC3 and AC8), the Gβγ stimulatory family (AC2, AC4 and AC7), the calcium and Gαi inhibitory forms (AC5 and AC6), the AC9 and lastly, the soluble adenylyl cyclase (Figure 1-5). The most recently identified soluble form is insensitive to activation by G proteins and forskolin. This isoform is found in the testis and is activated by bicarbonate [82].

The structure of adenylyl cyclases is similar in that all isoforms contain two transmembrane domains and two cytoplasmic domains (Figure 1-4). There is little homology in the transmembrane domains between isoforms, but the catalytic domains of adenylyl cyclases display 50%-70% similarity between their amino acid sequences. There are two catalytic domains within adenylyl cyclases and both are required for enzyme activity [83]. It has been shown that both the C1a and C2a domains bind ATP, while the C1a domain contains more of a

catalytic activity while the C2a domain contains a more regulatory role [84]. The catalytic domains come into close contact with each other upon activation through a conformation change in the enzyme.

The catalytic domains have been shown to be bound by both Gαs or the potent chemical activator forskolin. Forskolin is a potent activator of all mammalian isoforms of adenylyl cyclases, except AC9 and the soluble AC forms, through interactions within a hydrophobic pocket at their catalytic interface [85]. For AC2, AC4, AC5, AC6 and AC7 the effects of both forskolin and Gαs show a synergistic effect, while AC1, AC3, and AC8 show an additive effect [86]. Inhibition of adenylyl cyclases by Gαi subunits is not through direct competition with the Gαs subunit as forskolin-induced activation is inhibited by the Gαi subunits. Each subtype of the Gαi subunits (Gαi1, Gαi2, Gαi3, Gαo and Gαz) are selective for which isoforms they inhibit [87]. Production of cAMP is regulated through a feedback mechanism which results in the activation of phosphodiesterases (PDEs) which degrade cAMP. These phosphodiesterase enzymes can be inhibited with treatment of 3-isobutyl-1-methylxanthine (IBMX).

Production of cAMP leads to the activation of several downstream components. One well-known downstream substrate is protein kinase A (PKA). PKA is a kinase with a wide range of downstream targets and subsequent effects. Activated PKA can phosphorylate downstream substrates at serine and threonine residues, and has been shown to play a role in cAMP-response element binding protein (CREB) activation [88]. Other proteins activated by cAMP include the guanine exchange proteins EPAC-1 and EPAC-2. Activation of these proteins results in activation of the small Ras-like GTPase Rap-1 [89]. Another important component activated by cAMP include cyclic nucleotide-gated ion channels, nonselective cation channels that play a role in sensory transduction as well as hormone release and chemotaxis [90].

Each gene encoding the various isoforms of mammalian adenylyl cyclases is located on a different chromosome, all likely to be expressed by different promoters during distinct conditions [91-94]. Overstimulation of adenylyl cyclases has been associated with certain cancers including pituitary and thyroid adenomas [95, 96]. On the other hand, decreased adenylyl cyclase activity is associated with decreased cognitive function including memory and learning [97, 98]. Disregulation of adenylyl cyclases has also been observed in heart failure, longevity, and motor dysfunction [99-102]. Because there is such diversity in adenylyl cyclase expression and regulation, identifying selective stimulators or inhibitors for each adenylyl cyclase has been a challenge. Unraveling the biochemical pathways underlying LT-mediated inhibition of mammalian adenylyl cyclases promises to provide a new understanding of adenylyl cyclase regulation and may lead to new therapeutic approaches to a wide array of medical disorders.

In regards to cAMP and immune cell function, increased cAMP levels exert anti-inflammatory effects on immune cells. This suggests a mechanism utilized by pathogens which would impair the immune responses, facilitating the survival and proliferation of microbes. In leukocytes, it has been shown that cAMP suppresses the pro-inflammatory cytokines TNF-α and IL-12, and inhibits production of the chemokine macrophage inflammatory protein-1 (MIP-1) [103]. Phagocytosis is also suppressed in leukocytes, along with reactive oxygen intermediate release [104, 105]. T-cell proliferation has been shown to be inhibited by increased cAMP levels [106]. The overall inhibition of immune cell function by increased production of cAMP is utilized by numerous pathogens to help aid in their pathogenesis.

Infectious microbes known to manipulate host cAMP levels in order to help successfully establish and maintain infections include *Vibrio cholera, Bordetella pertussis, Pseudomonas*

aeruginosa, and *Yersinia pestis*. The gram-negative bacteria *V. cholera*, achieves its virulence through production of a toxin that ADP-ribosylates the stimulatory subunit Gαs of G proteins, resulting in an irrepressible synthesis of cAMP by adenylyl cyclase [107]. Another known ribosylating toxin produced by the gram-negative bacteria *Bordetella pertussis*, achieves increased cAMP levels through the ADP ribosylation of the inhibitory component Gαi resulting in unregulated production of cAMP [108]. Aside from modifications on host regulatory components, many microbes produce toxins which are themselves adenylyl cyclases, capable of direct synthesis of cAMP when coupled to specific host cofactors. These include the ExoY of *Pseudomonas aeruginosa*, the adenylate cyclase of *Yersinia pestis*, and the edema factor of *Bacillus anthracis* [54, 109, 110]. Many pathogenic microbes utilize mechanisms which elevate cAMP levels, thus how lethal toxin inhibits cAMP production is of great interest both clinically and physiologically.

Mitogen-Activated Protein Kinase Pathways

The MAPKKs are involved in a variety of host cell functions including cell proliferation, differentiation, migration, survival and immune responses. This pathway was first recognized in the early 1980's and is known to be activated through binding of mitogens, hormones, and neurotransmitters to tyrosine kinase receptors resulting in a downstream phosphorylation cascade of three subsequent protein kinases: the MAPK kinase kinase (MAPKKK/MEKK), the MAPK kinase (MAPKK/MEK) and the MAP kinase (MAPK) (Figure 1-2) [111, 112]. The MAPKs can be further divided in six groups: p38, extracellular regulated kinases (ERK1/2), Jun NH2 terminal kinases (JNK1/2/3), ERK7/8, ERK3/4, and ERK5. Three of these pathways, the p38 kinases, the ERK1/2, and JNKs, are the most well-known and widely studied pathways.

The MAPK pathways are activated upon activation of receptor tyrosine kinases by their cognate ligands. This leads to activation of small GTPases of the Ras and Rho family, among

other adaptor proteins, which subsequently phosphorylate downstream MAPK. All kinases within the MAPK family contain proline residues near the serine/threonine phosphorylation sites. The specificity of phophorylation is maintained by docking sites, either positively charged residues surrounded by hydrophobic residues, or a Phe-Xaa-Phe-Pro sequence [113, 114]. Because the MAPK signaling pathways are so diverse and the levels of MAP kinases do not fluctuate throughout the course of signal activation, the down regulation of these pathways is attributed to the action of serine/threonine phosphatases [115, 116].

The ERK1/2 pathway has been shown to be activated by growth factors, serum, cytokines, and microtubule disorganization [117-119]. The two isoforms of the ERK pathway, ERK 1 and ERK2 show an 83% similarity in amino acid sequences. Both isoforms are shown to be expressed in all tissues. Over 150 substrates have been identified for ERK, displaying a diverse range of physiological functions ranging from regulation of the cell cycle, cell survival and apoptosis to cell differentiation and development. Two kinases, MEK1 and MEK2, have been identified as upstream activators of ERK1/2 which phosphorylate a conserved Thr-Glu-Tyr motif within the ERK activation loop allowing relocalization to the nucleus (Figure1-2). Inhibition of MEK1/2 will prevent activation of ERK1/2.

The second pathway within the MAPK family includes the JNK pathway, named because of the role this pathway plays in the phosphorylation and activation of c-jun, a transcription factor. Ten different isoforms of JNK have been identified, and they are activated by numerous stimuli including cytokines, UV irradiation, growth factor deprivation, and DNA damaging agents [120-123]. The JNK pathway is phosphorylated by the upstream kinases MEK4 and MEK7 within an activation domain consisting of a Thr-Pro-Tyr motif (Figure 1-2). An important downstream effect of the JNK pathway is regulation of protein degradation. Higher levels of

JNK are also present in several cancer cell lines, while long term activation of JNK has been shown to mediate cell apoptosis, whereas transient activation leads to cell survival [124]. JNK has also been shown to play a role in T cell differentiation [125, 126].

The p38 pathway has four known isoforms: p38α/β/γ/δ. These isoforms show only a 60% similarity in sequences and the same stimuli that have been shown to activate the JNK pathway also activate the p38 pathway, which include environmental stress, inflammatory cytokines, and growth factors [127-129]. The p38 pathway is activated by MEK3 and MEK6 and has over 25 known substrates (Figure 1-2). Previous studies have shown p38 plays an important role in macrophage and neutrophil respiratory burst activity, chemotaxis, adherence, apoptosis and T-cell differentiation and apoptosis [130-134].

These kinases integrate and amplify signals with a wide variety of overlapping responses. LT cleaves the N-terminus of all MEKs (except MEK5), thereby inactivating downstream signaling of the p38, ERK, and JNK pathways. The consequences of LT's inhibition of the MEK pathways has been documented to have a wide range of effects on host cells. For example, LT has been documented to block cytokine release in macrophages, increase apoptosis and cell permeability in endothelial cells, block neutrophil motility, and decrease proliferation of lymphocytes [64, 70-75]. Here, we are providing an additional role of LT in which cleavage of the MAPKKs inhibits cAMP production. To date, inhibition of the MEK pathways has not been shown to effect cAMP production.

Monocytes

Blood monocytes are mononuclear cells that play a major role in the host immune response through regulation of inflammatory responses, secretion of cytokine and antimicrobial factors, and direct pathogen clearance [135]. They compose an important component of the innate immune system, along with neutrophils, macrophages, and dendritic cells. Monocytes are highly

dynamic cells that are capable of differentiation into multiple effector cells with distinct antimicrobial functions. Two main subsets of monocytes have been defined. During inflammation, a subset of monocytes containing the chemokine receptor 2 (CCR2) traffic to sites of infection in response to chemokine ligand 2 (CCL2) also known as monocyte chemotactic protein 1 (MCP1) and are referred to as inflammatory monocytes. The other subset of monocytes constitutively replenishes the tissue macrophage and dendritic cell pools in the absence of inflammation [136, 137], although more recent studies have shown some resident macrophages are maintained via local proliferation [138-140].

In humans, these monocyte subsets are defined based on the surface expression of CD14 or CD16. CD14+CD16- monocytes express CCR2 and compose roughly 90% of the circulating monocyte pool, while CD14+CD16+ and CD14++CD16+ are considered "patrolling" monocytes and display anti-viral responses [141]. Monocytes are derived from monoblasts in the bone marrow which are differentiated from haematopoietic stem cells, and circulate in the blood for 1-2 days before they migrate into tissues where they replenish the macrophage and dendritic pools [142-144]. It is estimated that nearly 340 million monocytes leave the periphery daily [145].

After monocytes leave the bone marrow and circulate throughout the periphery, they are recruited to various areas within the body, where their temporal localization dictate the type of cell they differentiate into, i.e. alveolar macrophages (lungs), kupffer cells (liver), microglia (brain), and osteoclasts (bone). The process by which monocytes migrate from the periphery to the tissues is termed diapedesis. Diapedesis begins with low-affinity attachment of monocytes to the endothelial via selectins, followed by high affinity anchoring of monocytes via integrins. After attachment, monocytes chemotax through the endothelium towards a chemokine gradient (MCP-1, IL-8) to sites of infection (Figure 1-6). Here they are activated and depending on the

surrounding microenvironment, differentiate into macrophages or dendritic cells to assist in clearance of invading pathogens. For differentiation into macrophages, granulocyte macrophage colony stimulating factor (GM-CSF) has been shown to play a role in this process while IL-4 and GM-CSF treated human peripheral monocytes have been shown to differentiate into dendritic cells.

Platelets

Platelets are anuclear fragments of megakaryocytes that aid in hemostasis and are found only in mammals. The function of the platelet is to maintain the vascular system's structure and prevent the loss of blood from the circulation. Platelets maintain the vascular integrity through the formation of thrombi, also known as a "platelet plug". Platelets are 3.0μM in width and circulate for ~10 days before being cleared by the spleen [146]. The events leading up to thrombosis is a highly regulated process, where a defect in platelet function can lead to severe blood loss or to diseases such acute myocardial infarction and ischemia.

Platelets are anuclear, so all of their molecular components must be pre-synthesized. They harbor storage granules, termed alpha or dense granules, which provide important components required for their function including receptors (P-selectin, glycoproteins), growth factors, coagulation proteins, integrins, and adhesion molecules. Upon activation, platelets begin to expand, release their granules, and bind to the plasma protein fibrinogen, forming a clot. Platelets can become activated, typically at sites of endothelial injury, by a number of agonists including adenosine diphosphate, thrombin, epinephrine, thromboxane A_2 serotonin, collagen, and prostaglandin E_2.

The activation of platelets by external stimuli is associated with a subsequent activation of the glycoprotein (GP) IIb-IIIa receptor, termed inside-out signaling. GPIIb-IIIa is also known as the fibrinogen receptor and its activation results in an increased affinity for fibrinogen [147].

Fibrinogen is a circulating plasma glycoprotein that binds platelets through their GPIIb-IIIa receptors. Binding of the GPIIb-IIIa receptor also leads to activation signals within platelets, termed outside-in signaling. This type of activation also requires integrin clustering [148].

The formation of a clot can be divided into three main events: adhesion of platelets to the endothelium, activation of bound platelets, and aggregation of platelets to form a thrombus (Figure 1-7). The initial low affinity interaction with platelets and the endothelium is through the platelet GPIb/IX/V with activated von Willebrand factor (vWF) on the endothelium. This initial interaction is termed "tethering", which is followed by additional interactions with platelet integrins (GPIa-IIa and GPIIb-IIIa) leading to a firm adherence of platelets to collagen [149, 150]. Platelets are then activated following receptor ligation, along with the potent platelet stimulus thrombin. This activation leads to degranulation of vWF, fibrinogen and ADP, resulting in platelet aggregation and formation of a platelet plug.

The platelet cytoskeleton plays a major role during activation of platelets. Platelets circulate in the periphery as disc shaped cells and eventually become activated, spreading out and flattening over damaged areas of endothelium. A major cytoskeleton component, actin, is the most abundant platelet protein. It forms 2000-5000 linear actin filaments in platelets with over 2 million molecules of actin per platelet [151, 152]. Previous studies with anthrax LT in neutrophils show LT indirectly sequesters actin from growing filaments, preventing formation of a functional actin cytoskeleton [76]. Because of the major contribution actin plays during platelet activation, and the known inhibitory effects of LT of actin assembly, we sought to determine the effects of anthrax LT on the platelet cytoskeleton.

Effects of Anthrax Toxins on Peripheral Blood Cells

LT is shown to have an overall inhibitory effect on immune cells. Unlike murine cells, human cells fail to undergo rapid lysis by LT, but in many cases LT does induce cell cycle arrest,

resulting in a halt in cell proliferation. It has been shown that differentiated human monocytic cell lines (U-937, HL-60 and THP-1), along with activated human mononuclear cells, are susceptible to LT-mediated apoptosis over longer time periods (up to 24 hours) [153, 154]. LT toxicity in dendritic cells varies with some reports showing resistance to LT-induced cell death up to 48 hours, while other reports of human DC show LT-induced apoptosis [69, 155].

ET has not been shown to induce cytotoxicity in DC, macrophages, T-cells, neutrophils, and human microvascular endothelial cells [156-161]. Production of cAMP has not been associated with cell death, in fact increased cAMP levels has been associated with an increase in macrophage survival, along with intricate pro- and anti-inflammatory effects on monocytes, DC and lymphocytes [162].

Many in vivo studies with LT and ET suggest animals succumb to anthrax toxins via vascular collapse. Toxin treated animals have shown hemorrhaging lesions, increased heart rate, reduced cardiac output and loss of circulatory fluids [68, 163-165]. Because platelets play a critical role in maintaining hemostasis, the effects of ET and LT on platelet function have been previously investigated. It was revealed that LT decreases human blood clotting time and decreases platelet's interactions with endothelial cells [166]. Another study using rabbit platelets and ET shows decreased thrombin-induced platelet aggregation and clotting [167]. The exact effects anthrax toxins are playing on human platelets and their contribution to vascular collapse requires further investigation.

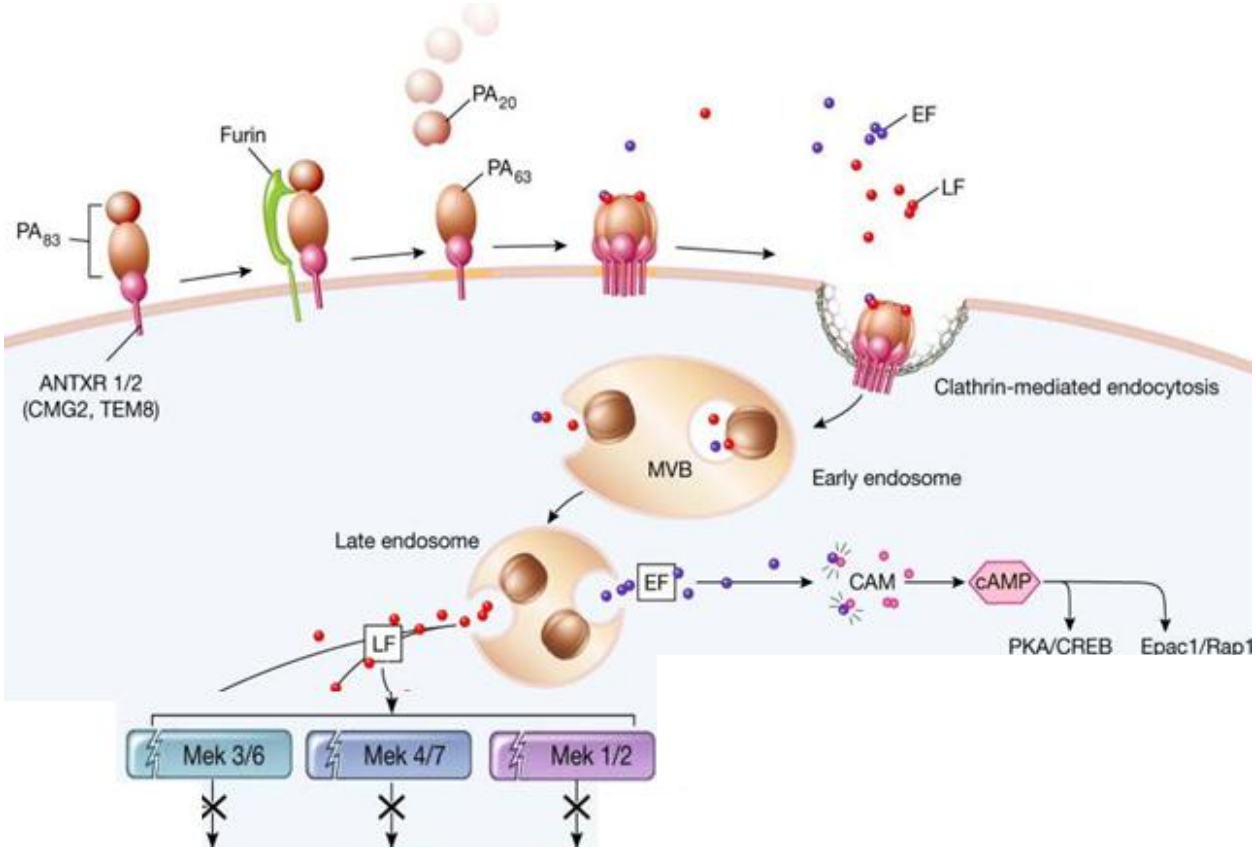


Figure 1-1. Anthrax toxins. *B. anthracis* secretes three exotoxins: protective antigen (PA), edema factor (EF) and lethal factor (LF). PA binds to the host cell, is cleaved by furin into its active conformation, and binds to host cell receptors leading to the formation of a heptamer complex. This complex is bound by LF and /or EF and is endocytosed into cells. The acidic compartments of the late endosomes induce a pore complex allowing EF/LF to enter the cell cytosol. Within the cytosol, LF cleaves the N-terminus of mitogen-activated protein kinase kinases, while EF increases intracellular levels of cAMP [168].

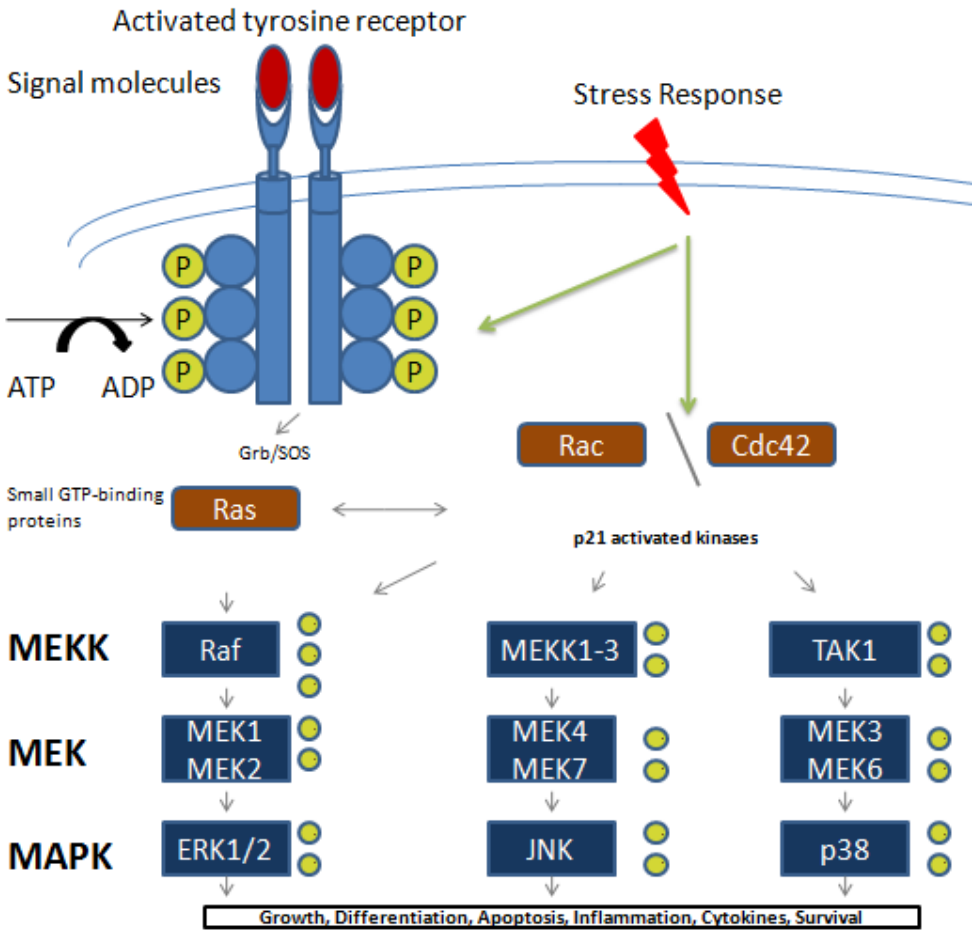


Figure 1-2. Mitogen Activated Protein Kinase Kinase (MAPKK or MEK) signaling pathway. Kinases function by phosphorylating downstream components. There are 3 MAPK pathways which are activated by the MAPKK pathway. This includes the ERK pathway which is activated by MEK1/2, the JNK pathway which is activated by MEK4/7, and the p38 pathway which is activated by MEK3/6. These pathways play a major role in response to stress and also contribute to cell growth, differentiation, apoptosis, and inflammation.

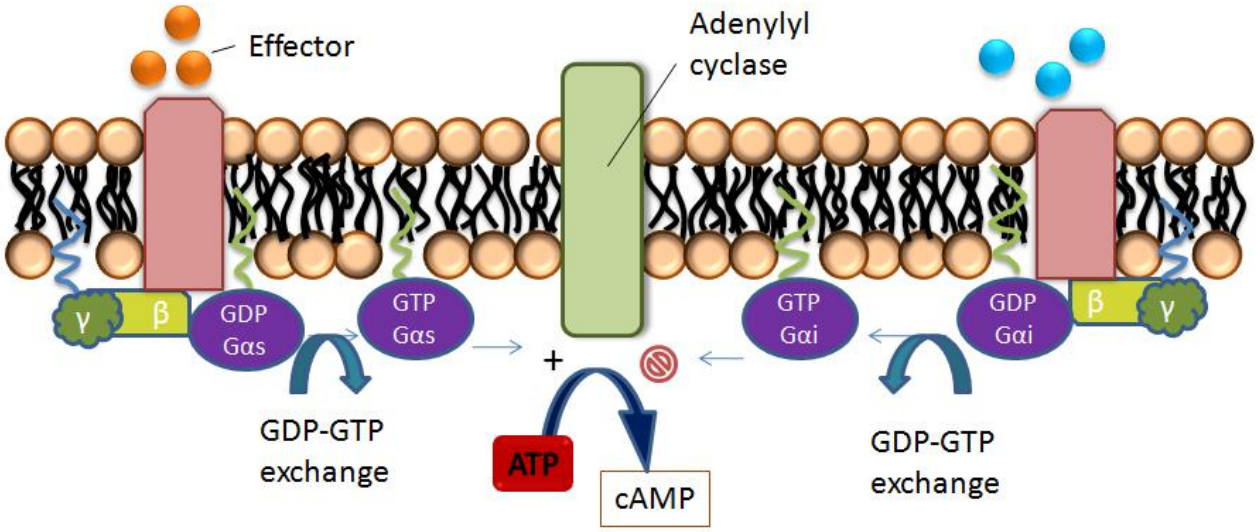


Figure 1-3. Regulation of adenylyl cyclases. Adenylyl cyclases are enzymes which produce cAMP from ATP and are embedded within the cell membrane. These enzymes are regulated by G-protein coupled receptors. Upon receptor stimulation the heterotrimeric G-protein complex dissociates into 2 subunits. An alpha subunit and a beta/gamma subunit. The G-alpha subunit is the subunit which interacts with adenylyl cyclases and can either activate or inhibit their activity. Inhibitory G-alpha subunits, denoted G-alpha i bind to adenylyl cyclases inhibiting the production of cAMP, while G-alpha stimulatory subunits, denoted G-alpha s, induce production of cAMP.

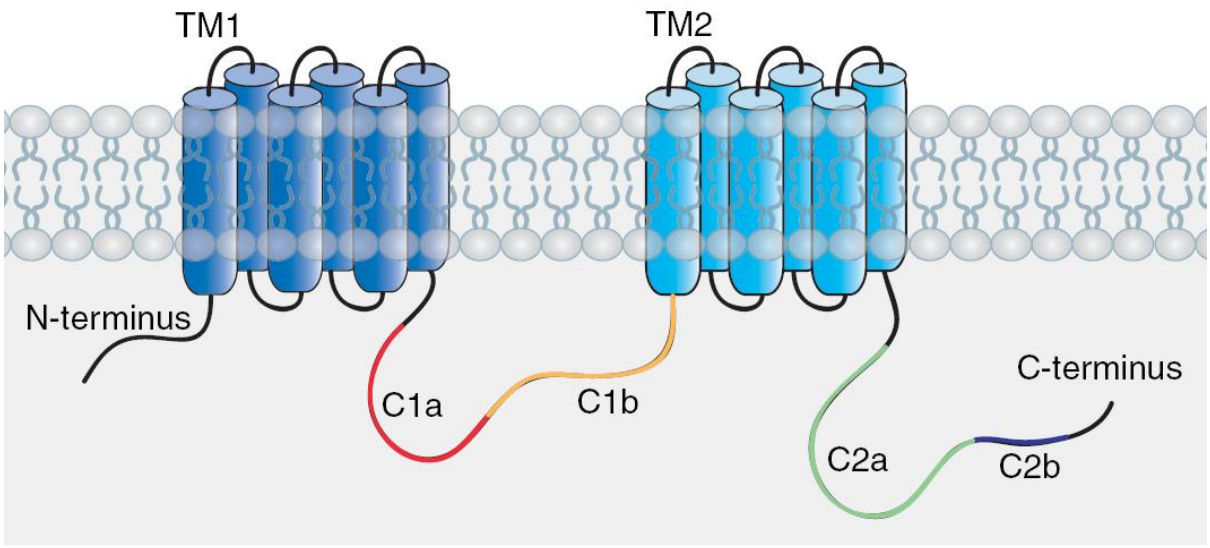


Figure 1-4. Molecular structure of adenylyl cyclase. The general structures of the nine transmembrane mammalian adenylyl cyclases have a calculated mass of 120-150 kD. They contain an N-terminal domain, two cytoplasmic catalytic domains and twelve transmembrane domains. The catalytic domains are 50-70% similar in amino acid sequence between mammalian adenylyl cyclases. Both catalytic domains are required for functional activity and activation of adenylyl cyclases involves a confirmation change in which both the catalytic domains (C1a and C2a) are in close proximity. The C1b and C2b domains are predicted to play a regulatory role as these domains are not required for catalytic activity [169].

	Response to cAMP signaling pathway component[a]					
AC isoform	**Gαs**	**Gαi**	**G$\beta\gamma$**	**FSK**	**Calcium**	**Protein kinases**
AC1	↑	↓ (CaM- or FSK-stimulated activities)	↓	↑	↑ (CaM) ↓ (CaM kinase IV)	↑ PKC (weak) ↓ (CaM kinase IV)
AC2	↑	→	↑ (when stimulated by Gαs)	↑		↑ (PKC)
AC3	↑	↓		↑	↑ (CaM) (in vitro) ↓ (CaM kinase II)	↑ (PKC) (weak) ↓ (CaM kinase II)
AC4	↑		↑	↑		↑(PKC)
AC5	↑	↓	↓ ($\beta1\gamma2$)	↑	↓ (<1 μM)	↓ (PKA) ↑ (PKCα/ζ)
AC6	↑	↓	↓ ($\beta1\gamma2$)	↑	↓ (<1 μM)	↓ (PKA, PKC)
AC7	↑		↑	↑		↑ (PKC)
AC8	↑	↓ (Ca^{2+} rises)			↑ (CaM)	→ (PKC)
AC9	↑	↓		↑ (weak)	↓ (calcineurin)	
sAC	→	→		→		

[a]↑, positive regulatory response; ↓, negative regulatory response; →, neutral response.

Figure 1-5. Isoforms of mammalian adenylyl cyclases. Ten isoforms of mammalian adenylyl cyclases exist, with each being differentially regulated and/or expressed. All isoforms except sAC are inhibited by P-site inhibitors, whereas all but isoform 9 and the sAC are stimulated by forskolin [170].

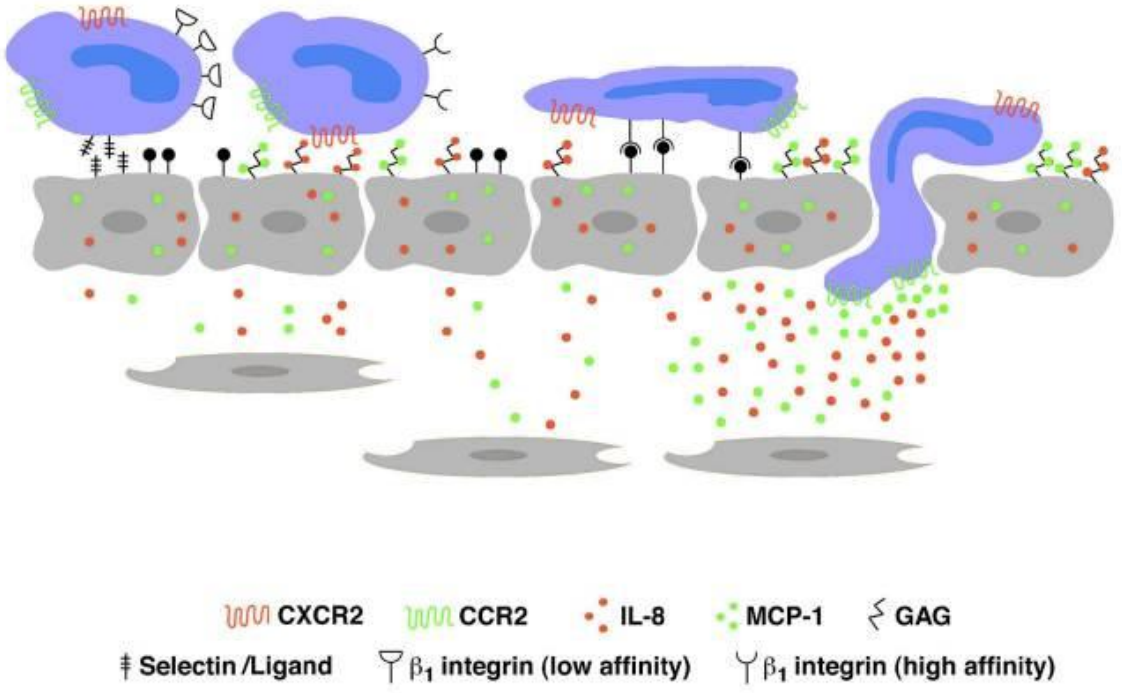


Figure 1-6. Monocyte attachment and diapedesis. Endothelial cells express multiple receptors facilitating attachment of monocytes (selectins, glycosaminoglycans). This low-affinity adhesion (selectins CCR2 and CXCR2) is followed by an increase in adhesion receptor and firm adhesion. Once monocytes have adhered to the endothelial, they diapedis through the endothelial towards areas of high chemokine production (MCP-1 and IL-8). Here, they replenish the macrophage and dendritic pool and function to assist in clearance of invading pathogens [171].

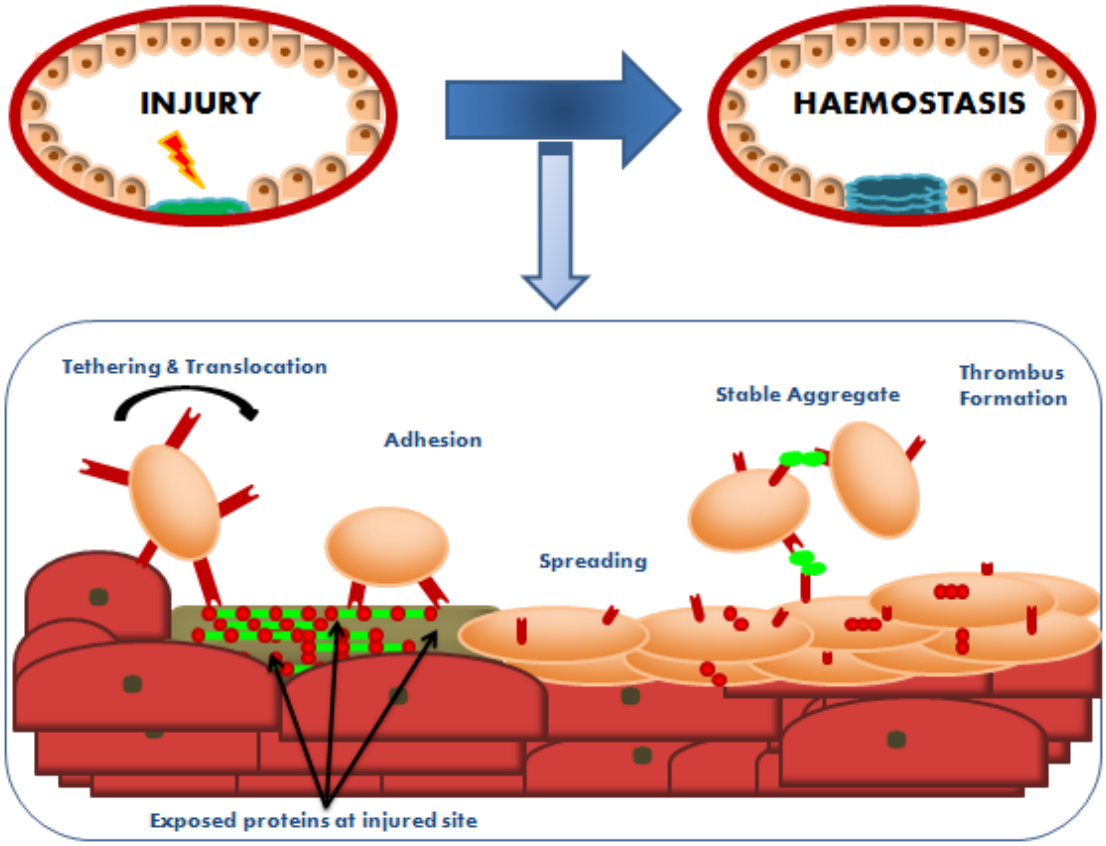


Figure 1-7. Platelet function. Platelets are small anuclear fragments of megakaryocytes which play a role in hemostasis. Upon injury of vesicles, platelets bind to exposed proteins on the severed endothelium. After adhesion, platelets are activated and their actin cytoskeletons begin to expand, sealing off the area of injury. Platelets bind to fibrin, allowing stable aggregates to form and this is known as a clot.

CHAPTER 2
ANTHRAX LETHAL TOXIN'S INHIBITION OF ANTHRAX EDEMA TOXIN

Overview

An extensive body of research has unraveled the mechanisms of independent actions of lethal toxin and edema toxin; however, the affects of combined toxins has never been fully elucidated. Our lab has found a very surprising effect after dual anthrax toxin treatment that has never been reported. We have found that treatment of human neutrophils and monocytes with lethal toxin and edema toxin combined leads to a decrease in cAMP production (unpublished data).

To date, there has only been one study measuring cAMP levels after dual toxin treatment. This study concluded a lower level of cAMP production by ET after LT treatment, and attributed this result to a decrease in ET entry, due to binding of the same toxin component required for entry into cells [54]. Another study has shown that mice found to be previously resistant to the effects of LT can become susceptible after treatment with sublethal doses of ET [50]. Other studies have found that dual toxin treatment worsens shock in a rat infusion model, but no studies have focused on the underlying signaling events [163]. Here, we determine the effects of dual toxin treatment on host signaling pathways and further elucidate the mechanisms behind these effects.

Materials and Methods

Toxin Purification

Edema factor (EF) was expressed and purified from E. coli as previously described [172]. Protective antigen (PA) and lethal factor (LF) were purified from *B. anthracis* as previously described [173]. Briefly, culture media was filtered through a 0.22 µM filter, followed by diethylaminoethyl cellulose (DEAE) anion exchange chromatography. The toxins were then

subjected to gel filtration and hydrophobic interaction fast protein liquid chromatography (FPLC) and highly purified toxins were confirmed by coomassie blue staining. ET refers to the combination of EF plus PA while LT refers to the combination of LF plus PA.

Chemicals Used

Positive controls for cAMP include Forskolin (Fsk, Sigma-Aldrich), and 3-isobutyl-1-methylaxthine (IBMX, Sigma-Aldrich). MEK inhibitors included the p38 inhibitor SB203580 (Calbiochem), the Erk1/2 inhibitors is PD98059 (Calbiochem) U-0126 (Cell Signaling), and CI-1040, and the JNK inhibitor is SP600125 (Sigma). Antibodies used were anti-MEK antibody (Cell Signaling). The metalloprotease inhibitor GM-6001 was obtained from Millipore.

Primary Cell Isolation and Treatment with ET

Human neutrophils and monocytes were purified using a Ficoll-Hypaque gradient as previously described [72]. For monocyte isolation, a negative selection antibody cocktail (Roche) was mixed with whole blood before separation over a Ficoll-Hypaque gradient. The study followed US Department of Health and Human Services guidelines and was approved by the Institutional Review Board at the University of Florida. Healthy volunteer donors ranged in age from 25-58 years old and included both males and females. Purified neutrophils and monocytes were re-suspended in RPMI with L-glutamine (Mediatech), and adjusted for 1 X 10^6 cells/ml. Cells were treated with toxins for the indicated time points at 37°C while gently rotating the cells to prevent clumping or cell activation. Control cells were incubated with buffer alone. All isolated cells were used immediately after isolation and neutrophil studies were completed within five hours of blood drawing.

Cell Culture

HeLa cells were grown in Dulbecco's modified eagles media (DMEM) with 4.5g/L glucose supplemented with 10% fetal bovine serum (FBS) and 5% penicillin and streptomycin

(Cellgro). THP-1 cells (ATCC) were grown in RPMI-1640 (Cellgro) supplemented with 10% fetal bovine serum (FBS) and 5% penicillin and streptomycin (Cellgro) and used at a concentration of 1 x 10^6 cells/mL.

Intracellular Cyclic-AMP Levels

Cyclic-AMP levels were determined using either an enzyme-linked immunoassay (Amersham Biosciences or Arbor Assay) or a cAMP Glo-Assay (Promega), as previously described [158]. Cells were treated with varying concentrations of ET as indicated for 2 hours or with 10μM forskolin plus 100μM IBMX for 20 minutes. All incubations were carried out at 37°C. Cells were pelleted, lysed, and sonicated. Intracellular cAMP levels were measured in a 96-well plate and samples were run in duplicates.

Western Blot Analysis

Cell extracts were subjected to western blot analysis using an antibody directed against the amino-terminus MEK, as described previously [58]. This antibody binds epitopes on the first seven amino acids of MEK, the region cleaved by LT, and loss of antibody reactivity indicates proteolysis by LT. Cells were treated with toxins, as indicated, lysed in lysis buffer (1% Triton X-100, 50mM TrisCl, 150mM KCl, 50mM EDTA, 0.2% NaA2, 200mM imidazole, 100mM NaFl, 100mM Na3VO4) plus complete mini protease inhibitor cocktail tablet (Roche), sonicated, and 40ug total protein was loaded to gels. Gels were ran for 1 hour at 100 V and transferred to a polyvinylidene fluoride (PVDF) membrane. All blocking and antibody incubation was in Tris-Buffered Saline and Tween 20 containing 5% milk.

Extracellular ATP and cAMP Measurement

HeLa cells were treated for 2 hours with buffer alone, 500ng/mL EF plus 500ng/mL PA or with 500ng/mL EF plus 2000ng/mL PA plus 500ng/mL LF at 37°C. For Triton samples, 0.1% Triton was added and cells were sonicated. All samples were pelleted at 800 x g for 30 seconds

and supernatant were collected, while cell pellets were lysed and sonicated. Both intracellular and extracellular levels of ATP was assayed using an ELITEN® ATP assay (Promega), while cAMP was measured using an ELISA (Arbor Assays).

LT Metalloprotease Inhibitor

HeLa cells or THP-1 cells were treated 2 hours at 37°C with buffer alone, 500ng/mL EF plus 500ng/mL PA, with 500ng/mL EF plus 2000ng/mL PA plus 500ng/mL LF or with 500ng/mL EF plus 2000ng/mL PA plus 500ng/mL LF which had been pre-incubated for 10 minutes with 44uM GM-6001. Cells were assayed for intracellular cAMP levels (Arbor Assays) or lysed and analyzed for MEK1 cleavage using Western Blot Analysis, as previously described above.

HeLa Cell Extracts and cAMP Production

HeLa cell extracts were prepared by sonication of cells. Extracts were incubated with varying concentrations (250ng or 500ng) of EF at varying time point (2 minutes, 10 minutes, 20 minutes). The optimal concentration and time point for cAMP production of 500ng for 10 minutes was used for dual toxin treatment experiments. For LT experiments, HeLa cells were pre-treated with 500ng/mL LT for 2 hours. Cell extracts were then treated with buffer alone, 500ng EF, or 500ng EF plus 500ng LF and cAMP production was measured.

2-D gel analysis

For 2-D analysis of HeLa cells, cells were treated with either 500ng/mL EF plus 500ng/mL PA or with 500ng/mL EF plus 2000ng/mL PA plus 500ng/mL LF for 4 hours. Inhibition of cAMP production was confirmed using an ELISA. HeLa cells were lysed and 50 µg each of Cy3- ET treated and Cy5-ET plus LT treated extracts were subjected to isoelectric focusing in a 24 cm pH 3 to 10 IPG strip at 10 kVolt for 98 kilovolt-hours before analyzed in a 24 x 24 cm 8 to 16% SDS PAGE.

Results

Anthrax ET is Inhibited by Anthrax LT

It is well-known that ET increases intracellular cAMP levels, but here we show that this increase in cAMP is inhibited by LT. As the concentration of LT is increased, there is a dose-dependent inhibition of cAMP production. HeLa cells were treated for 2 hours with 500ng/mL EF plus varying concentrations of LF (50ng/mL, 100ng/mL, 250ng/mL, and 500ng/mL) plus 2000ng/mL PA at 37°C. It is known that 2-3 subunits of EF and/or LF are capable of binding to PA [53]. Previous studies investigating dual toxin treatment predicted a decrease in ET-induced cAMP production due to decreased internalization of EF by competition with LF [54]. In order to assure PA concentration was not a limiting factor in EF entry, a higher concentration (2000ng/mL) of PA was utilized. As the concentration of LF reaches 250ng/mL, there is a maximum reduction in cAMP production (Figure 2-1A), as measured by a competitive ELISA.

To confirm our results using an alternative assay, we employed an indirect assay to measure cAMP production through downstream activation of PKA. HeLa cells were treated for 2 hours with 500ng/mL EF plus 500ng/mL LF plus 500ng/mL PA at 37°C. We confirm that ET increases cAMP levels in HeLa cells, while addition of LT along with ET inhibits cAMP production by ET (Figure 2-1B). Because cAMP is derived from ATP and cells undergoing cell death may have reduced ATP levels, we next measured viability in HeLa cells after dual toxin treatment. We find that treatment with both 500ng/mL EF plus 500ng/mL PA or with 500ng/mL EF plus 2000ng/mL PA plus 500ng/mL LF after a 2 hour treatment does not induce cell death as cells from all conditions remain viable (Figure 2-1C). Cell death was measured using an MTT viability assay.

We next sought to determine if inhibition of cAMP could be attributed to inhibition of the MEK pathways. Using chemical inhibitors to mimic the actions of LT inhibition of MAPKK, we

show that treatment with 3 different chemical inhibitors SB203580, U-0126, and CI-1040 also inhibit ET-induced cAMP production (Figure 2-1D). SB203580 competitively inhibits the p38 signaling pathway while U-0126 and CI-1040 non-competitively inhibit the ERK signaling pathway. Previous work in our lab has shown the JNK inhibitor also inhibits cAMP production (unpublished data). We conclude that an intact MAPKK signaling pathway positively regulates cAMP production by ET and when these pathways are inhibited, cAMP production is also inhibited.

Because our results only focus on HeLa cells, an epithelial cancer cell-line, we wanted to confirm our findings in other cell types. We tested LT inhibition of ET induced cAMP production in THP-1 cells, a human acute monocytic leukemia cell line, and primary human monocytes. Both cell types were treated for 2 hours with buffer alone, 500ng/mL EF plus 500ng/mL PA or with 500ng/mL EF plus 2000ng/mL PA plus 500ng/mL LF (Figure 2-1 E and F). Primary human monocytes and THP-1 cells showed increased production of cAMP after treatment with ET, while cells treated with ET plus LT showed an inhibition in cAMP production. We conclude inhibition of cAMP by LT is not dependent on cell type, as this phenomenon is seem in primary human monocytes and THP-1 cells.

Inhibition of cAMP is Through LT's Metalloprotease Activity

LT is known to cleave MEK 1-7, with the exception of MEK5. Some studies have predicted that LT has additional activity outside of cleavage of MEKs so we next sought to determine if inhibition of cAMP is through LT's metalloprotease activity. The metalloprotease inhibitor GM-6001 was used to block LT activity. Previous studies have shown inhibition of LT activity after treatment with GM-6001 [174]. HeLa cells were treated with buffer alone, ET, LT or LT that had been pre-incubated for 10 minutes with 44μM GM-6001 to inactivate the metalloprotease activity plus ET and intracellular cAMP levels were measured. As expected, ET

increased cAMP levels while dual toxin treatment inhibited this increase in cAMP. The ET/LT/GM sample had comparable levels of cAMP to ET treated indicating that LT's inhibition of cAMP is through its metalloprotease activity. GM-6001 treated HeLa cells did not increase cAMP levels. The samples were run in duplicate each time and the duplicate samples were assayed for MEK1 cleavage. After dual toxin treatment, MEK1 had been cleaved while in the presence of GM-6001, the levels of MEK1 returned to higher levels. This confirms the metalloprotease activity of LT had been inhibited in the presence of GM-6001. The same experiment was performed in THP-1 cells confirming LT's inhibition of cAMP production is through its metalloprotease activity (Figure 2-2C and D).

LT Does Not Cause Release of ATP or cAMP

LT is a zinc dependent metalloprotease that has been previously shown to open ATP connexin channels leading to release of ATP in mouse macrophages [175]. Because ATP is the substrate for cAMP, we next sought to determine if LT caused a similar release of ATP in HeLa cells. HeLa cells were treated with buffer alone, 500ng/mL EF plus 500ng/mL PA or with 500ng/mL EF plus 2000ng/mL PA plus 500ng/mL LF at 37°C for 2 hours. An additional sample with 500ng/mL EF plus 500ng/mL PA was treated with 0.1% Triton X-100 and sonicated before cells were pelleted to show maximal levels of extracellular ATP. The extracellular and lysed pellets (intracellular) were collected and assay for ATP. The extracellular levels of ATP were approximately 0.5 µM ATP in the control, ET and ET/LT samples, while treatment with ET/Triton showed extracellular levels of ATP near 1.3 µM (Figure 2-3A). The intracellular levels of ATP in the control, ET and ET/LT samples all showed similar levels again, while the ET/Triton contained insignificant levels of intracellular ATP (Figure 2-3B). It can be concluded from this experiment that dual toxin treatment does not lead to the release of ATP, thereby

depleting the substrate for cAMP. Therefore, LT's inhibition of cAMP cannot be attributed to a depletion in the substrate ATP.

We next sought to determine if LT was causing a release of cAMP, which could explain the lower levels of intracellular cAMP after treatment with LT. A previous study has found that cAMP can be secreted out of cells [176]. HeLa cells were treated with buffer alone, 500ng/mL ET or 500ng/mL ET plus 500ng/mL LT at 37°C for 2 hours. A fourth sample containing 500ng/mL ET was incubated for 2 hours at 37°C followed by the addition of 0.1% TritonX-100 and sonication. All samples were centrifuged, supernatants were collected, and pellets were lysed. Both intracellular and extracellular levels of cAMP were assessed. Intracellular levels of cAMP remained low in the control, ET/Triton, and dual toxin treated samples, while ET treatment caused a rise in cAMP levels as expected (Figure 2-3D). The extracellular levels of cAMP were increased in both the ET and ET/Triton samples, while in the control and dual toxin treated samples levels of cAMP were lower (Figure 2-3C). Based on these experimental results, we can conclude that dual toxin treated cells are not releasing cAMP into the extracellular media.

Forskolin Induced cAMP production is Inhibited by Anthrax LT

To better understand the extent of cAMP inhibition, we induced cAMP by exposing cells to forskolin (Fsk), a known stimulus of mammalian adenylyl cyclases, along with 3-isobutyl-1-methylxanthine (IBMX), a known phosodiesterase inhibitor. HeLa cells were treated with buffer alone, 10uM Fsk plus 100uM IBMX, or for 2 hours with 500ng/mL LF plus 500ng/mL PA followed by 10uM Fsk plus 100uM IBMX for 20 minutes. cAMP levels were measured using a PKA based assay and revealed that LT is also capable of inhibiting Fsk induced cAMP (Figure 2-2A). We next determined if inhibition of cAMP by Fsk was dose dependent. HeLa cells were treated with buffer alone or increasing concentrations of LF (50ng/mL, 100ng/mL, 250ng/mL)

plus 500ng/mL PA for 2 hours followed by the addition of 10uM Fsk plus 100uM IBMX for 20 minutes. Intracellular cAMP levels were measured and showed a dose-dependent inhibition of cAMP (Figure 2-2C).

Next, inhibitors were used to determine if they too could block production of Fsk induced cAMP. HeLa cells were treated with buffer alone, 10µM Fsk plus 100µM IBMX or with 10µM Fsk plus 100µM IBMX and one of the following inhibitors: 100µM SB203580, 20µM U-0126, or 100nM CI-1040. Intracellular levels of cAMP were measured and Fsk/IBMX induced cAMP levels as expected, while samples containing Fsk/IBMX plus inhibitors all showed lower levels of cAMP (Figure 2-2B). These results indicate the inhibition of the MAPKK pathways, using chemical inhibitors or LT, leads to an inhibition in Fsk induced cAMP production.

LT Does Not Inhibit cAMP production by EF in Cell Extracts

Next, an *in vitro* assay was used to determine if LT could also inhibit cAMP production in cell extracts. For these experiments, HeLa cell extracts were utilized. First, cell extracts were treated for 2 minutes, 10 minutes, or 20 minutes with buffer alone, 250ng EF or 500ng EF and cAMP levels were assessed. It was found that optimal production of cAMP by EF occured after a 10 minute incubation with 500ng EF (Figure 2-5) using extracts from an initial Hela cell concentration of 2×10^5 cells (Figure 2-6A). This time and concentration of EF is consistent with a previous study measuring EF activity *in vitro* [54]. After activity of EF in cell extracts was confirmed, HeLa cells were pelleted, sonicated then treated for 10 minutes with buffer alone, 500ng EF, 500ng EF plus 500ng LF, or with 500ng EF after pre-treatment of HeLa cells with 500ng/mL LF plus 500ng/mL PA at 37°C for 2 hours. The control sample showed no production of cAMP, while EF induced high levels of cAMP, as expected. Surprisingly, Hela cells that had been pre-treated with LT still showed high levels of EF activity in cell extracts and extracts that contained both LF and EF had cAMP levels comparable to EF treated cell extracts alone (Figure

2-6B). These unexpected results show that cells must remain intact and LF is incapable of inhibiting cAMP production in cell extracts.

2-D Gel Analysis of ET and ET plus LT HeLa Cells

Because previous results indicated no inhibition of cAMP production by LT in cell extracts, we predicted a potential post-translational modification may be playing a role in the ability of LT to inhibit of cAMP production. It is known that LT inhibits the MAPKK pathways which phosphorylate downstream components. With this in mind, a 2-D gel was employed to help identify intermediate proteins which exhibit a decrease in phosphorylation levels after dual toxin treatment. These intermediate proteins could explain the mechanism behind LT's inhibition of cAMP. Hela cells were treated with either 500ng/mL EF plus 500ng/mL PA or with 500ng/mL EF plus 2000ng/mL PA plus 500ng/mL LF and subjected to isoelectric focusing before being run over SDS-PAGE. Results identified over 30 potential proteins (Table 1-1). These proteins are involved in a wide range of activities from transcriptional regulation, protein turnover, and regulation of the cytoskeleton to heat shock responses and glycolysis.

Discussion

The innate immune response is essential for successful clearance of invading pathogens. *B. anthracis* secretes the exotoxins ET and LT capable of impairing the host response during an anthrax infection. The MAPKK signaling cascade is essential for successful production of host immune responses including oxidative burst, pro-inflammatory cytokine expression, and cell motility [72, 177]. By cleaving all but one MAPKK, LT shuts down these signaling cascades. ET has also been shown to inhibit host cell responses including impairment of cytokine secretion, phagocytosis and neutrophil chemokinesis [56, 178-180]. Both ET and LT possess unique catalytic activities that aid in *B. anthracis* survival within host cells. The current hypothesis on the effects of anthrax toxins is that increased cAMP levels and inhibition of the MAPKK

signaling cascade combine to decrease immune cell function and allow survival of *B. anthracis* within the host [181].

During systemic anthrax infections, cells are exposed to both LT and ET. Studies to date have focused on the immunological consequences of either LT or ET. It is of interest that we now find that one toxin, LT, inhibits the actions of another, ET, in a dose dependent manner (Figure 2-1A). This phenomenon cannot be attributed to cell death, as treatment for up to 4 hours with both toxins does not induce cell death (Figure 2-1C). One possible explanation for this effect could be that continuously high levels of cAMP induced by ET may be harmful to *B. anthracis,* and therefore a mechanism to shut down this production could provide a survival advantage.

Because LT is known to block the MAPKK pathway, it was intriguing to discover that blocking of the individual pathways via chemical inhibitors was able to block the production of cAMP. Both non-competitive and competitive chemical inhibitors of ATP were employed to assure no indirect effects on cAMP production. We discovered that chemical inhibition of any individual MAPKK pathway decreased cAMP production by ET, suggesting that all three major pathways must be actively functioning to promote cAMP production (Figure 2-1D). The interaction between MAPKK pathways and cAMP is further supported by the observation that the ERK pathway can be activated by increased cAMP levels. This finding suggests a potential feedback mechanism regulated by cAMP levels could be applicable to all MAPKK pathways [182, 183].

Next, we determined if LT inhibition of cAMP affected mammalian adenylyl cyclases, in addition to ET, a bacterial adenylyl cylase. For these experiments, forskolin, a known inducer of mammalian adenylyl cyclase, was employed. Forskolin activates isoforms 1-8 of mammalian

adenylyl cyclases, while weakly activating isoform 9. Using HeLa cells, we found that LT inhibited forskolin induced cAMP levels and was dependent on the concentrations of LT (Figure 2-2C). We conclude that LT is capable of blocking not only ET induced cAMP levels, but also forskolin induced cAMP levels.

Because the chemical inhibitors of the MAPKK pathway, SB203580, U-0126, and CI-1040, blocked ET-induced cAMP production, we next determined if these inhibitors were also capable of blocking forskolin induced cAMP production. We found that all chemical inhibitors were able to block cAMP production induced by forskolin (Figure 2-2B). These experiments show that the MAPKK signal transduction pathways must remain intact in order to maintain successful production of cAMP by mammalian adenylyl cyclases. We are the first to report that the MAPKK pathways regulate the production of cAMP by both ET and forskolin.

Mechanistically, it is important to assure the results obtained are a consequence of LT's metalloprotease activity. Previous reports have indicated that LT can alter cell functions that are independent of its ability to cleave MAPKKs [184, 185]. To address this concern, the metalloprotease activity of LT was blocked and cAMP levels were assessed. It was found that pre-treatment of LF with the metalloprotease activity GM-6001 blocked cleavage of MEK1, and also blocked its inhibitory effects on cAMP production (Figure 2-2C and Figure 2-2D). This finding is consistent with the hypothesis that cleavage of MEKs is required for inhibition of cAMP production.

Previous reports have shown LT is capable of inducing the release of ATP extracellularly. This study indicated that LT blocks the phosphorylation of an ATP channel connexin-43 that, in turn, resulted in an opened channel and release of ATP [175]. This release in ATP led to a six fold increase in extracellular levels. Because the substrate for cAMP is ATP, it

was imperative to determine if these studies using mice macrophages would translate mechanistically to human cells. Using HeLa cells, we found that ATP was not being secreted from cells, as previously shown in mice macrophages (Figure 2-3A). We therefore concluded that the intracellular ATP pools were not being depleted by LT (Figure 2-3B) and the decreased cAMP production could not be explained by a loss of substrate.

Along with depleted ATP levels, it was essential to determine if LT induces a release of cAMP. Previous studies of skeletal muscle cells treated with forskolin, have demonstrated a release of cAMP into the extracellular medium [186]. This could be a plausable explanation for why intracellular cAMP levels are decreased after LT treatment. Using HeLa cells, we found that LT did not cause a release of cAMP outside of cells (Figure 2-3C). We conclude that the effects of anthrax LT are not due to the depletion of either ATP or cAMP.

In the hopes of unraveling the mechanisms behind LT inhibition of cAMP production, cell extracts were used. EF was capable of inducing cAMP levels in cell extracts, even in the presence of LF or in cell extracts that had been pre-treated with LT (Figure 2-6B). The findings indicate that cells must remain intact in order for cAMP production to be inhibited by LT.

Our findings raised another possibility that LT inhibition of cAMP production could be mediated by post-translation modifications of one or more regulatory proteins. To determine potential candidates for this process, 2DIGE gels of HeLa cell extracts were analyzed. Twenty-eight potential proteins were identified that could be playing a role in LT's inhibition of cAMP (Table 1-1). These candidate proteins are involved in actin assembly, glycolysis, stress response, and heat shock responses. Of interest is a protein involved in the regulation of phosphatases known as immunoglobulin-binding protein 1(IGBP-1). Phosphatases are enzymes which remove phosphate groups from other proteins/enzymes. IGBP-1 is associated with a family of

serine/threonine phosphatases known as protein phosphatase 2A. This family of phosphatases play a role in the regulation of various signal transduction cascades including MAPKK signaling, cell cycle regulation, and cell morphology and development [187-193].

EF has been shown to be associated with the endosomal membrane after translocation, while mammalian adenylyl cyclases remain within lipid rafts at the plasma membrane [194-197]. By disrupting adenylyl cyclase localization with the membrane, LT may be diminishing enzyme activity. It is interesting that multiple proteins were identified using 2DIGE that play a role in lipid transport, lipid metabolism, and membrane structure. These proteins included annexin A1, apolioprotein L2, endoplasmic reticulum resident protein 29, and enoyl-CoA hydratase. By disrupting the membrane structure required for successful enzyme activity, LT may be inactivating adenylyl cyclase activity.

Both endosomes and lipid rafts are in close contact with the actin cytoskeleton. Multiple proteins involved in actin regulation were identified by 2DIGE after dual toxin treatment. These proteins included isoform 1 of LIM and SH3 domain protein 1, tropomodulin-3, isoform beta of LIM domain and actin-binding protein 1, pirin, isoform 1 of plectin, heat shock protein beta-1, Src substrate cortactin, and isoform 2 of filamin-A. These proteins modify the actin cytoskeleton by regulating actin elongation and the branching of actin filaments (Table 1-1). Disruption of these actin regulatory proteins after dual toxin treatment could be preventing adequate spatial localization of adenylyl cyclases, thereby rendering them inactive.

Here, we have identified numerous protein targets modified by dual toxin treatment. Further investigations into how these proteins contribute to cAMP regulation will help further our understanding of this complex process. The proteins identified here have the potential to

provide new therapeutic targets for the treatment of infections that utilize cAMP, and may also provide new treatment options for other diseases associated with aberrant cAMP responses.

Table 1-1. Description of proteins excised for identification after treatments with ET + LT versus ET alone. ID refers to the spot(s) sent for identification. Some spots were pulled together to assure higher protein levels for the identification process

ID	Spot Number	Average Ratio after ET + LT	pI	MW
1	533	-1.04	5.52	81213
2	534	1.25	5.54	81529
3	1307	-1.42	5.93	42715
3	1313	-1.25	5.95	42715
4	1429	-1.31	5.34	37775
4	1435	-1.09	5.47	37775
5	1565	-1.35	6.02	32915
5	1568	-1.46	5.95	32915
6	1573	-1.69	6.27	32862
6	1574	-1.11	6.45	32705
7	1741	-1.36	5.85	24537
7	1743	-1.44	5.78	24468
7	1745	-1.14	5.88	24676
8	1807	-1.5	5.69	22804
8	1809	1.06	5.84	22777
8	1817	-1.14	5.74	22645
9	1929	-1.4	4.36	16806
9	1931	-1.09	4.49	16774

Table 1-2. Description of proteins differentially expressed in HeLa cells after 4 hour treatment with dual toxin LT +ET versus ET alone

Fold change	Protein	Accession Number	MW	Function
-1.69	Isoform 1 of LIM and SH3 domain protein 1	IPI00000861	30	Regulation of dynamic actin-based, cytoskeletal activities
-l.31	Tropomodulin-3	IPI00005087	40	Blocks the elongation and depolymerization of actin filaments
-1.04	Isoform Beta of LIM domain and actin-binding protein 1	IPI00008918	85	Increases actin stress fibers and inhibits membrane ruffling
-1.50	Peroxiredoxin-4	IPI00011937	31	Regulates the activation of NF-kappa-B in the cytosol
-1.42	Adenosylhomocysteinase	IPI00012007	48	
-1.50	Serine/Arginine-rich splicing factor 9	IPI00012340	26	Required for pre-mRNA splicing
-1.69	Pirin	IPI00012575	32	May play a role in the regulation of cell migration
-1.69	Isoform 1 of Heterogenous nuclear ribonucleoprotein H3	IPI00013877	37	__
-1.04	Isoform 1 of Plectin	IPI00014898	532	Role in cytoskeleton structure
-1.4	Prostaglandin E synthase 3	IPI00015029	19	Promoting disassembly of transcriptional regulatory complexes
-1.31	Immunoglobulin-binding protein 1	IPI00019148	39	Involved in regulation of phosphatases
-1.50	Endoplasmic reticulum resident protein 29	IPI00024911	29	Processing of secretory proteins within the endoplasmic reticulum
-1.50	Enoyl-CoA hydratase	IPI00024993	31	Lipid metabolism
-l.31	Cation-dependent mannose-6-phosphate receptor	IPI00025049	31	Transport of lysosomal enzymes to lysosomes

-1.50	Heat shock protein beta-1	IPI00025512	23	Involved in stress resistance and actin organization
-1.50	NADH dehydrogenase iron-sulfur protein 3	IPI00025796	30	Functions in the transfer of electrons from NADH to the respiratory chain
-1.04	Src substrate cortactin	IPI00029601	62	Organization of the actin cytoskeleton and cell structure
-1.69	Aldo-keto reductase family 1 member C1	IPI00029733	37	Converts progesterone to its inactive form
-1.46	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	IPI000218236	37	Essential for cell division
-1.46	Annexin A1	IPI00218918	39	Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis
-1.69	Glyceraldehyde-3-phosphate dehydrogenase	IPI00219018	36	Role in glycolysis
-1.69	L-lactate dehydrogenase B chain	IPI00219217	37	Role in glycolysis
-1.69	Isoform M1 of Pyruvate kinase isozymes M1/M2	IPI00220644	58	Glycolytic enzyme
-1.04	Isoform 2 of Filamin-A	IPI00302592	280	Promotes branching of actin filaments
-l.31	Serpin B6	IPI00413451	46	Protease inhibitor
-1.04	Heat shock protein HSP 90-beta	IPI0041676	83	Involved in stress response
-1.69	Isoform 2 of Annexin A2	IPI00418169	40	Involved in heat-stress response
-l.31	Isoform 2 of Suppressor of G2 allele of SKP1 homolog	IPI00791573	38	Role in ubiquitination
-1.46	Apolipoprotein L2	IPI00877964	37	Lipid transport

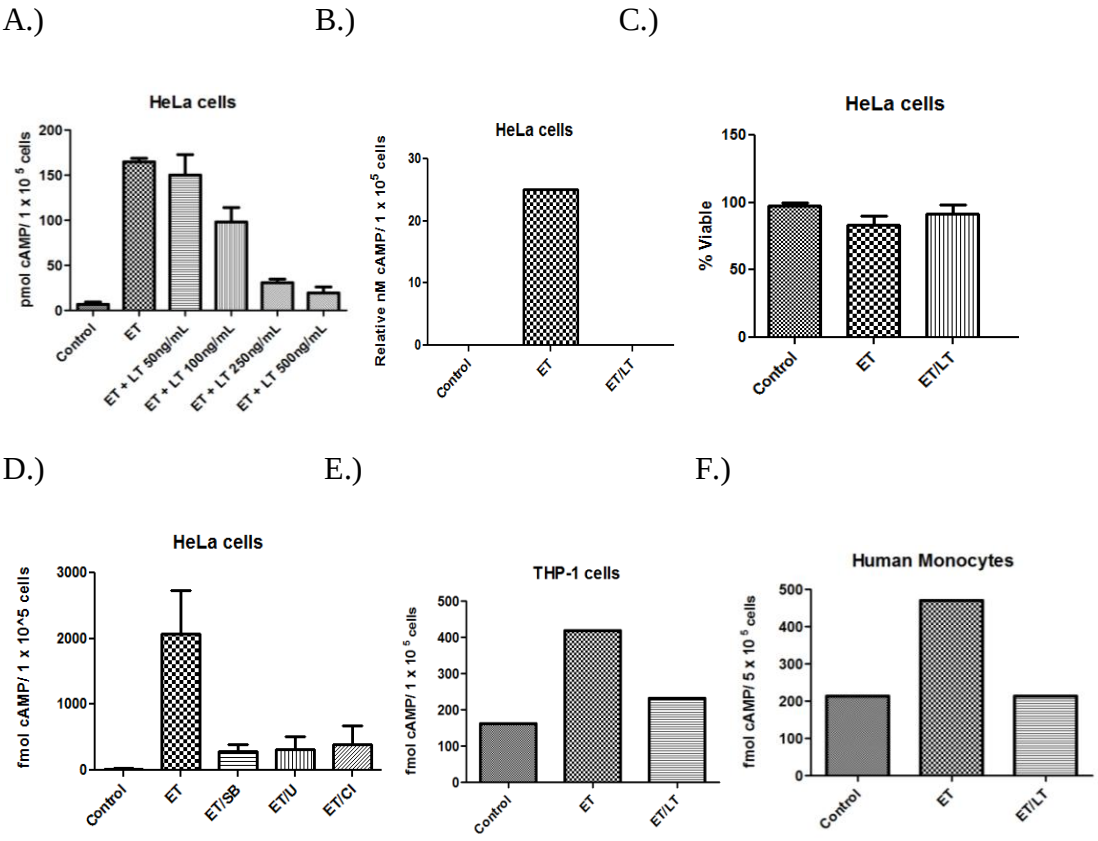
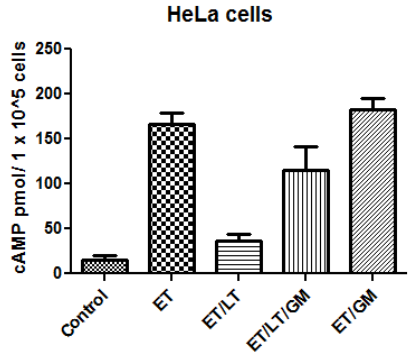
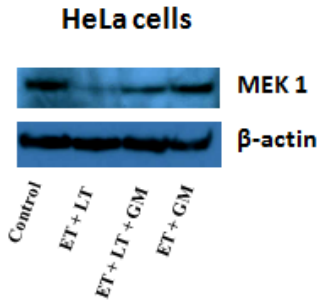


Figure 2-1. Effects of anthrax LT and MAPKKs inhibitors on cAMP production after treatment with anthrax ET. A.) Dose response of HeLa cells with increasing concentration of LT. Cells were treated with buffer alone (Control), 500ng/mL ET, or 500ng/mL ET with increasing concentration, 50ng/mL, 100ng/mL, 250ng/mL or 500ng/mL of LT. Error bars indicated the standard error of means (SEM) of 3 different experiments. C.) cAMP levels after treatment of HeLa cells with buffer alone (Control), 500ng/mL ET or 500ng ET plus 500ng LT. B.) Percentage of viable HeLa cells after treatment with buffer alone (Control), 500ng/mL ET or 500ng ET plus 500ng LT. Error bars indicate the SEM of 2 experiments. D.) cAMP levels in HeLa cells after treatment with buffer alone (Control), 500ng/mL ET or 500ng ET plus inhibitors (SB203580, U-0126, or CI-1040). Error bars represent the SEM of 3 different experiments. E.) Confirmation of inhibition of cAMP in another cell line. cAMP levels in THP-1cells after treatment with buffer alone (Control), 500ng/mL ET or 500ng ET plus 500ng LT. D.) Confirmation of cAMP inhibition in primary cells. cAMP levels in human monocytes after treatment with buffer alone (Control), 500ng/mL ET or 500ng ET plus 500ng LT.

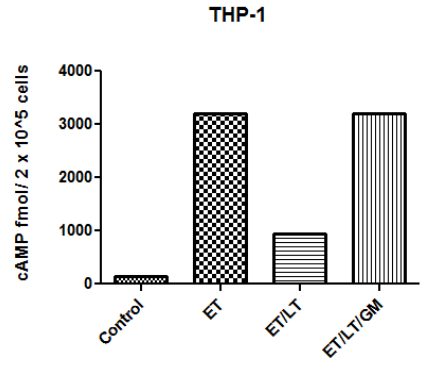
A.)



B.)



C.)



D.)

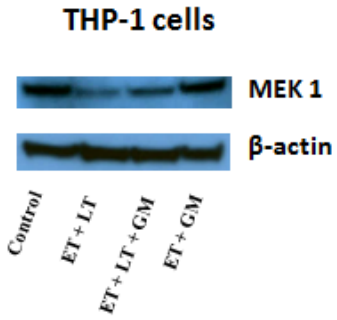


Figure 2-2. Effects of the inhibition of LT metalloprotease activity on ET induced cAMP production. A.) HeLa cells were treated with buffer alone (Control), 500ng/mL ET, 500ng ET plus 500ng/mL LT, 500ng/mL ET plus 500ng/mL LT plus 44µM GM-6001, or 500ng/mL ET plus 44µM GM-6001 and cAMP levels were determined. Error bars represent the SEM of 3 different experiments. B.) HeLa cells were treated with buffer alone (Control), 500ng/mL ET, 500ng ET plus 500ng/mL LT, 500ng/mL ET plus 500ng/mL LT plus 44µM GM-6001, or 500ng/mL ET plus 44µM GM-6001 and cleavage of MEK1 was measured using Western Blot analysis. C.) THP-1 cells were treated with buffer alone (Control), 500ng/mL ET, 500ng ET plus 500ng/mL LT, 500ng/mL ET plus 500ng/mL LT plus 44µM GM-6001, or 500ng/mL ET plus 44µM GM-6001 and cAMP levels were determined. Error bars represent the SEM of 3 different experiments. D.) THP-1 cells were treated with buffer alone (Control), 500ng/mL ET, 500ng ET plus 500ng/mL LT, 500ng/mL ET plus 500ng/mL LT plus 44µM GM-6001, or 500ng/mL ET plus 44µM GM-6001 and cleavage of MEK1 was measured using Western Blot analysis.

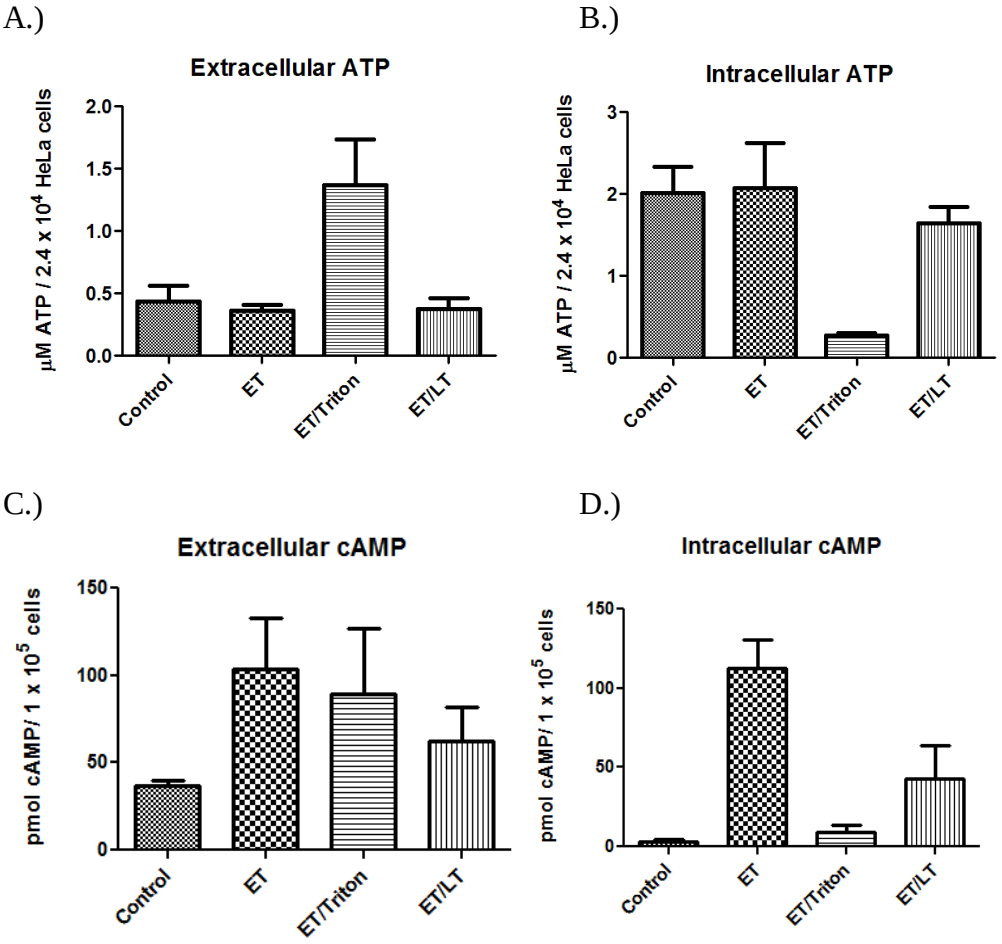
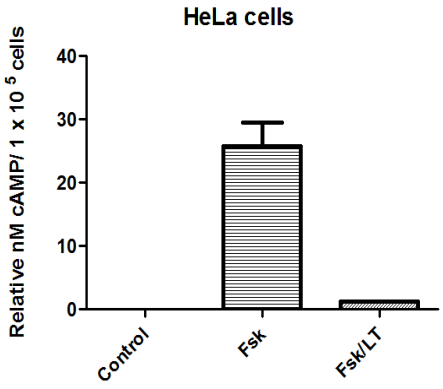


Figure 2-3. Effects of LT on the release of ATP or cAMP. A.) Extracellular ATP levels were measured in HeLa cells after treatment with buffer alone (Control), 500ng/mL ET, 500ng/mL ET plus 1% Triton, or 500ng ET plus 500ng LT. Error bars represent the SEM of 3 different experiments. B.) Intracellular ATP levels were measured in HeLa cells after treatment with buffer alone (Control), 500ng/mL ET, 500ng/mL ET plus 1% Triton, or 500ng ET plus 500ng/mL LT. Error bars represent the SEM of 3 different experiments. C.) Extracellular cAMP levels were measured in HeLa cells after treatment with buffer alone (Control), 500ng/mL ET, 500ng/mL ET plus 1% Triton, or 500ng ET plus 500ng LT. Error bars represent the SEM of 3 different experiments. D.) Intracellular cAMP levels were measured in HeLa cells after treatment with buffer alone (Control), 500ng/mL ET, 500ng/mL ET plus 1% Triton, or 500ng ET plus 500ng LT. Error bars represent the SEM of 3 different experiments.

A.)



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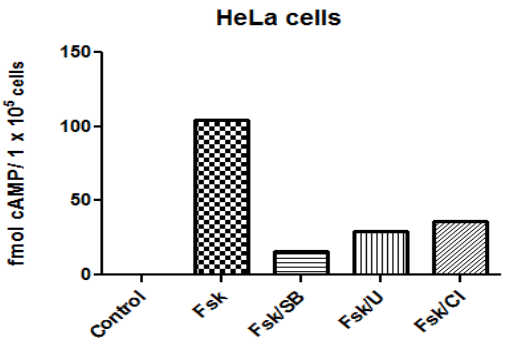


C.)

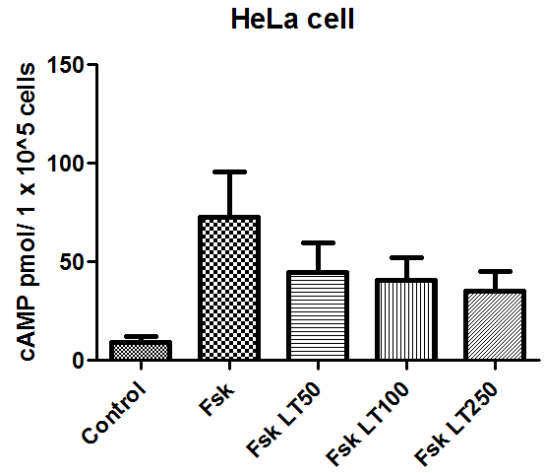
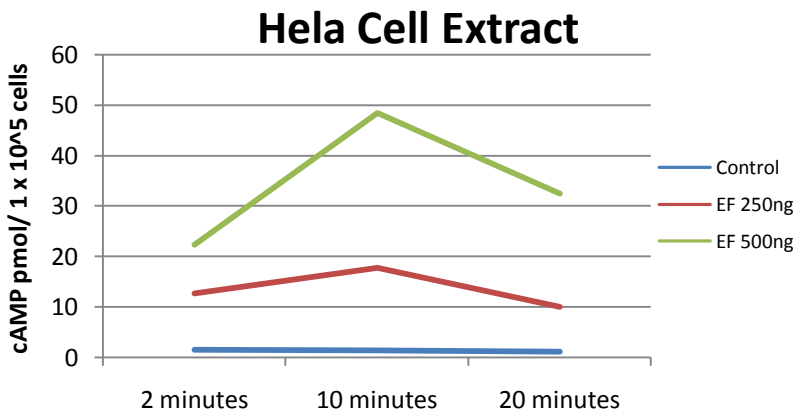


Figure 2-4. Effects of anthrax LT and inhibition of MAPKKs on cAMP production by Forskolin (Fsk). A.) cAMP levels in HeLa cells after treatment with buffer alone (Control), 10µM Fsk or 10µM Fsk plus 500ng LT. Error bars represent the SEM of 2 experiments. B) Inhibition of Fsk induced cAMP production by chemical inhibitor of MAPKKs in HeLa cells. HeLa cells were treated with buffer alone (Control), 10µM Fsk or 10µM Fsk plus inhibitors of MAPKKs (SB203580, U-0126, or CI-1040). C.) Dose response of HeLa cells treated with varying concentrations of LT. HeLa cells were treated with buffer alone (Control), 10µM Fsk or 10µM Fsk plus varying LT concentrations (50ng/mL, 100ng/mL, 250ng/mL or 500ng/mL). Error bars represent the SEM of 3 different experiments.

A.)



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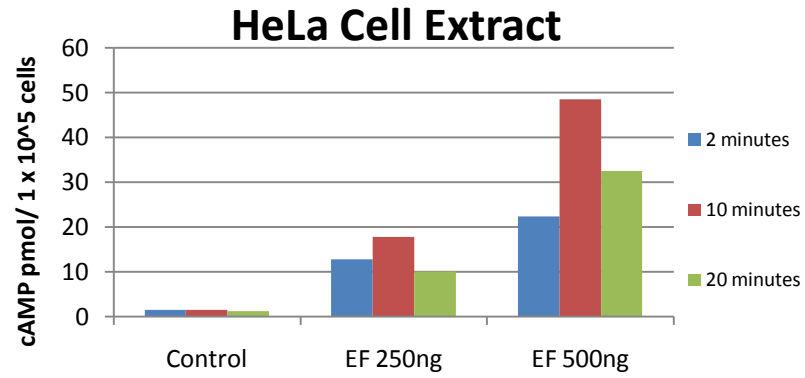
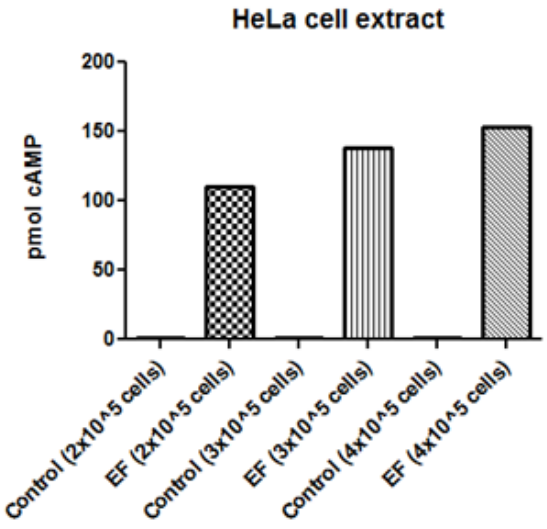


Figure 2-5. Adenylate cyclase activity of EF in HeLa cell extracts. A.) and B.) Production of cAMP after treatment of HeLa cell extracts with buffer alone, 250ng/mL EF or 500ng/mL EF. cAMP levels were measured at 2 minutes, 10 minutes and 20 minutes after addition of EF showing highest activity of EF at a concentration of 500ng and at 20 minutes. Graphs show cAMP levels and are plotted in relation to time on the x-axis (A) or treatment conditions (B).

A.)



B.)

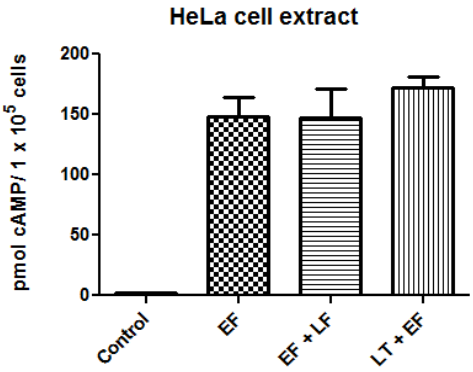
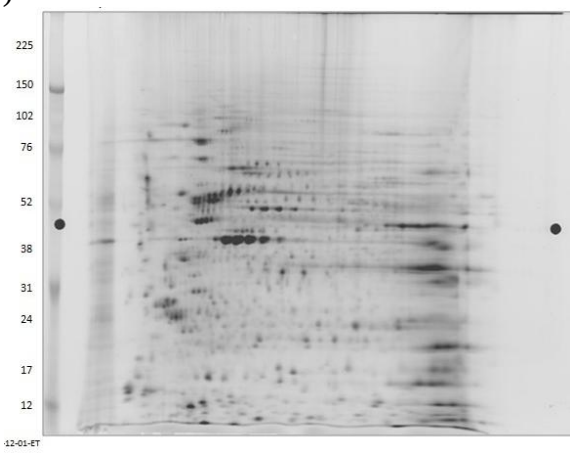


Figure 2-6. EF activity in HeLa cell extracts with addition of LT. A.) cAMP produced in HeLa cell extract using increasing numbers of cells. Extracts were treated for 10 minutes with buffer alone (Control) or 500ng/mL EF. B.) Production of cAMP after treatment of HeLa cell extracts for 10 minutes with buffer alone (Control), 500ng EF, 500ng EF plus 500ng LF, or pre-treatment with LT for 2 hours followed by treatment of extracts with 500ng EF.

A.)



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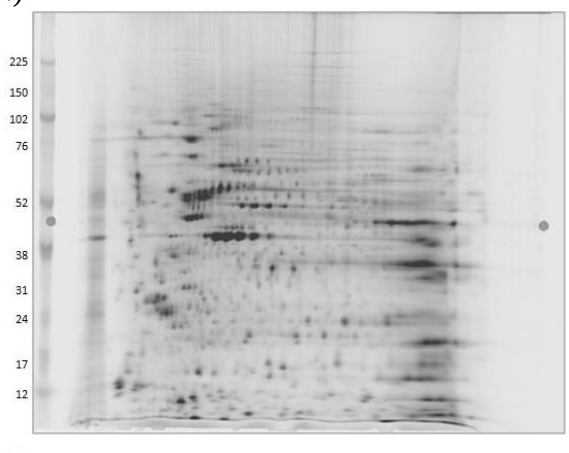

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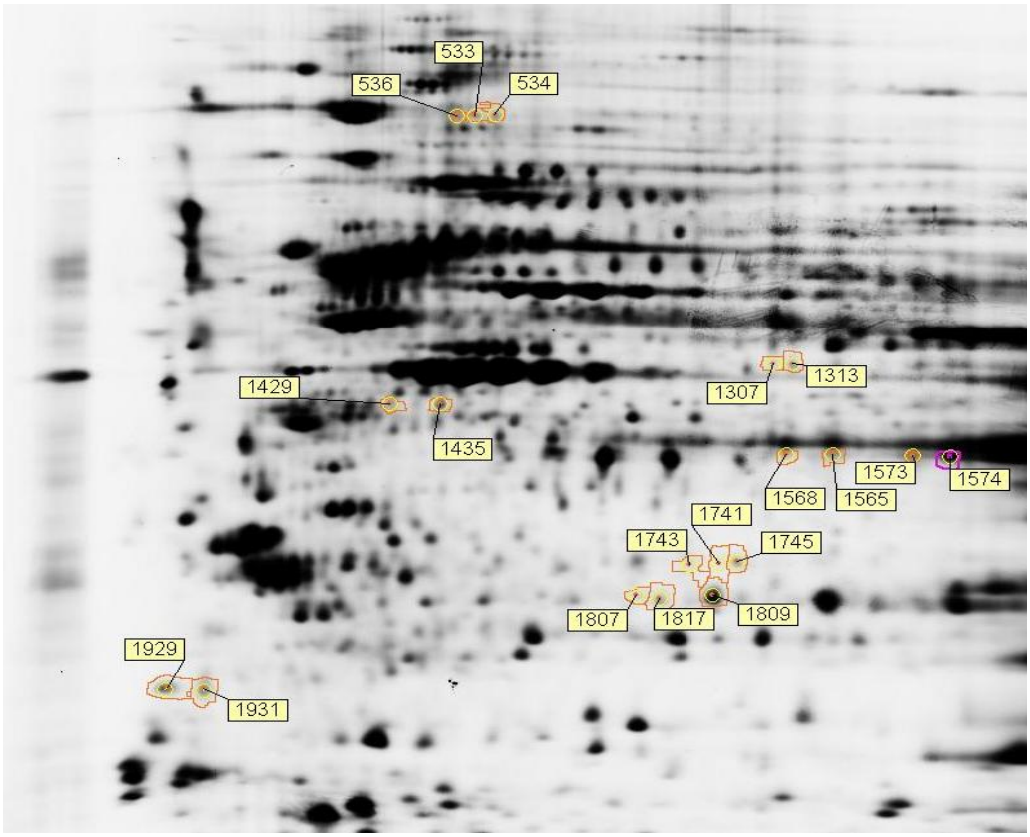


Figure 2-7. Proteomic analysis of anthrax treated HeLa cells. A.) Gray scale image of ET-treated HeLa cell total protein expression. B.) Gray scale image of ET plus LT treated HeLa cell total protein expression. C.) Gray scale image of spots excised for identification.

CHAPTER 3
TRANSCRIPTIONAL ANALYSIS OF HUMAN MONOCYTES TO ANTHRAX LETHAL TOXIN

Overview

Monocytes play a pivotal role in clearance of infections by replenishing the macrophage and dendritic pools. Early studies using murine macrophages have shown some strains which contain alterations in the kinesin motor protein Kif1C, show resistance to LT induced cytotoxicity [185, 198]. The cytotoxicity induced in susceptible cells by LT is through inactivation of the MAPK, specifically the p38 pathway, causing cells to undergo apoptosis [199]. Other studies using anthrax animal models have documented resistance to anthrax lethal toxin (LT) through depletion of host macrophages, suggesting that these cells play a critical role in anthrax LT induced lethality [66, 67].

In human studies, the effects of LT on mononuclear cells have included suppression of cytokine responses by peripheral blood mononuclear cells, induction of macrophage apoptosis, and prevention of monocyte proliferation and differentiation [153, 154, 200]. Inhalation anthrax cases present clinical manifestations indicative of host immune collapse in humans and in nonhuman primate studies [201-203]. However, more recent studies investigating human monocytes and macrophages have suggested human alveolar macrophages are resistant to LT, and undifferentiated human monocytic cell lines are resistant to LT-induced death [153, 204]. LT's targeting of human monocytes/macrophages could help to explain the rapid onset of fatal symptoms and host demise during an inhalation anthrax infection, but the exact effects LT exerts on human monocytes, along with the mechanisms underlying the impairment of the host immune cell's responses, have yet to be fully determined.

The impact of LT on host gene expression will help to further elucidate the ways in which we treat and prevent anthrax. Studies using murine macrophages and LT have shown a broad range in transcriptional effects. This study concluded LT induced changes in macrophage inflammation, signaling, and transcription factors, along with changes in the immune response by macrophages including down regulation of CD137, shown to play a role in monocyte proliferation in response to LPS, and up regulation of plasminogen activator inhibitor type I, which results in fibrin deposits, massive imbalances in coagulation, and can lead to multiorgan failure [205, 206].

This study is the first to determine direct human monocyte susceptibility, along with the analysis of the transcriptional responses, to anthrax LT. The mechanisms of LT impairment on human peripheral monocytes will help elucidate the roles monocytes contribute during the host immune system collapse documented during an anthrax infection. The transcriptional analysis will serve to not only unravel the mechanisms behind the rapid onset of lethality documented in anthrax victims, but may provide new targets for controlling inflammation and enhancing host defense.

Materials and Methods

Monocyte Isolation and Toxin Treatment

Human monocytes were isolated using a negative selection antibody technique followed by separation over a Ficoll gradient. Whole blood was collected by venous puncture from healthy human volunteers into 8mL vacutainer tubes containing Ficoll (BD Biosciences). The study followed US Department of Health and Human Services guidelines and was approved by the University of Florida Institutional Review Board. Healthy volunteers ranged in age from 24-58 years and included both male and females. Whole blood was incubated with a monocyte negative selection antibody (Stem Cell Technologies) for 20 min., centrifuged 1700 x g for 25 minutes at

RT, no brake over. Monocytes were re-suspended in 10mL RPMI (Mediatech) complete media, centrifuged at 250 x g for 9 min. to remove platelets, and re-suspended to 7-9 x 10^5 cells/mL in RPMI. Monocytes were used immediately following isolation. Monocytes were treated with 500ng/mL LF and 500ng/mL PA for 4 hours at 37°C while rotating in a cell inverter to prevent the cells from clumping.

Toxin Purification

Lethal factor (LF) and protective antigen (PA) were kind gifts from Dr. Conrad Quinn (Center for Disease Control) and purified as previously described [173]. Both PA and LF were purified from culture supernatants of *B. anthracis* cultures. Bacterial culture media was filtered through a 0.22 µM filter followed by diethylaminoethyl cellulose (DEAE) anion exchange chromatography. The toxins were then subjected to gel filtration and hydrophobic interaction fast protein liquid chromatography (FPLC), as previously described [173]. Cultures of 15 liters were found to produce approximately 8mg of PA and 13mg of LF. Toxin purity was assessed using coomassie blue staining and yielded 90% purity.

Monocyte Purity and Apoptosis Analysis

Monocyte apoptosis was assessed using Annexin-V-FLUOS Staining Kit (Roche), in accordance with the manufacturer's protocols. Purified monocytes were inverted at 37°C with 500ng/mL LF and 500ng/mL PA for 4 hours, stained with CD-14 Pac Blue (BD Biosciences), Annexin-V-Fluorescein and propidium iodide. The cell population was gated first for CD14-Pac-Blue followed by analysis of the relative amount of Annexin (FL1) and PI (FL2) using flow cytometry FACScan (BD). The same gating parameters were used for all samples and 10,000 cells were collected, and analyzed by FCS Express (De Novo).

MEK Cleavage

Purified monocytes were incubated at 37° C with 500ng/mL lethal toxin for 4 hours. Cells were lysed in lysis buffer (1% Triton X-100, 50mM TrisCl, 150mM KCl, 50mM EDTA, 0.2% NaA2, 200mM imidazole, 100mM NaFl, 100mM Na3VO4) plus complete mini protease inhibitor cocktail tablet (Roche), sonicated, and protein concentrations were determined using bicinchoninic assay (Pierce). Forty micrograms of protein was separated by 10% SDS-PAGE gel (Pierce), transferred to a Polyvinylidene fluoride (PVDF) membrane (Bio-rad) and probed for MEK1 (Upstate) at 1:1000 overnight at 4°C, washed in TBS-t and incubated with 1:10,000 goat anti-rabbit (Sigma) for one hour at room temperature. Membranes were washed, and detected using SuperSignal Chemiluminescence detection system (Pierce). Membranes were then stripped and probed overnight with 1:1000 MEK3 antibody (Santa Cruz). β-actin (Sigma) was used to check consistent loading amounts.

RNA Isolation

Purified monocytes from 4 healthy volunteers were incubated at 37° C with media alone or with 500ng/mL LF plus 500ng/mL PA for 4 hours at 37°C while rotating in a cell inverter to prevent the cell clumping. Total RNA was collected using RNAeasy mini kit (Qiagen). Samples were lysed, homogenized using the QIAshredder and ethanol was added provide optimal binding conditions. The lysate was loaded onto the RNeasy silica membrane and all contaminants were washed out of the column. Pure concentrated RNA was eluted using 30µL water. RNA quantity and quality was assessed using the A260/A280 ratio measured using NanoDrop (Thermo Scientific) technology.

Microarray Procedure

One-hundred nanograms of total RNA was labeled using Affymetrix Gene Chip® 3' IVT Express Kit for each replicate. Amplified labeled RNA was purified, fragmented, then

hybridized for 16 hours on Affymetrix Gene Chips® (HG U133 plus 2.0) representing approximately 22,000 well-characterized human genes. Arrays were washed using Affymetrix Gene Chip® Fluidics Station FS450 and scanned using Gene Chip® Scanner 3000 7G.

Microarray Analysis

Low-level analysis was performed using dChip modeled-based expression matrix (dChip 2007 (DNA-Chip Analyzer), Build date: Jan 4, 2008). Unsupervised analysis – probes sets whose hybridization signal intensity exhibited a coefficient of variation of greater than 0.5 were analyzed by unsupervised hierarchical cluster analysis using algorithms implemented in dChip. Supervised analysis – significant probe sets between treatment groups were identified using a paired t-test (by donor) at a significance threshold of $p < 0.001$. Leave-one-out-cross-validation using 4 prediction models was used to test the ability of probe sets significant at $p < 0.001$ to distinguish between the treatment groups. Microarray analyses were done using dCHIP and BRB-Array Tools by Richard Simon (http://linus.nci.nih.gov/BRB-ArrayTools.html).
The microarray data for this study was deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) [30] with accession numbers GSM848717 through GSM 848724. The microarray data are also available in a series with accession number GSE34407.

Quantitative Real Time-PCR (qRT-PCR)

RNA was collected using RNAeasy mini kit (Qiagen), quantitated using a Nanodrop system (Thermo Scientific), and 233µg total RNA was used for cDNA synthesis using Super Script III First-Strand Synthesis (Invitrogen). cDNA was quantitated using SYBR Green Jump Start Taq Ready Mix (Sigma) and 10mM forward and 10mM reverse primers were used for each indicated reaction. Primers used were as follows: ACTB-forward TCACCGAGCGCGGCT, ACTB-reverse TAATGTCACGCACGATTTCCC, GAPDH-forward

GGTGAAGGTCGGAGTCAACG, and GAPDH-reverse AGAGTTAAAAGCAGCCCTGGTG. All other primers are listed in Table 3-1. Reactions were run on the MJR Opticon Continuous Fluorescence detector (Bio-Rad) and analyzed with Opticon Monitor Software 1.08 (Bio-Rad).

Results

Microarray Setup

Human peripheral monocytes were treated for 4 hours with 500ng/mL LF + 500ng/mL PA or media alone, and microarray analysis was performed using four biological replicates from healthy volunteers. A total of 8 microarray hybridizations were employed and analyzed on Affymetrix Gene Chips® (HG U133 plus 2.0). The chips contained 54,675 probe sets and identified multiple differentially regulated pathways and genes by human peripheral monocytes after LT treatment.

Monocyte Purity and Apoptosis

In order to first determine monocyte cell purity, isolated cells were analyzed using flow cytometry and gated using forward and side scatter, along with the monocytic marker, CD14. It was found that monocytes were isolated with a >85% purity (Figure 3-1A and 3-1B). Because previous reports have documented LT induced cell apoptosis, it was important to assure the transcriptional response of LT treated monocytes would be indicative of the monocyte's responses to LT outside an induction of apoptotic response genes. This was accomplished through the analysis of the necrosis and apoptosis markers, propidium iodide (PI) and Annexin V, on human peripheral monocytes. Using flow cytometry, it was found that human peripheral monocytes showed 99% viability after a 4 hour treatment of LT (Figure 3-1C and 3-1D).

Susceptibility to Anthrax LT

In order to explore the actions of LT on human peripheral monocytes, a Western Blot analysis was performed and MEK1, along with MEK3, cleavage was determined after a 4 hour

treatment with LT. It was found that human peripheral monocytes were susceptible to the actions of MEK cleavage by LT (Figure 3-1E). HeLa cells were used as a positive control and β-actin was used to assure equal loading controls.

Unsupervised Analysis

Unsupervised hierarchical analysis was used to assess the noise in the array experiments. First, probe sets whose signal intensity varied most in the data set were selected by applying a variation filter. Probes sets which displayed a coefficient of variation of greater than 0.5 were subjected to hierarchical analysis. The clustering dendrogram showed the major node of separation between control and LT treated samples (Figure 3-2A).

To identify specific genes responsive to LT treatment, a paired t-test (by donor) was performed at a significance threshold of $p < 0.001$. Genes specified by 820 probe sets were found to be significant among the treatment groups. The hierarchical cluster pattern of the significant probe sets is shown (Figure 3-3A). Of these probe sets, multiple gene products known to play a role in monocyte function were discovered (Figure 3-2B). The ability of probe sets significant at $p < 0.001$ to function as a classifier between treatment groups (LT treated vs. control) was established by leave-one-out-cross-validation and Monte Carlo simulations. Using 4 different prediction models, the classifier performed flawlessly. Of the significant genes identified, many are known to play a role in monocyte function (Figure 3-2C).

Pathway Analysis

Using the Gene Set Expression Comparison Analysis, as implemented in BRB Array tools, the Biocarta pathways which were associated with the differentially regulated genes were identified. Over 60 differentially regulated pathways were discovered in monocytes in response to LT treatment. As expected, the most significant pathway affected by LT treatment was the MAPK signaling pathway, with the p38 MAPK signaling pathway being most impacted with 103

genes affected (Figure 3-3B). Additional pathways altered by LT at the $p < 0.001$ significance level included the IL-18, Toll-Like Receptor, IFN alpha, and G-Protein Family signaling pathways. It is interesting to note that a previous study measuring the transcriptional response of human alveolar macrophages to anthrax spores detected an activation of the TLR pathways [207], and our results indicated anthrax LT targets multiple genes within the TLR signaling pathway (Figure 3-3B).

Genes Associated with Monocyte Function

RGS14 is a protein involved in the regulation of G-protein signaling through attenuation of G-protein heterotrimer signaling, thereby inactivating this signaling cascade. The Affymetrix microarrays revealed that RGS14 expression in LT treated monocytes showed a 6 fold increase in expression (Table 3-1). This is a potentially significant finding in that RGS14 inhibits G-proteins important for chemotaxis. Therefore LT could be impairing chemotaxis not only by blocking Hsp27 phosphorylation through disruption of the P38 pathway [76], but also by inducing the over-expression of RGS14, thereby inhibiting G-protein mediated signaling required for actin-based motility.

RGS14 expression has been shown to be down-regulated during the maturation of monocytes to dendritic cells [208] and over-expression of this G-protein regulator would be expected to block monocyte maturation. RGS14 levels are also known to decrease in dendritic cells exposed to *Leishmania major* or *Toxoplasma gondii*, suggesting that RGS14 down regulation may be an important step in a normal immune response, and up-regulation of RGS14 by LT could be contributing to LT's immunosuppressive effects [209].

Three chemokine receptors were also altered after LT treatment, suggesting that LT may be inducing functional defects in monocyte response signaling. IL-8 receptor beta (CXCR2) was up-regulated after LT treatment (Table 3-1). CXCR2 transduces signaling through a G-protein

activated second messenger system. This receptor is important for monocyte transendothelial migration, and up-regulation of CXCR2 could serve to enhance the delivery of monocytes to tissues.IL-1 receptor type II (IL-1R2), was found to be markedly down-regulated. IL1-R2 is a decoy receptor for IL-1 that functions either at the cell surface or in a soluble form [210]. The decreased expression of the decoy receptor would presumably increase IL-1a levels and increase the febrile response of the host potentially at least in part explaining the high fever that common accompanies systemic anthrax [211]. CCR5 is a receptor for the monocyte chemokines RANTES and MIP. The down-regulation of CCR5 by LT could reflect an inability of toxin-treated monocytes to differentiate into macrophages [212] (Table 3-1).

In addition to an alteration in the chemokine response by LT, an additional enzyme, heparanase (HPSE), was found to be decreased in LT-treated human monocytes. This enzyme is an endoglycosidase that degrades heparin sulfate, resulting in disassembly of extracellular barriers required for cell migration [213]. Heparanase has also been postulated to play a role in inflammation [214] and our results showed a 2.6 fold decrease in heparanase gene expression (Table 3-1). One study has concluded that an in vivo siRNA against heparanase, along with an inhibitor of its enzymatic activity, results in a diminished inflammatory response [215]. Thus LT-mediated inhibition of heparanase expression could also contribute to the inhibition of the host immune response during an anthrax infection.

Microarray Validation by qRT-PCR

In order to confirm the microarray data, an external verification method using quantitative real-time PCR was utilized. The eight genes corresponding to RGS14, IL8RB, TLR5, PPM1H, CD47, SYK, CCR5, and IL1R2 were chosen for microarray confirmation due to their statistical significance and roles in immunity. CCR5 and IL1R2 were confirmed to be down-regulated at 4 hours after LT treatment, reinforcing the microarray data, while the other six genes were up-

regulated, again confirming the microarray data (Table 3-2). Results were performed in duplicates and fold values were normalized to GAPDH.

Discussion

There has been some conflicting data suggesting monocytes, along with monocyte-derived cells, are not susceptible to the actions of anthrax LT. One study utilized human monocytic cell lines and found that undifferentiated monocytic cells did not undergo LT-induced cytotoxicity, while the differentiated cells were susceptible [153]. Another study investigating human alveolar macrophages (AM) found that these cells were relatively resistant to the actions of LT. This study ascertained that LT failed to suppress human AM cytokine responses, cleave MEKs, and induce apoptosis [204].

Our investigations show human peripheral monocytes are susceptible to the actions of anthrax LT and do not undergo LT-mediated cytotoxicity after a four hour toxin treatment. We also find that LT induces several genes involved in previously unidentified pathways including the TLR pathway, IFN alpha pathway, and G-Protein family signaling pathways. The identification of several previously unappreciated gene products including RGS14, IL8 receptor beta, CD47, TNF ligand, IL-16, Syk, CCR5, and IL-1 receptor II adds to our understanding of how LT impacts the immune response. Our pathway analysis reveals that anthrax LT targets multiple normal immune-regulatory pathways that would be expected to protect the host against anthrax infection. Our findings should encourage further investigation into how these pathways converge functionally to impair normal monocyte function and these discoveries promise to provide many new insights into host defense and the regulation of inflammation.

Table 3-1. Predicted effects of LT on monocyte function

Gene	Microarray	Effects
RGS-14	5.61	Blockade of monocyte maturation to dendritic cells, inhibition of chemotaxis
CXCR2	5.04	Increased monocyte transendothelial migration into tissues
HPSE	-2.58	Diminished inflammatory response
CCR5	-2.33	Reduced responsiveness to the inflammatory mediators RANTES, MIP1 beta
IL1R2	-12.5	Increased IL-1 alpha responsiveness and increased fever

Table 3-2. q-RT-PCR of LT-induced genes

Probe ID	Microarray	q-RT-PCR	Gene Name	Primer Sequence
38290_at	5.61	7.40	RGS14-F	CAGGGATCTGTGAGAAACGAG
			RGS14-R	AGGTGATCCTGTTTTCCAGC
207008_at	5.04	7.50	IL8RB-F	GTCTAACAGCTCTGACTACCAC
			IL8RB-R	TTAAATCCTGACTGGGTCGC
210166_at	3.90	2.24	TLR5-F	TTTTCAGGAGCCCGAGC
			TLR5-R	AGCCCGAGATTGTGTCACTG
212686_at	2.65	3.85	PPM1H-F	GAGTACAGAGAAAGGAGCTTGG
			PPM1H-R	TCCAATAGTTGCCATTACCCG
226016_at	2.38	1.60	CD47-F	TTTGCTATACTCCTGTTCTGGG
			CD47-R	TGGGACGAAAAGAATGGCTC
209269_s_at	2.15	1.56	SYK-F	CAAGTTCTCCAGCAAAAGCG
			SYK-R	CATCCGCTCTCCTTTCTCTAAC
206991_s_at	-2.66	-2.33	CCR5-F	CCAAAAGCACATTGCCAAACG
			CCR5-R	ACTTGAGTCCGTGTCACAAGCC
205403_at	-12.5	-28.0	IL1R2-F	TGGCACCTACGTCTGCACTACT
			IL1R2-R	TTGCGGGTATGAGATGAACG

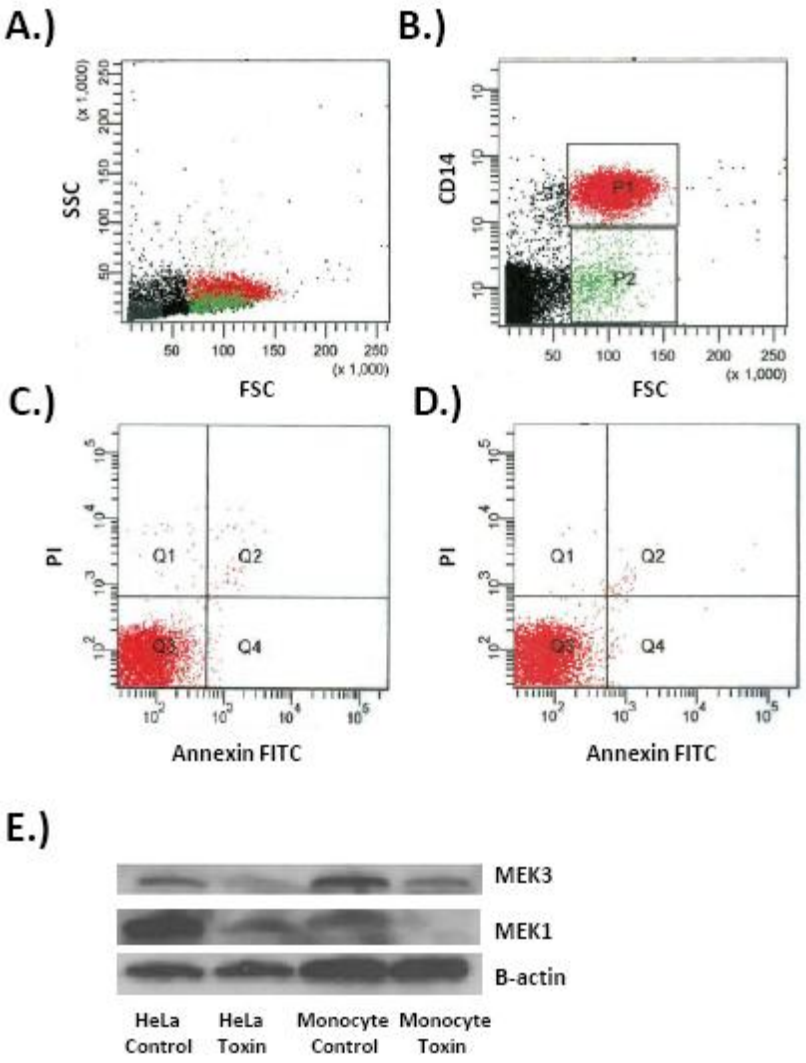
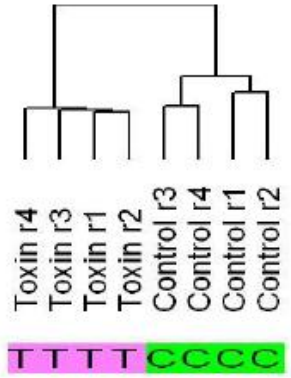


Figure 3-1. Monocyte purity, apoptosis, and susceptibility to LT. Red = CD14plus monocytes. Green = CD14- lymphocytes. A.) Forward and side scatter analysis of purified fixed human monocytes showing the monocyte population as compared to total population. B.) CD14 Pacific Blue and forward scatter analysis of fixed purified human monocytes showing >85% monocytes. C.) PI and Annexin-FITC analysis of CD14 plus monocytes after a 4 hour incubation showing 99.0% viable cells indicated in quadrant 3. D.) PI and Annexin-FITC analysis of CD14 plus monocytes after a 4 hour LT treatment showing 99.1% viable cells indicated in quadrant 3. HeLa cells or human monocytes were left untreated or treated with 500ng/mL LT for 4 hours at 37ºC. Samples were lysed, ran on SDS-PAGE, transferred to PVDF membrane, and probed with indicated antibodies. Both MEK3 and MEK1 were cleaved by LT while control cells showed no MEK cleavage. β-actin loading controls shows equivalent loading of both control and LT treated cells.

A.)



B.)

Affymetrix Probe ID	Gene or gene product name	Fold change	P-value
38290_at	Regulator of G-protein signaling 14 (Rgs14)	5.94	0.0009308
207008_at	Interleukin 8 receptor, beta	5.04	0.0008868
210986_s_at	Tropomyosin 1 (alpha)	3.49	0.0006411
212686_at	Protein phosphatase 1H (PP2C domain containing)	2.65	0.0001409
226016_at	CD47 molecule	2.40	0.0002531
211495_x_at	Tumor necrosis factor (ligand) superfamily, member 13	2.25	0.0001799
209828_s_at	Interleukin 16 (lymphocyte chemoattractant factor)	2.19	0.0001590
209269_s_at	Spleen tyrosine kinase (Syk)	2.15	0.0007547
229497_at	Ankyrin repeat and death domain containing 1A	2.1	0.0002478
206991_s_at	Chemokine (C-C motif) receptor 5 (CCR5)	-2.66	0.0009506
205403_at	Interleukin 1 receptor, type II	-12.04	0.0001070

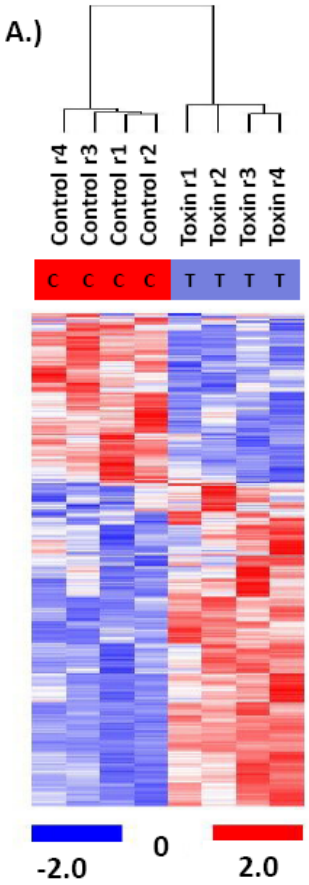
Figure 3-2. Unsupervised microarray analysis. A.) Hierarchical clustering dendrogram showing similarities between expression patterns within each condition. Specimens were paired based on donor, using 4 separate donors as indicated in replica r1 through r4. B.) Significant genes ($p < 0.001$) up or down regulated after LT treatment, along with their fold change, p-value and probe ID. C.) Leave-one-out-cross validation was used to calculate mis-classification rate which yielded a 100% correct classification between pairs.

C.) **Performance of classifiers during cross-validation.**

Pair ID	Mean Number of genes in classifier	Diagonal Linear Discriminant Analysis Correct?	1- Nearest Neighbor Correct?	3-Nearest Neighbor Correct?	Nearest Centroid Correct?
1	466	Yes	Yes	Yes	Yes
2	261	Yes	Yes	Yes	Yes
3	197	Yes	Yes	Yes	Yes
4	199	Yes	Yes	Yes	Yes
Correct Classification:		100%	100%	100%	100%

Figure 3-2. Continued

A.)



B.)

Biocarta Pathway	Pathway Description	# of genes	P-value
h_mapkPathway	MAPKinase Signaling Pathway	228	0.00001
h_p38mapkPathway	p38 MAPK Signaling Pathway	103	0.00001
h_il18Pathway	IL 18 Signaling Pathway	9	0.00004
h_tollPathway	Toll-Like Receptor Pathway	87	0.00028
h_ifnaPathway	IFN alpha signaling pathway	25	0.00043
h_gpcrPathway	Signaling Pathway from G-Protein Families	70	0.00044
h_p53hypoxiaPathway	Hypoxia and p53 in the Cardiovascular system	67	0.00191
h_arenrf2Pathway	Oxidative Stress Induced Gene Expression Via Nrf2	51	0.00418

Figure 3-3. Supervised microarray analysis. A.) Hierarchical cluster analysis showing the 820 probe sets which were differentially expressed at the 0.001 significance level. The arrays clustering on the left are from control samples, whereas the cluster on the right shows the LT treated samples. Up-regulated genes are shown in red and down-regulated genes are shown in blue. B.) Biocarta pathway analysis showing the pathways most significantly affected by LT, along with the number of genes and p-value within each pathway that were affected.

CHAPTER 4
ANTHRAX LETHAL AND EDEMA TOXINS FAIL TO DIRECTLY IMPAIR HUMAN PLATELET FUNCTION

Overview

Platelets are anuclear fragments of megakaryocytes that aid in hemostasis. Previous studies investigating rabbit platelets have shown that ET suppresses platelet aggregation, induces a rise in cAMP and activates Protein Kinase A (PKA) [167]. Additional investigations have shown anthrax LT inhibits arachidonic acid induced whole blood aggregation, inhibits platelet P-selectin expression, and increases mortality in mice receiving aspirin and rhodostomin [166].

Other studies using anthrax animal models have documented severe hemostasis abnormalities including: hemorrhagic lesions, fibrin deposits, thrombocytopenia, increased prothrombin time, elevated activated partial thromboplastin time (APTT), vascular permeability, pleural effusions, and decreased fibrinogen levels [65, 203]. In human cases, hemorrhagic lesions, capillary and vascular lesions consisting of fibrin deposits, pleural effusion, blood vessel leakage, and disseminated intravascular coagulopathy (DIC) have been documented [202]. These clinical manifestations suggest *B. anthracis* is capable of inducing severe defects in hemostasis; however, the exact mechanisms underlying these manifestations remain to be fully defined.

Material and Methods

Platelet Isolation and Toxin Treatment

Human whole blood was collected by venous puncture from healthy volunteers into Aster-Jandl anticoagulant, following US Department of Health and Human Services guidelines and approved by the University of Florida Institutional Review Board. Healthy volunteer donors (total of 6 subjects) ranged in age from 23-58 years, and included both males and females. Nine milliliters of whole blood was drawn into a 10cc syringe containing 1mL Aster-Jandl

anticoagulant (85 mM sodium citrate, 69 mM citric acid, 111 mM glucose, pH 4.6). Human platelet rich plasma (PRP) was obtained by centrifugation of whole blood at 100 x *g* for 10 min and purified platelets were re-suspended to 1 x 10^6 platelets/mL in Tyrode-Hepes buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM Hepes, 5 mM glucose, 1 mM $MgCl_2$ (pH 7.3)). Platelets were treated with toxin as indicated. Up to 1µg of ET and LT was used for a time of up to 12 hours. For mouse platelet studies, mice were anesthetized with CO_2, followed by cervical displacement. Cardiac puncture was performed and approximately 1mL of whole blood was obtained per mouse. PRP was obtained using the same methods as described above.

Cell Culture

HeLa cells (ATCC) were grown in Dulbecco's modified eagles media (DMEM) with 4.5 grams/L glucose supplemented with 10% fetal bovine serum (FBS) and 5% penicillin and streptomycin (Cellgro). Cells were grown to 75% confluence and used at a passage between 5-20. THP-1 cells (ATCC) were grown in RPMI-1640 (Cellgro) supplemented with 10% fetal bovine serum (FBS) and 5% penicillin and streptomycin (Cellgro) and used at a concentration of 1 x 10^6 cells/mL.

Chemicals Used

Reagents used were thrombin receptor agonist peptide (TRAP), forskolin (Fsk), and 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich), streptavidin conjugated DyLight 488 (Thermo Scientific), SB203580 (Calbiochem) and CD62P-FITC (BD Bioscience).

Toxins

Lethal factor (LF) and protective antigen (PA) were kind gifts from Dr. Conrad Quinn (Center for Disease Control) and purified as previously described [173]. Both PA and LF were purified from culture supernatants of *B. anthracis* cultures. Bacterial culture media was filtered

through a 0.22 µM filter followed by diethylaminoethyl cellulose (DEAE) anion exchange chromatography. The toxins were then subjected to gel filtration and hydrophobic interaction fast protein liquid chromatography (FPLC), as previously described [173]. Cultures of 15 liters were found to produce approximately 8mg of PA and 13mg of LF. Toxin purity was assessed using coomassie blue staining and yielded 90% purity.

Edema factor (EF) was expressed and purified from *E. coli* and was a generous gift from Dr. Wei-Jen Tang (University of Chicago) [53]. *E. coli* cultures were induced for four hours with Isopropyl-β-D-thio-galactoside (IPTG) and purified with a 50% Ni-nitrilotriacetic acid resin, followed by Sepharose cation-exchange purification and stored in -80°C aliquots.

Western Blot Analysis

For Western blot analysis, cells were lysed in lysis buffer (1% Triton X-100, 50mM TrisHCl, 150mM KCl, 50mM EDTA, 0.2% NaA2, 200mM imidazole, 100mM NaFl, 100mM Na3VO4) plus complete mini protease inhibitor cocktail tablet (Roche), sonicated, and protein concentrations were determined using bicinchoninic assay (Pierce). Forty micrograms of protein was separated by 10% SDS-PAGE gel (Pierce), transferred to a Polyvinylidene fluoride (PVDF) membrane (Bio-rad) and probed for MEK1 (Upstate), TEM-8 (Abcam), or LRP6 (Santa Cruz). β-actin (Sigma) was used to assure equal loading amounts.

Intracellular cAMP Measurements

For cAMP analysis, platelets were treated with cAMP agonists: 10µM forskolin plus 100µM IBMX for 20 minutes, or varying concentrations of ET as indicated for 2 hours. All incubations were carried out at 37°C. Platelets were pelleted, lysed, and sonicated. Intracellular cAMP levels were measured using an enzyme-linked immunoassay (Amersham Biosciences and Arbor Assays) a 96-well plate in duplicates, following kit protocol [158].

Measurement of P-selectin

For P-selectin expression, platelets were treated for 2 hours with either 1000ng/mL EF, 1000ng/mL LT, 1000ng/mL PA, 1000ng/mL ET or 1000ng/mL LT at 37ºC for 2 hours or for 20 min at 37ºC with SB203580 (400µM) or with Forskolin (10µM) and IBMX (100µM). Platelets were then stimulated with TRAP (30µM) for 2 minutes, fixed in a 1:1 ratio of 3.7% formaldehyde, probed for P-selectin, and analyzed using FACScan (BD) and FCS Express (De Novo). The relative amount of FITC-P-selectin was determined using flow cytometry analysis using the same gating parameters for all samples and a using a total of 10,000 cells.

Toxin Binding and Internalization

PA was labeled with biotin (Thermo Scientific), according to the manufacturer's instructions. THP-1 cells or human platelets were treated for 2 hours with 1000ng/mL PA-biotin at 37ºC, 4ºC or with buffer alone. Cells were fixed in a 1:1 ratio of 3.7% formaldehyde, probed with FITC-streptavidin and analyzed using FACScan (BD) and FCS Express (De Novo). The relative amount of PA binding and internalization was determined using the same gating for either platelets or THP-1 cells. A total of 10,000 cells were analyzed.

Anthrax Receptor Expression

For surface expression of LRP6 and TEM8/CMG2, human platelets or THP-1 cells were fixed in a 1:1 ratio of 3.7% formaldehyde, incubated for 1 hour with primary antibodies against either isotype control, TEM8/CMG2 or LRP6, followed by the secondary Alex 488 anti-rabbit IgG antibody. All experiments were performed in triplicate, using a minimum of three platelet donors for each study. Results were analyzed using Accuri C6 (BD Biosciences) and FCS Express (De Novo). Fold changes in mean fluorescent intensity of anthrax receptors: TEM8/CMG2 and LRP6 in THP-1 cells and human platelets were determined by comparing each sample to its isotype control.

Results

LT Activity in Human Platelets and HeLa cells

To determine LT's efficacy, human platelets or HeLa cells were treated with 500ng/mL LT. Because LT is known to cleave the N-terminus of MEK and prevent phosphorylation of a downstream substrate, Hsp27, a Western Blot was performed to determine LT activity. LT did not cleave MEK1 or prevent Hsp27 phosphorylation in human platelets (Figure 4-1A and 4-1B), while HeLa cells were susceptible to MEK1 cleavage after LT treatment (Figure 4-1B).

ET Activity in Human and Mouse Platelets

We next sought to determine the effects of ET on human and mouse platelets. It is well documented ET increases intracellular cAMP levels [54]; thus, measurement of cAMP after ET treatment is an additional assay used to measure toxin entry and activity. Treatment of human platelets with increasing concentrations of ET, surprisingly, failed to increase intracellular cAMP levels, while platelets treated with the positive control Fsk/IBMX, demonstrated at least a 5-fold increase in cAMP (Figure 4-1C). Mouse platelets also failed to show an increase in cAMP levels after a 500ng/mL treatment of ET, while the positive control lead to an increase in cAMP levels (Figure 4-1D). Based on these known anthrax toxin activities, we demonstrate here that anthrax LT and ET do not directly affect platelets.

Toxin Binding and Internalization

To determine if a lack of cellular responses to anthrax toxin was due to ineffective toxin internalization or binding, a PA-biotin conjugate was utilized. Purified platelets and THP-1 cells were treated with PA-biotin at 4º C, to determine toxin binding, or 37º C, to determine toxin internalization. PA-biotin was probed with streptavidin-FITC, followed by flow cytometry analysis. THP-1 cells showed an increase in PA binding at 4ºC and subsequent warming to 37ºC revealed internalization of the toxin (Figure 4-2C), while purified platelets failed to exhibit PA

binding at 4ºC, or internalization at 37°C (Figure 4-2B). The failure of PA to bind to the platelet surface indicated a defect in anthrax toxin binding to its cognate receptor.

P-selectin Expression After Toxin Treatment

To determine the direct functional significance of LT and ET on platelet activation, we measured platelet P-selectin expression after toxin treatment. P-selectin is a glycoprotein that is translocated to the platelet surface from α granules upon cell activation and is used as a marker for platelet activation[216]. Platelets were treated with anthrax toxins, followed by activation with TRAP, a known P-selectin stimulus, and P-selectin levels were determined. Pre-treatment with both Fsk/IBMX, that represents a positive control for ET by raising intracellular cAMP levels, and SB203580, which represents a positive control for LT by preventing activation of the p38 pathway, inhibited TRAP induced P-selectin surface expression, while pre-treatment with LT or ET failed to block TRAP induced P-selectin expression on human platelets (Figure 4-2A). P-selectin surface expression is an important component for shear-induced aggregation and aggregate formation of platelets. These results indicate neither LT nor ET directly inhibit TRAP-induced platelet activation.

Expression of Anthrax Receptors

We next sought to determine if the anthrax receptors, TEM-8 or CMG-2, or the co-receptor LRP6, were present on human platelets. LRP6 is required for anthrax toxin lethality and has been shown to play a role in toxin binding and internalization [217]. Western blot analysis confirmed the presence of the anthrax receptors in human platelets (not shown). Flow cytometry was utilized to measure the anthrax receptor's surface expression. THP-1 cells were used as a positive control and showed significantly high levels of the anthrax receptors, TEM8 and CMG2, along the co-receptor LRP6, compared to isotype control (Figure 3-2B), while human platelets exhibited low levels of TEM8/CMG2, and displayed no surface expression of LRP6 (Figure 4-

3C). Human platelets demonstrated two populations of TEM8/CMG2 surface expression. One population (47%) exhibited a 1.02 fold increase in TEM8/CMG2 expression, and a second population (53%) displayed a 9.00 fold increase in TEM8/CMG2 expression, while LRP6 expression was at a 1.25 fold increase. THP-1 cells exhibited a 39.5 fold increase in TEM8/CMG2 receptor expression and a 6.74 fold increase in LRP6 surface levels. We conclude human platelet do not express the anthrax co-receptor LRP6, and have reduced surface expression of TEM8/CMG2.

Discussion

Hemostatic abnormalities have been well documented during anthrax infections, but the precise underlying mechanisms have yet to be identified. Pathologic findings, including hemorrhagic lesions, fibrin deposits and prolonged clotting times, suggest a defect in platelet function [202, 203]. Based on our extensive analysis of the interaction of anthrax toxin with human platelets, we conclude that both anthrax LT and ET do not have a direct effect on human platelet function.

We demonstrate here that anthrax LT failed to cleave MEK1 and did not prevent phosphorylation of a downstream substrate, Hsp27, indicating LT has no direct effects on human platelets. ET was also unable to increase intracellular cAMP levels, which is a well-known activity of this toxin [54]. Functional studies revealed that pre-treatment of human platelets with LT or ET failed to inhibit TRAP induced P-selectin surface expression, which is required for platelet aggregation [218], indicating that neither LT nor ET can directly impair platelet the component required for platelet aggregation.

Anthrax PA failed to bind to human platelets, an initial event critical for entry of LT and ET. This led us to speculate whether human platelets expressed the anthrax toxin receptors. CMG2 and TEM8 are the two receptors known to facilitate toxin entry into cells, while LRP6, a

co-receptor is also required for entry of the toxins [217, 219-221]. We found human platelets exhibited low levels of the TEM8 and CMG2 anthrax receptors, and displayed no surface expression of the co-receptor LRP6. Anthrax LT and ET therefore, are not able to directly affect human platelets due to the fact that they lack the anthrax receptors. This indicates that the toxins are unable to enter into platelets and have no direct catalytic activity towards these cells.

Despite anthrax toxin's inability to directly affect human platelets, additional factors may be indirectly contributing to the haemostatic abnormalities documented in anthrax victims. It has been reported that LT induces vascular dysfunction, depletes antithrombin levels, induces a loss of cytoskeletal integrity, and disrupts the cadherin junctional complexes resulting in loss of intercellular contacts [222, 223]. Additional anthrax proteolytic enzymes have also been identified from *B. anthracis* and have been shown to play a role in: impairing fibrinolysis, decreasing plasma D-dimer circulating levels, cleaving von Willebrand Factor, and impairing platelet aggregation, suggesting bacterial components outside of LT and ET are contributing to hemorrhage and thrombosis during an anthrax infection [224]. This is the first study to demonstrate that LT and ET do not have a direct effect on human platelet function. These findings should guide future investigators to focus on alternative mechanisms to explain defective hemostasis in systemic anthrax.

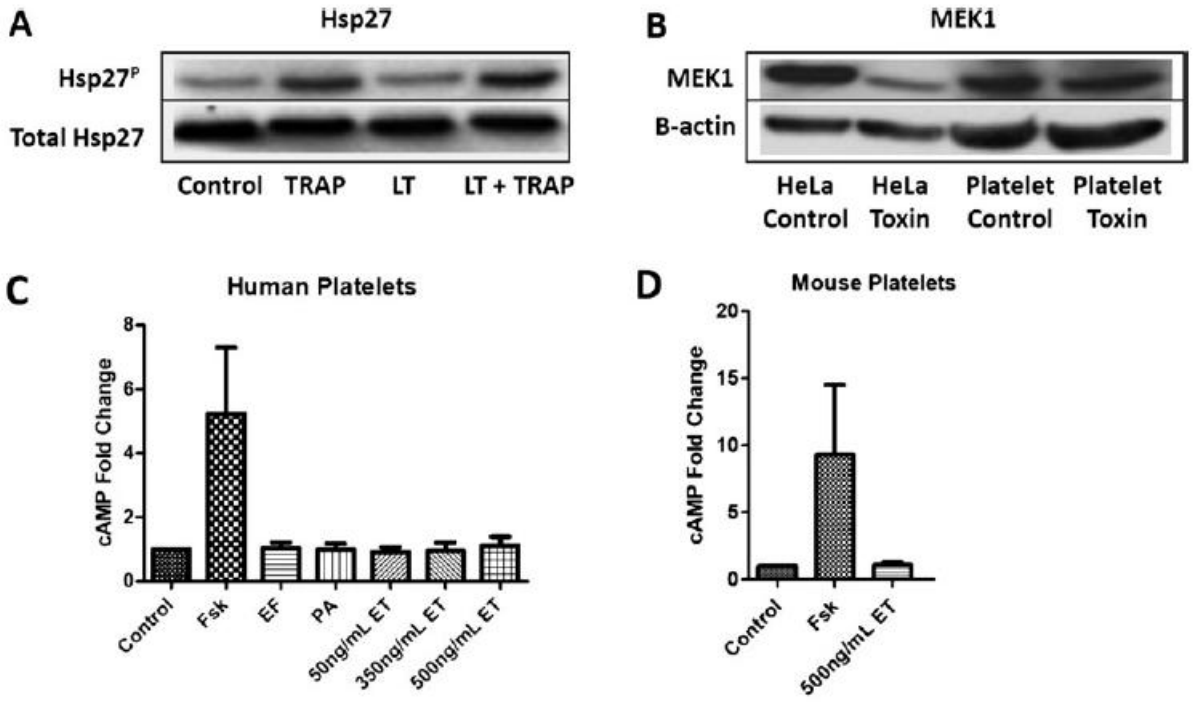
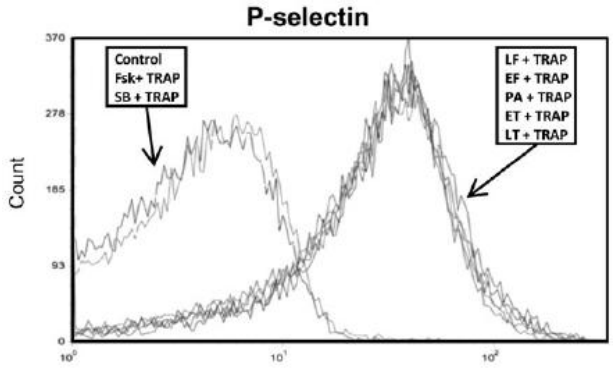


Figure 4-1. Effects of LT and ET on human platelets. (A) Western blot showing phosphorylated Hsp27 in human platelets treated with 500ng/mL LT for 4 hours at room temperature and stimulated with 30µM TRAP for 2 minutes or left unstimulated. Blots were stripped and re-probed with a primary antibody to total Hsp27 to show equal loading. (B) Western blot showing MEK1 in human platelets or HeLa cells treated with 500ng/mL LT for 4 hours at room temperature or left untreated. Blots were stripped and re-probed with a primary antibody to β-actin to show equal loading. (C and D) Enzyme-linked immunoassay measuring intracellular cAMP levels in human platelets (C) treated with 50, 350, or 500 ng/mL ET for 2 hours at 37ºC. Negative control platelets were treated with 500ng/mL EF or 500ng/mL PA for 2 hours at 37ºC. Positive control platelets were treated with Fsk (10µM) and IBMX (100µM) for 20 min at 37ºC. Mouse platelets (D) were treated with 500ng/mL ET for 2 hours at 37ºC. Error bars indicate SEMs from three separate donors.

A.)



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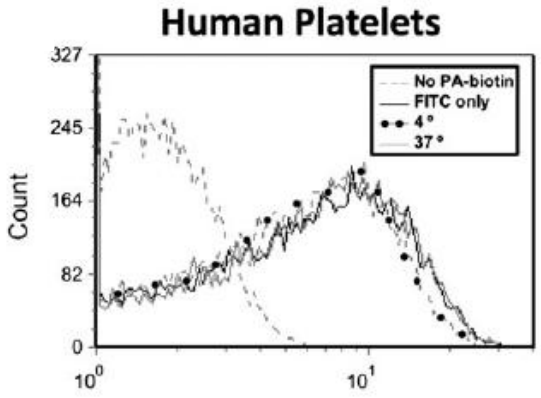


C.)

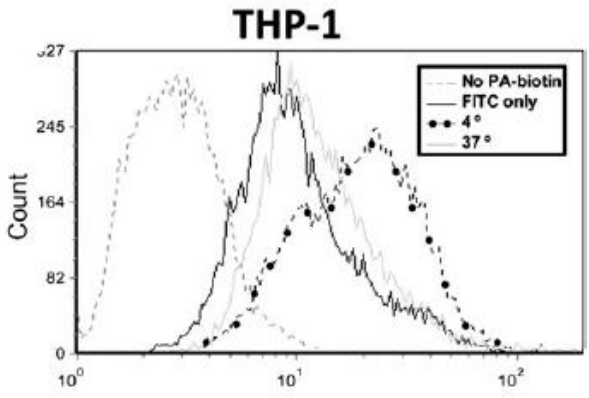
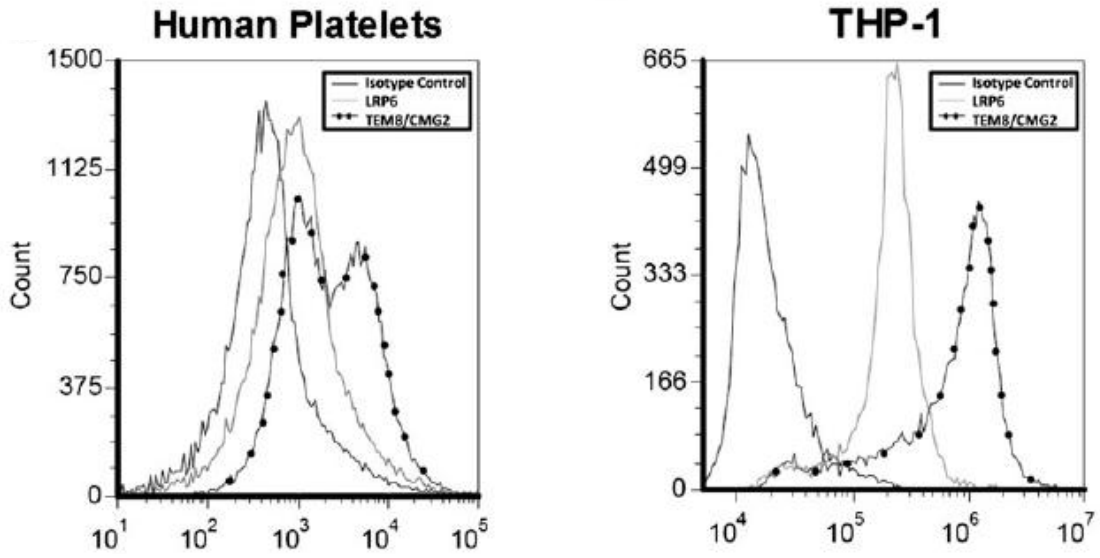


Figure 4-2. Functional significance of ET and LT treated human platelet and anthrax receptor detection. (A) Flow cytometry measuring P-selectin levels in human platelets after 2 hour toxin treatment at 37ºC with either 1000ng/mL EF, 1000ng/mL LT, 1000ng/mL PA, 1000ng/mL ET or 1000ng/mL LT, or for 20 min at 37ºC with SB203580 (400µM) or with Forskolin (10µM) and IBMX (100µM). Platelets were then stimulated with TRAP (30µM) for 2 minutes, fixed, probed for P-selectin, and analyzed using FACScan (BD) and FCS Express (De Novo). (B and C) Flow cytometry measuring PA binding and internalization in THP-1 cells (C) or human platelets (B) after 2 hour treatment with 1000ng/mL PA-biotin at 37ºC, 4ºC or buffer alone. Cells were fixed in a 1:1 ratio of 3.7% formaldehyde, probed with FITC-streptavidin and analyzed using FACScan (BD) and FCS Express (De Novo).

A.) B.)



C.)

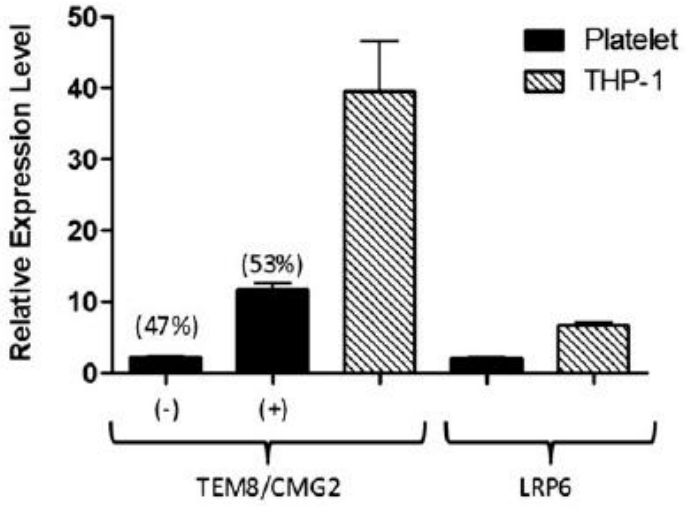


Figure 4-3. Anthrax receptor expression. Flow cytometry measuring anthrax receptor surface expression in THP-1 cells (B) or human platelets (A). Cells were fixed, probed for TEM8/CMG2, LRP6, or an isotype control, followed the secondary antibody anti-rabbit Alexa488, and analyzed using Accuri C6 (BD Biosciences) and FCS Express (De Novo). (C) Relative expression levels of mean fluorescent intensity of anthrax receptors: TEM8/CMG2 and LRP6 in THP-1 cells and human platelets. The fold change in mean fluorescence intensities were calculated by comparing each sample to its isotype controls.

CHAPTER 5
FUTURE WORK

Functional Impairment of Monocytes by Anthrax toxins

Peripheral blood monocytes play a pivotal role in linking the macrophage and dendritic pools to the bone marrow. Monocytes are produced in the bone marrow, circulate in the periphery, and supply the tissues with macrophages and dendritic cells (DCs). Mechanisms used to maintain the tissue pools of macrophages and dendritic cells include a continuous recruitment and differentiation of circulating peripheral blood monocytes. Recruitment of monocytes through the blood vessels from the circulation is termed diapedesis. Diapedesis occurs through two main steps: monocyte adhesion through integrin cross-linking to the endothelium, followed by migration from the periphery to the tissue.

Previous studies have shown that ERK and the p38 pathways contribute to monocyte adhesion to the endothelium and subsequent activation [225, 226]. This was accomplished through the activation of the ERK and p38 pathways following very late antigen 4 (VLA-4), LFA-1 (CD11a/CD18) and MAC-1 (CD11b/CD18) integrin binding and activation. During an anthrax infection, production of LT would block this MAPKK-dependent activation leading to reduced recruitment of monocytes to areas of infection.

Another important component of monocyte diapedesis is actin assembly. Previous studies using human neutrophils show LT inhibition of the p38 pathway leads to a sequestering of an important actin component, Hsp27 [76]. This leads to reduced neutrophil chemokinesis and chemotaxis. Other studies utilizing the p38 inhibitor SB203580 have shown decreased diapedesis in monocytes [227] . This would indicate LT mediated blockage of monocyte diapedesis would subsequently reduce the recruitment of monocytes to areas of infection. Because LT production

by *B. anthracis* is also accompanied by ET, it would also be interesting to determine what role dual toxin treatment has on monocyte function.

Mechanism of cAMP Inhibition

Many microbes utilize cAMP to establish infections within a host. *B. pertussis* secretes an adenylate cyclase which is capable of inhibiting immune cell function, attributing to a lack in neutrophil response, an absence of fever, and a high incidence of pneumonia [228]. Without its adenylate cyclase toxin, *B. pertussis* has been shown to be avirulent in mouse models [229]. Other studies using bacterial toxins which increase cAMP levels have shown inhibition of phagocytosis, decreased cytokine production, impaired chemotaxis, and decreased bacterial killing [230-233]. We have shown LT inhibition of ET and forskolin induced cAMP production. The mechanisms behind this inhibition would prove to have a broad impact on developing new treatment options for multiple pathogens which increase cAMP levels.

Because LT inhibits the bacterial produced adenylyl cyclase of *B. anthracis* ET, along with mammalian adenylyl cyclases, it seems the mechanism of inhibition would be a common component in both types of enzymes. For *in vitro* activity, ET requires calmodulin in a calcium dependent manner [55]. Looking at the requirements for functional activity in both ET and mammalian adenylyl cyclase, a few common components are noted. Both adenylyl cyclases are regulated by calmodulin, sensitive to calcium and can be inhibited by nucleotide analogues that mimic ATP. Additionally, our results indicate the mechanism of cAMP inhibition is downstream of the MAPKK pathway. It is possible that the MAPKK pathway positively regulates cAMP production through calmodulin binding, calcium concentration or an unknown intermediate component. Another possibly for cAMP inhibition by LT is through binding to the ATP site of adenylyl cyclases.

Here, we have shown that production of cAMP by ET and forskolin requires an intact MAPKK pathway. As LT concentrations were increased, cAMP levels were decreased. This suggests that inhibition of the MAPKK pathways leads to a decrease in cAMP production. It was also shown that all chemical inhibititors of the MAPKK pathway inhibited cAMP production so the mechanism for this inhibition would have to be a component downstream of all the pathways: p38, ERK and JNK. This is important in that all pathways are required for successful production of cAMP by both host mammalian adenylyl cyclases and the bacteria produced adenylyl cyclase ET. We also show the substrate ATP, required for cAMP production, was not being secreted out of the cell, but it may be possible that ATP is being prevented from binding to the adenylyl cyclases after MAPKK inhibition.

ATP is required for successful production of cAMP. If LT is capable of causing a modification or induction of a component that bound to the substrate site of adenylyl cyclase, production of cAMP would be blocked. Previous studies have shown that blocking the substrate site on both ET and adenylyl cyclases blocks production of cAMP. The nucleotide analogue, adefovir, binds to the catalytic site of EF, blocking endogenous binding of the substrate ATP and inhibits cAMP production [52, 234, 235]. Other studies show adenosine containing ligands capable of binding the ATP site on mammalian adenylyl cyclases block their catalytic activity [235-239]. Because these enzymes are able to be blocked at their site of ATP binding, it may be possible that LT is causing a modification allowing binding to the catalytic site of adenylyl cyclases.

We have also shown that cells must remain intact in order for LT to inhibit ET-induced cAMP levels. This may suggest membrane localization of adenylyl cyclases may be playing an important role in their regulation through an interaction between adenylyl cyclases and

membrane components. It has been predicted that after translocation out of the late endosomes, EF refolds in the cytosol side of the endosomal membrane, remaining associated with the membrane [240, 241]. Mammalian adenylyl cyclases are also localized to the plasma membrane within lipid rafts [194-197]. This could indicate LT is impairing these localization events required for both ET and mammalian adenylyl cyclases, possibly through a defect in cytoskeletal rearrangements. Remaining associated with the membrane may be a critical regulatory mechanism required for adenylyl cyclases and through disruption of the actin cytoskeleton, this association may be diminished resulting in inactive enzyme activity.

We have shown using 2D DIGE analysis that over 30 proteins are differentially regulated after dual toxin treatment, as compared to ET alone. The results yielded numerous proteins which were involved in actin regulation. This finding indicates a defect in the actin cytoskeleton through dysfunction of specific actin regulatory proteins after treatment with LT could be leading to diminished cAMP production. Another possibility is a post-translational event caused by LT which would be inhibiting the activity of ET. A potential intermediate protein may require phosphorylation by all three components of the MAPKK pathway in order for successful production of cAMP.

Calcium plays a regulatory role in both ET and mammalian adenylyl cyclases. The mammalian adenylyl cyclase isoforms 1, 3 and 8, along with ET, are sensitive to regulation by calcium. One study investigating the effects of calcium on mammalian adenylyl cyclases activity in HeLa cells noted elevated calcium levels in HeLa cells treated with trichosanthin (TCS), a chemical which increases cytosolic calcium, suppresses cAMP production through inhibition of adenylyl cyclase activity [242-244]. The calcium requirements for EF have previously been studied. It is shown that physiological intracellular calcium levels or slightly elevated calcium

concentrations (0.1-0.4μM) stimulate EF activity [245]. Although, when calcium levels reach above 1μM, EF activity is inhibited [172]. If LT was causing an influx of calcium above physiological levels, the inhibition of cAMP by LT could be explained.

Calmodulin is a calcium sensor protein shown to regulate over 100 different proteins. *In vitro* activity of EF requires calmodulin for activity. The concentration of calmodulin for optimal ET activity is ≥ 10μM [172]. One study has shown LT requires calmodulin, as calmodulin inhibitors protect cells against the action of LT [246]. LT may be sequestering calmodulin away from ET during intoxication, but no studies to date have investigated this mechanism. This could also explain inhibition of mammalian adenylyl cyclases as isoforms 1,3 and 8 require calmodulin for their activity. Although not all mammalian adenylyl cyclases require calmodulin, it may be possible that these isoforms could be contributing to the majority of cAMP production. Another relevant detail concerning LT inhibition of cAMP production is the fact that calmodulin contains phosphorylation sites shown to increase its affinity for its peptide [247]. This is interesting in that EF and some mammalian adenylyl cyclases require calmodulin for activity. Another way LT may be blocking calmodulin binding to adenylyl cyclases it the prevention of calmodulin phosphorylation via an intermediate component downstream of MAPKKs, thereby, decreasing calmodulin's affinity for adenylyl cyclase.

Our results suggest a new area of investigation in which MAPKKs regulate the production of cAMP. These findings can potentially lead to new therapeutic interventions for pathogens manipulating host cAMP levels including *V. cholera*, and *E. coli*, as well as other diseases associated with overproduction of cAMP including certain cancers, heart failure, and neurodegenerative diseases. Our results indicate an overlap in which the MAPKK pathway

affects the important secondary messanger cAMP. We are the first to show that an intact p38, JNK and ERK pathway are required for successful production of cAMP.

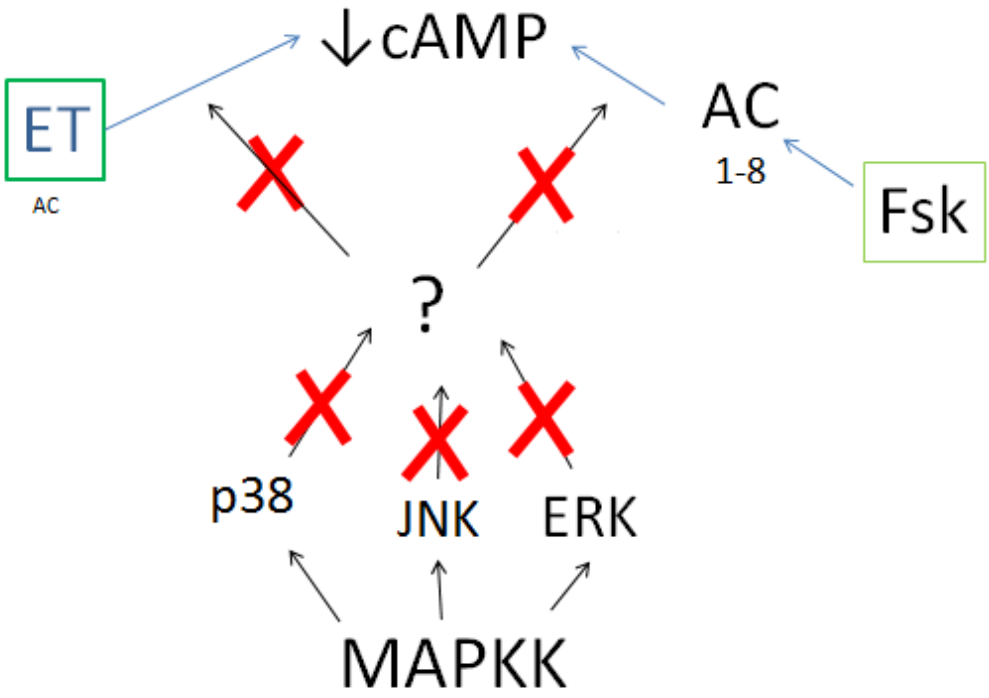


Figure 5-1. Mechanism of cAMP inhibition. Blocking the MAPKK signaling cascade leads to decreased production of cAMP. ET is an adenylyl cyclase which is blocked by LT and chemical inhibitors of the MEK pathway. Forskolin (Fsk) stimulates mammalian adenylyl cyclases (AC) and is also blocked by LT and chemical inhibitors.

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BIOGRAPHICAL SKETCH

Kassidy Chauncey was born in Dade City, Florida. Kassidy grew up the only daughter and middle child. Kassidy began her higher educational career in 2003 at the University of South Florida in Tampa, FL and transferred to the University of Florida where in May of 2006 she received her BS in microbiology and cell sciences. After graduation, Kassidy decided to enter the biomedical research field and began working at an infectious disease lab at the University of Florida.

During this time as a lab technician, Kassidy's research interests flourished and she decided to continue an education in biomedical research and was accepted into the University of Florida's master's program in biomedical sciences in the summer of 2007. During her first year in the master's program, Kassidy continued to enjoy the challenges of research and was encouraged to apply for the PhD program, where she was accepted in the summer of 2008 and worked to obtain her PhD in immunology and microbiology. Kassidy has taken opportunities to teach a dual enrollment class to high school students, along with accepting continuous opportunities to educate high school teachers on biomedical research topics.