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Cellular and Systemic Effects of Anthrax Lethal Toxin and Edema Toxin

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Abstract

Anthrax lethal toxin (LT) and edema toxin (ET) are the major virulence factors of anthrax and can replicate the lethality and symptoms associated with the disease. This review provides an overview of our current understanding of anthrax toxin effects in animal models and the cytotoxicity (necrosis and apoptosis) induced by LT in different cells. A brief reexamination of early historic findings on toxin *in vivo* effects in the context of our current knowledge is also presented.

1. Introduction

The three toxin polypeptides encoded by the *Bacillus anthracis* pXO1 plasmid combine in binary combinations to form two toxins, lethal toxin (LT) and edema toxin (ET), which are responsible for the lethality and symptoms associated with anthrax disease. Although the pXO2 plasmid-encoded capsule is an important virulence factor for the establishment of disease, the symptoms associated with anthrax are the result of toxin production following septicemia. Thus, antibiotics which clear bacteria from infected hosts cannot protect against toxin effects.

Beyond the replication of disease symptoms and lethality by the toxins, proof of their essential and singular role in anthrax also comes from the multitude of animal studies which have shown that immunization against the protective antigen (PA) component of anthrax toxin and antibodies against this protein are sufficient for protection from lethality.

PA is the receptor binding component in LT and ET, for which it facilitates the delivery, respectively, of lethal factor (LF) or edema factor (EF) into cells. PA binds to two cellular receptors, is cleaved and oligomerizes to form binding sites for the EF and LF enzymatic moieties (Figure 1). Detailed analyses of PA interaction with cellular receptors, endocytosis and LF/EF translocation through the PA heptamer pore are not the subjects of this review, and can be found in reviews by Young and Van der Goot as well as Collier in this volume.

LF is a zinc metalloproteinase (Klimpel *et al.*, 1994) which cleaves the N-terminus of a number of mitogen activated protein kinase kinases (MAPKKs, or Meks) (Duesbery *et al.*, 1998; Pellizzari *et al.*, 1999; Vitale *et al.*, 1998; Vitale *et al.*, 2000). LF cleavage of Meks disrupts

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the ERK1/2, JNK/SAPK and p38 signaling pathways. These pathways play important roles in numerous cellular functions ranging from proliferation and cell cycle regulation, to immune modulation and survival against toxic insults. The pharmacological inhibition of the Mek pathways has yielded a large volume of data implicating them in these functions in different cell types, and as expected, LF treatment of cells often replicates these inhibitor effects in the same cells.

EF is a potent calmodulin-dependent adenylate cyclase (Leppla, 1982) and cAMP production by this toxin also affects cell signaling pathways. The literature on regulation of cell function by this important second messenger is even more substantial and it is expected that many cAMP-mediated effects already described in different cell types would also occur universally in response to ET as almost all tested cells have functional PA receptors.

It is interesting that both anthrax toxins target such ubiquitous cell signaling pathways with wide-reaching effects in almost every cell type. The potential modulation of so many different pathways by these toxins makes the identification of the relevant *in vivo* targets a crucial goal for the understanding of their role in human disease.

This review will provide an overview of our current understanding of anthrax toxin toxic effects *in vivo* as well as primarily focusing on cytotoxic effects for different cells. A brief reexamination of the early historic findings on toxin *in vivo* effects in the context of our current knowledge is also presented. The important nontoxic immunomodulatory effects of the toxins are covered in detail by other reviews in this volume.

2. *In vivo* studies

2.1. Lethality

Studies with highly pure *Bacillus anthracis*-derived, endotoxin-free LT in animal models confirm discoveries made in the 1960s (see later section on historic studies). LT induces an atypical vascular collapse in mice and rats without classic hallmarks of endotoxic shock, marked by absence of thrombosis or cytokine involvement (Cui *et al.*, 2004; Moayeri *et al.*, 2003). In contrast, ET used at doses that are lethal for mice is associated with hemorrhaging lesions in many organs accompanied by both hypotension and bradycardia (Firoved *et al.*, 2005). In fact, early *in vivo* studies which often used PA or LF preparations contaminated with EF also did note capillary thrombosis (Beall and Dalldorf, 1966). ET also induces shock in rats in a manner distinctly different than that caused by LT, accompanied by hemorrhage (Kuo *et al.*, 2008), increased heart rate (Cui *et al.*, 2007; Watson *et al.*, 2007a), and reduced cardiac output without altered myocardial contractility (Watson *et al.*, 2007a; Watson *et al.*, 2007b). ET effects on the heart are likely secondary to general loss of circulatory fluids in many tissues.

The striking rapid death LT induces certain inbred rats was first observed decades ago (Beall *et al.*, 1962). As little as 7.0 µg of each toxin component (injected IV) can kill numerous strains while other strains are completely resistant to doses as high as 250 µg. All thus far tested rat strains can be divided into absolute sensitive and resistant groups (M. Moayeri, unpublished data). Sensitive rat strains (including Fischer, Sprague Dawley and Brown Norway) can die as rapidly as 37 minutes (Ezzell *et al.*, 1984; Gupta *et al.*, 2008) when given a saturating dose of LT (100 µg each of PA and LF, IV) and never take longer than 60 minutes to succumb. In contrast, inbred mice die over days (48–120 hours).

Not surprisingly, there are no histopathological changes in LT-treated rats, and almost all histopathological observations in LT-treated mice are simply secondary to vascular insufficiency. While the absence of classic anthrax hemorrhagic pathology previously attributed to LT's effect is a distinct difference from anthrax disease, other LT resultant effects

such as pleural edema and rapid shock closely parallel what is seen with infection in humans and numerous animal models. Despite early hypotheses on LT-induced endotoxic-like shock occurring due to macrophage lysis and cytokine (TNF- α /IL-1 β) induction by LT (Hanna *et al.*, 1993), we now know cytokine shock is not required for LT-mediated vascular collapse in rodents and mice with resistant macrophages also succumb to LT (Cui *et al.*, 2004; Moayeri *et al.*, 2003). The rapidity of the hemodynamic changes in the rat model also supports a unique LT-mediated event which differs from most classic shock states. Amazingly, we still do not know how LT induces vascular collapse in animals. Endothelial cells may be targeted but *in vivo* evidence for their involvement is lacking. Recent data indicates the heart may actually be the primary target for LT in rats and the pulmonary edema associated with LT treatment may simply result from severe heart failure (Kuo *et al.*, 2008; Watson *et al.*, 2007a; Watson *et al.*, 2007b). Our recent electron microscopy analyses show striking early LT-mediated pathological changes in murine heart myocytes and endothelium as early as 6 hours after toxin treatment, accompanied by changes in major cardiac markers (Moayeri *et al.*, 2009). These changes, not detectable by light microscopy analyses, suggest an early targeting of the murine heart and endothelial cells by LT. It will be important to find out if electron microscopy can also detect *in vivo* toxin targeting of endothelial cells in other tissues.

Over recent years, a Sprague Dawley rat infusion model has provided information on the novel vascular shock induced by LT, underlining its differences from cytokine-mediated endotoxic shock (Cui *et al.*, 2004; Cui *et al.*, 2006; Sherer *et al.*, 2007). LT infusion causes blood pressure and heart rate changes in these rats, but cytokines, nitric oxide or histopathological changes are absent. Classic shock therapies such as fluid support either worsen outcome in LT-mediated rat shock (Sherer *et al.*, 2007) or have no beneficial effect, as seen with norepinephrine treatment (Li *et al.*, 2009). Once again, these findings support the concept that LT-related shock is independent of classic vasopressor-mediated *in vivo* changes and essentially different than most bacterial-induced shock.

Two issues arise in the use of LT-sensitive rodents as models for toxin-mediated effects *in vivo*. First, mice succumb to toxin-deficient encapsulated *B. anthracis* strains (by multiple routes) similarly to wild type strains (Heninger *et al.*, 2006), making these animals a poor model for *B. anthracis* bacterial infection studies assessing the role of toxin. However, toxin knockouts in the non-encapsulated Sterne strain (pXO1+ pXO2-) are attenuated (Pezard *et al.*, 1991). While rodents are clearly not the best animal model for the study of anthrax infection, purified LT can reproduce major symptoms or anthrax such as pleural edema in these animals, making them valuable for assessment of *in vivo* toxin mechanisms (Ezzell *et al.*, 1984; Firoved *et al.*, 2005; Moayeri *et al.*, 2003).

The second important issue when using a reductionist approach in studying toxin effects *in vivo* is the question of dose relevance. The amounts of LT used as a bolus in mice are based on older data collected on terminal levels of toxin in various animals (Turnbull, 1990). Until recently, however, ET was often only measured as trace levels in infected animals and the levels in terminal infection were unknown. A recent detailed analysis of *in vivo* toxin production levels in anthrax infected rabbits found a range of LF (10–35 μ g/ml) by 48 hours and a 5:1 ratio of LF:EF in most samples (Dal Molin *et al.*, 2008). This finding actually confirms what was reported for *in vitro* toxin production (PA:LF:EF, 20:5:1) (Sirard *et al.*, 1994) as well as findings in another *in vivo* study of Ames spore infection in rabbits (Mabry *et al.*, 2006). Based on the 5:1 ratio of LF:EF found in real infection models, it is likely lethal doses of ET for mice are actually not achieved in infection (Firoved *et al.*, 2005). However the pathology associated with lower nonlethal doses of ET which does include nonlethal hemorrhaging in multiple organs and extreme changes in adrenal glands are more likely the relevant ET effects in infection (Firoved *et al.*, 2005). Clearly, further investigation of the timing and levels of toxins produced in early stages of infection will be important

Finally, the wide range of mouse and rat strain susceptibilities to LT and ET, which cannot be attributed to receptor expression, suggest an interesting role for genetic background in susceptibility to these toxins (see Tables 1–3). Such genetic-based toxin sensitivity differences may also have important consequences in the human population. BALB/cJ mice succumb to LT (LD100 is 35 µg, IV, 100 µg IP), while the AKR/J, NOD/J and DBA/2J strains are resistant to these doses of toxin. The C57BL/6J strain which has intermediate resistance to LT relative to BALB/cJ, is highly susceptible to ET compared to most other mice. In the case of LT, there appear to be multiple loci involved in determining susceptibility to toxin in mice, only one of which has been identified. The *Nalp1b* (also known as *Nlrp1b*) locus which determines macrophage sensitivity to LT can contribute to increased sensitivity to toxin, but only in certain strains (Moayeri *et al.*, 2004)(see next section). Recombinant inbred mice derived from DBA/2J and Balb/cJ mice suggested at least three loci controlling murine sensitivity (McAllister *et al.*, 2003). The identification of genes contributing to LT and ET sensitivity in animals may be important in understanding the widely differing levels of disease found in humans exposed to similar spore infections.

2.1.1 The role of macrophage sensitivity in LT induced rodent death—

Macrophages from select inbred mouse strains undergo a rapid lysis in response to LT, while other strains harbor LT-resistant macrophages (Friedlander, 1986; Friedlander *et al.*, 1993) (see later sections). LT induced vascular shock kills mice with LT resistant macrophages as well as macrophage-deficient mice (Moayeri *et al.*, 2003; Moayeri *et al.*, 2004). The strong IL-1β and cytokine burst resulting from macrophage lysis (and only occurring in mice with sensitive macrophages) can exacerbate LT-induced vascular collapse (Moayeri *et al.*, 2004), but only in some strains. Certain inbred mice such as those in the C3H background (independent of their Tlr4 status) have macrophages that rapidly lyse following LT treatment, but the mice are resistant to toxin doses that kill Balb/cJ mice. C57BL/6J mice, however, can be converted to the same sensitivity of Balb/cJ mice if harboring macrophages that lyse (Moayeri *et al.*, 2004). Recombinant inbred mice derived from DBA/2J and Balb/cJ mice support these findings and suggest at least three loci controlling murine sensitivity (McAllister *et al.*, 2003). Genetic background may provide protection against the cytokine burst seen in mice harboring LT-sensitive macrophages (Moayeri *et al.*, 2004) and it is tempting to hypothesize that genes involved in regulation of endocrine responses may be involved in sensitivity differences (Moayeri *et al.*, 2005). In addition to the LT macrophage sensitivity locus, *Nalp1b* (Boyden and Dietrich, 2006) another chromosome 11 susceptibility locus originally hypothesized to be inducible nitric oxide synthase (iNOS)(McAllister *et al.*, 2003) was ruled out through mouse knockout studies (Moayeri *et al.*, 2004). The identification of the genetic loci influencing host resistance is important to understanding the role of toxin sensitivity differences in human disease, because thus far tested human macrophages are resistant to Nalp1 mediated macrophage lysis (see later sections).

Unlike mice, intermediate sensitivity states are absent in rats (our unpublished data). Rats either succumb to toxin after injection of 10 µg PA + 10 µg LF by 60–90 minutes or are completely resistant to 25-fold higher doses. In rats survival is thought to be controlled by a single locus (Nye *et al.*, 2007), and a correlation to macrophage sensitivity has been noted, although the susceptibility locus has not yet been mapped. The rapid death in rats is both similar (induction of pleural edema) and very different (death as rapidly as 37 minutes) than lethality in mice (which occurs over days). Thus care should be taken in extrapolating findings from the rat toxin lethality model to mice.

2.1.2. Toxin specific activity and lethality models—While the advances made in purification of high quality recombinant LF and PA from *B. anthracis* have allowed for the assessment of LT effects *in vivo* without the endotoxin issues that plagued *E.coli*-derived preparations, moderate differences in specific activity of toxin preparations can still alter both

in vitro and *in vivo* analyses of toxin effects. For LT the most sensitive assay for testing potency of LF and PA preparations is the minutes to death in the Fischer rat, much in the manner the assay was used for verification of toxin purity in the 1960s. While most labs use macrophage lysis assays to assess potency of toxin preparations, that assay is not sensitive enough to pick up differences that severely alter responses in animals (i.e., a one well difference in a two-fold dilution curve is invariably associated with loss of LT lethal activity at receptor saturating toxin concentrations in mice). Our laboratory has accumulated extensive data on the potency (as time to death, TTD) of toxin preparations in the Fischer rat model, and how it correlates to LT effects in other species such as mice. 10 µg LT (10 µg LF + 10 µg PA) generally kills a rat in 55–90 min and a toxin preparation with a lower specific activity may prolong TTD in rats and not kill mice at receptor-saturating doses. More importantly, *in vitro* studies with low activity preparations lead to changes in the dominant pathway of cell death, or altered immune responses (see later sections). Thus, in our view, the many highly variable results obtained both *in vitro* and *in vivo* for a good number of reports on LT can be directly linked to the potency of the toxin used which can often translate to orders of magnitude difference in “dose” of toxin treatment. Hence one laboratory kills a sensitive macrophage with 200 ng/ml of each toxin component in under 90 min and another requires 5 hours. The ramifications of simply using higher doses of toxin to compensate for low specific activity include considerations of receptor clearance effects by PA which further complicate analyses.

Perplexingly, all *B. anthracis*-derived recombinant EF preparations (produced commercially or in various laboratories) to date have specific activities that are orders of magnitude lower than those for EF produced in *E. coli* or from the original Sterne and Ames *B. anthracis* strains (unpublished observations). The basis for the variability in EF preparations is unrelated to protein breakdown or sequence alterations and is currently unknown.

2.2. Glucocorticoid receptor inhibition

LT, through its effects on the p38 pathway, inhibits glucocorticoid receptor (GR)-mediated activation, but not repression of transcription factors (Webster *et al.*, 2003; Webster and Sternberg, 2005). The importance of the hypothalamic-pituitary-adrenal (HPA) axis and the (GR) response in inflammation during infection is well established and inhibition of GR activation by LT has been demonstrated in mice (Webster *et al.*, 2003). Thus, endocrine response differences to underlying infections may explain variation in mouse sensitivities observed for one strain of mouse in different animal facilities. Supporting this hypothesis, LT resistance in mice can be reversed by adrenalectomy or glucocorticoid feeding/injections (Moayeri *et al.*, 2005). Stress and animal housing conditions must be considered when performing LT studies in mice. Proposed LT mouse susceptibility loci may involve endocrine-related pathways (McAllister *et al.*, 2003; Moayeri *et al.*, 2004). As the preferred treatment for shock in the clinic often involves steroid-based control of inflammatory responses, special attention to potential exacerbation of LT-mediated effects may be warranted.

2.3. LT/ET combinatorial effects in vivo

Sublethal doses of ET which do not induce pathological changes can sensitize LT-resistant DBA/2J mice (Firoved *et al.*, 2007). We now know that LPS associated with the EF used in these studies may exacerbate this sensitization effect by ET in the context of the high cAMP response (unpublished work). ET's striking effects on the adrenal gland (Firoved *et al.*, 2005) and the resulting endocrine perturbation known to alter LT sensitivity in murine models (Moayeri *et al.*, 2005) may account for sensitization. In the rat infusion model, ET was shown to increase heart rate (unlike LT, which decreases heart rate), but the toxins cooperate together to worsen shock (Cui *et al.*, 2007). Care should be taken in interpreting any results of combinatorial ET/LT *in vivo* studies, as the actual relevant doses of each toxin produced in different animal models through various stages of disease may have very different resultant

effects. A better understanding of the contribution of each toxin to the other may be possible through comparing the effects of toxin-isogenic mutants in animal models.

2.4. Other *in vivo* studies

While most studies of the immunomodulatory effects of the toxins are performed *in vitro* using cell lines or primary cells, there are a small number of studies in which immunomodulatory effects were also verified *in vivo*. These studies are covered by other reviews in this volume.

3. Cytotoxicity

The discovery that LT induces a rapid and striking lysis of murine macrophages from some inbred mice (Friedlander, 1986; Friedlander *et al.*, 1993) resulted in a singular pursuit of this cell type as the target of LT function for over a decade. The discovery of LT's cellular substrates has now led to increased focus on the likely more relevant noncytotoxic immunomodulatory effects this toxin induces in many cell types (see the review of Tournier *et al.* in this volume). Despite the fact that the classic rapid lysis of murine macrophages has little relevance to human anthrax disease (human macrophages do not undergo LT-induced rapid lysis) the bulk of the scientific literature on LT's cytotoxic effects still focuses on murine macrophage lysis. Thus, this review will also provide a detailed review of LT cytotoxicity in this particular cell type. It is far more likely, however, that LT effects on another target are responsible for its lethality to the host. Ironically, a classic inverse relationship that has been noted between host macrophage sensitivity to LT and susceptibility to spore infection is often ignored (Lincoln *et al.*, 1967; Welkos *et al.*, 1986).

In recent years many reports of LT 'cytotoxicity' to other cell types have left the impression that this toxin induces cell death in many targets. LT's inactivation of the Mek1/2 pathway, however, is likely to lead to cell cycle arrest in many of these cells, and an accompanying inhibition of proliferation that is often mistaken for cell death in assays that are performed over days. Thus, it is important to review the literature on LT-mediated effects on other cell types very carefully.

Unsurprisingly, ET is not cytotoxic to any cell type.

3.1. Rapid Lysis of Murine Macrophages

Macrophages from inbred mice and rats can be divided into LT-sensitive (examples for mice include Balb/cJ, C3H/HeJ, CBA/J, FVB/NJ, SWR/J and for rats Brown Norway and Fischer) and LT-resistant groups (examples for mice include DBA/2J, AKR/J, SJL/J, A/J, C57BL/6J and for rats Lewis and Wistar-Kyoto) (Nye *et al.*, 2007; Roberts *et al.*, 1998). Sensitive macrophages succumb to LT by 90 minutes if subjected to saturating fully active toxin concentrations (1 ug/ml each component). "Resistant" macrophages that do not succumb to the rapid lysis by LT undergo a slower apoptotic death (Muehlbauer *et al.*, 2007; Park *et al.*, 2002) which can even be observed in LT-sensitive macrophages when sublytic doses of toxin or low activity toxin preparations are used (Park *et al.*, 2002; Popov *et al.*, 2002b). The ability to undergo apoptosis, therefore, appears to be universal to all macrophages, but masked by the rapid lysis/necrosis pathway which dominates in LT-sensitive macrophages. We discuss each type of macrophage death in the following sections (SUMMARIZED IN FIGURE 1).

3.1.1 Nalp1b, caspase-1 and the inflammasome—The identification of *Nalp1b* as the LT sensitivity locus has been the most important leap in the understanding of LT-mediated rapid macrophage lysis (Boyden and Dietrich, 2006). Initially mapped to a single locus on mouse chromosome 11, *Ltxs1* (Roberts *et al.*, 1998) and first misidentified as *Kif1c* (Watters *et al.*, 2001), the *Nalp1b* gene was definitively implicated as the sensitivity allele through a

series of experiments. Expression of a *Nalp1b^S* containing BAC (bacterial artificial chromosome) in *Nalp1b^R* containing resistant mouse macrophages demonstrated that this single gene was dominant in conferring sensitivity and *Nalp1b^S* silencing protected macrophages from rapid lysis. To date, five polymorphic *Nalp1b* alleles have been described in mice (Boyden and Dietrich, 2006).

Nalp1b is a member of the NLR (Nod-like receptor) protein family. These proteins function in a complex known as the “inflammasome” which is responsible for recruitment and proteolytic activation of caspase-1, which in turn activates the pro-inflammatory cytokines IL-1 β and IL-18 (Martinon and Tschopp, 2007). NLRs act as intracellular pattern recognition sensors for danger signals in the cytoplasm (Martinon and Tschopp, 2007; Tschopp *et al.*, 2003). Inflammasome-mediated caspase-1 activation is not only involved in the innate immune response leading to cytokine processing and release, but also cell death in response to many stimuli (Yu and Finlay, 2008).

Early *in vivo* studies showed that rapid transcription-independent IL-1 β release only occurred in LT-injected mouse strains harboring sensitive macrophages (Moayeri *et al.*, 2004; Moayeri *et al.*, 2003). We now know that LT activates caspase-1 in LT-sensitive (*Nalp1b^S*) but not LT-resistant (*Nalp1b^R*) macrophages (Boyden and Dietrich, 2006; Cordoba-Rodriguez *et al.*, 2004) and that this activation is required for rapid lysis (Muehlbauer *et al.*, 2007; Wickliffe *et al.*, 2008b). The *Nalp1b^S* inflammasome is similar to other inflammasomes in requiring an upstream potassium efflux event (Fink *et al.*, 2008; Petrilli *et al.*, 2007; Wickliffe *et al.*, 2008b) and different in that its formation also requires proteasome activity (Fink *et al.*, 2008; Squires *et al.*, 2007; Wickliffe *et al.*, 2008b). Late addition of proteasome inhibitors even after Mek cleavage has occurred can rescue macrophages as long as caspase-1 activation has not occurred (Alileche *et al.*, 2006; Squires *et al.*, 2007; Wickliffe *et al.*, 2008b). Furthermore, the *Nalp1b^S* inflammasome complex also differs from most inflammasome complexes in lacking the adaptor protein ASC (Nour *et al.*, 2009). Caspase-1 activation alone is clearly insufficient for LT-mediated macrophage lysis, as it can be induced in any macrophage, and must occur in the context of a *Nalp1b^S* inflammasome for rapid lysis to ensue (Wickliffe *et al.*, 2008b). We do not know what LT-induced events activate the *Nalp1b^S* inflammasome, or how caspase-1 activation leads to rapid macrophage lysis and what role, if any, Mek cleavage plays in this death pathway. Earlier studies showed no correlation between Mek cleavage and macrophage sensitivity to LT (Pellizzari *et al.*, 2000; Pellizzari *et al.*, 1999). It is possible, however, that the cellular events downstream of Mek cleavage (which occurs equally in *Nalp1b^S* and *Nalp1b^R* macrophages) are sensed differently by *Nalp1b^S* and *Nalp1b^R* proteins. In such a scenario Mek may indeed be the only LF substrate. Alternatively, unidentified LT substrates including potential components of the inflammasome pathway may be involved in initiating cell death. The cleavage of these substrates may also lead to their targeting to the proteasome.

In a recent report NOD2 has been found to be associated with the *Nalp1*-caspase1 complex and a potential role for NOD2 was suggested for LT-mediated caspase-1 activation (Hsu *et al.*, 2008). Because LT effects were only tested in *Nalp1b^R* macrophages in which LT does not activate caspase-1, it is difficult to interpret the role of LT in the *B. anthracis*-induced IL-1 β release observed in these macrophages. The response to bacterial infection in the LT-resistant macrophages clearly depended on NOD2 and *Nalp1b^R* and was attenuated when LT-deficient mutant bacteria were used, but this finding could have more relevance to the general effects of LT deficiency on bacteria (i.e., growth, division, etc.). It remains to be seen if NOD2 is required for LT-mediated caspase-1 activation in *Nalp1b^S* macrophages.

3.1.2 Other discoveries related to macrophage lysis—While the recent discovery of an inflammasome activation requirement for rapid LT-mediated macrophage death has

redirected research on this topic, it is useful to revisit the numerous reports on LT effects in sensitive macrophages for potential links to the inflammasome-mediated cell death.

Early reports on LT-mediated superoxide production and antioxidant protection against LT (Hanna *et al.*, 1994) suggest a role for reactive oxygen species (ROS) in LT-mediated cell death. ROS which have been linked to inflammasome activation (Cruz *et al.*, 2007; Meissner *et al.*, 2008) may also control LT-mediated caspase-1 activation. ROS production may be linked to the mitochondrial membrane potential loss that occurs in response to LT (Alileche *et al.*, 2006). Mitochondrial Bcl-2 family proapoptotic Bnip3 proteins have been implicated in both apoptotic and rapid necrotic LT-mediated macrophage death pathways and resistance in *Nalp1b^S* macrophages is correlated with induction of these proteins (Ha *et al.*, 2007a) (see later section). It is unknown if LT-mediated mitochondrial potential loss occurs due to plasma membrane perturbations or if LT directly targets mitochondria.

Among the many treatments reported over the years that protect against LT-mediated macrophage lysis only those which do not effect LF binding, entry and translocation (thus not effecting Mek cleavage), may provide clues to the mechanism of cell death. For this reason the many studies on compounds protecting cells by preventing LF delivery to the cytosol are not discussed here. Among the many treatments protecting against LT-lysis at a step downstream of Mek cleavage, proteasome inhibitors have been the most interesting. The historic observation of macrophage protection by proteasome inhibitors (Tang and Leppla, 1999) has now clearly been linked to LT-mediated inflammasome and caspase-1 activation (Fink *et al.*, 2008; Squires *et al.*, 2007; Wickliffe *et al.*, 2008b). The identity of the proteasome substrate(s) involved in LT-mediated lysis and Nalp1b inflammasome function is unknown, but this target is subject to the N-end rule of protein breakdown, as type-2 destabilizing amino acid derivative inhibitors of this pathway (e.g., phenylalanine amide) are protective and can also synergize with the aminopeptidase inhibitor bestatin methyl ester (Wickliffe *et al.*, 2008a). The only currently identified N-end rule substrate targeted for proteasome breakdown by LT is c-IAP1, a member of the inhibitor of apoptosis protein (IAP) family, although its breakdown is not required for activation of the Nalp1b inflammasome (Wickliffe *et al.*, 2008a) and may be more important to the slower apoptosis induced by LT (see next section).

Recent studies show that heat shock protects against LT lysis at a step downstream of Mek cleavage. Heat shock may be a universal inhibitor of caspase-1 activation through trapping pro-caspase-1 in a large complex as both LT-mediated Nalp1b inflammasome activation and LPS/nigericin-mediated Nalp3 (Nlrp3 or cryopyrin) inflammasome activation can be inhibited by heat shock with no effect on caspase-1 enzymatic activity (Levin *et al.*, 2008).

A series of studies have shown protection with phosphatase inhibitors, calcium channel blockers/antagonists, phospholipase A2 inhibitors, genistein, neomycin, protein kinase C inhibitors, and phospholipase C inhibitors (Bhatnagar *et al.*, 1989; Bhatnagar *et al.*, 1999; Kau *et al.*, 2002; Panchal *et al.*, 2007; Shin *et al.*, 1999; Shin *et al.*, 2000). While in the case of phosphatase inhibitors Mek targeting may be affected, many of these studies need to be revisited to see how the compounds impact the inflammasome pathway. It bears mentioning that the inhibitor dosing used in some these older studies is often not relevant to their physiological effects.

Finally, despite the many reports on transcriptional and translational responses to LT treatment of sensitive macrophages, continued protein synthesis is not needed for LT-induced inflammasome-mediated lysis (Pellizzari *et al.*, 1999; Alileche *et al.*, 2006; Levin *et al.*, 2008). The targets reported in these studies to be up or downregulated often represent the cell's protective responses to severe stress and cell death rather than LT-specific events. Perplexingly, the time points utilized in many of these studies are often when a substantial percentage of

cells are already dead, even by each study's own death response curves, (Comer *et al.*, 2005b; Kuhn *et al.*, 2006; Sapra *et al.*, 2006), and even when time points are selected where a smaller number of cells are dead, the lytic event has clearly already commenced (Chandra *et al.*, 2005). The only study investigating responses at very early times after LT treatment and prior to any cell death found altered expression of genes under GSK-3 β (glycogen synthase kinase 3 β) regulation and inhibition of this kinase sensitized both *Nalp1b^S* and *Nalp1b^R* macrophages (Tucker *et al.*, 2003). The upregulation of genes controlled by GSK-3 β may be a survival response in the apoptotic death pathway LT induces in "resistant" macrophages (see next section), as GSK-3 β inhibition is normally a *protective* event, but it is difficult to speculate on how this kinase plays a role in caspase-1 mediated lysis. GSK-3 β inhibition has also been linked to protection from Mek-cleavage mediated cell arrest induction by LT (Ha *et al.*, 2007b).

3.2. Apoptotic death of macrophages

Almost all tested macrophages from numerous species which do not succumb to LT through the rapid caspase-1 lysis are subject to toxin-induced apoptosis.

3.2.1 "Resistant" *Nalp1b^R* macrophages—*Nalp1b^R* macrophages succumb to LT over a 16- to 72-hour period through an apoptotic pathway dependent on Mek cleavage (Muehlbauer *et al.*, 2007; Park *et al.*, 2002) and LPS can sensitize them to an even more rapid 8-hour death (Park *et al.*, 2002). The apoptotic death by LT was shown to require inhibition of the p38 protective pathway (Park *et al.*, 2002). Surprisingly, LPS-primed *Nalp1b^S* macrophages in the same study were also shown to undergo apoptosis after LT treatment, possibly because the LF/PA63 mixture used had a low specific activity allowing observation of this apoptosis in the absence of rapid caspase-1 mediated lysis (Park *et al.*, 2002). A third caspase-independent necrotic mechanism of macrophage involving TNF- α has also been suggested for *Nalp1b^R* macrophages sensitized to LT (Kim *et al.*, 2002). Various microbial products were shown to result in a slow LT-mediated necrotic death, which in contrast to the LPS-mediated LT sensitization to apoptosis (Park *et al.*, 2002), did not require classic apoptotic caspase activation (Kim *et al.*, 2002). Interestingly, LT-resistant human macrophages were also sensitized through a TNF- α dependent pathway, but no effect was seen on *Nalp1b^S* murine macrophages (Kim *et al.*, 2002). It is attractive to speculate that macrophage sensitization to LT may occur in real infections through apoptosis or a novel slow necrosis different than the murine caspase-1 dependent rapid lysis and this cell death could have consequences for the infectious process in humans.

3.2.2. Resistance induction in *Nalp1b^S* macrophages—Subpopulations of *Nalp1b^S* macrophages become resistant to LT after multiple passages (Ha *et al.*, 2007a) or by treatment with sublytic toxin doses (Salles *et al.*, 2003). *Nalp1b^S* macrophages in the phenotypically "resistant state" have reduced expression of two related proteins, mitochondrial Bcl2/adenovirus E1B-interacting proteins Bnip3 and Bnip3L (Ha *et al.*, 2007a). The p38 shutdown required for LT killing of LPS-sensitized cells (Park *et al.*, 2002) is actually essential to LT-mediated induction of *resistance* in *Nalp1b^S* macrophages such that p38 inhibitors can mimic LT-induced resistance. It is believed the p38 shutdown leads to reduction of the Bnip3 proteins. Bnip3 overexpression in *Nalp1b^S* macrophages imparts resistance, while siRNA mediated shutdown of expression sensitizes. It is unclear if Bnip3 controls caspase-1 mediated cell death in *Nalp1b^S* macrophages or if it primarily serves a protective role in resistance. Bnip3 proteins may alter mitochondrial protective responses downstream of *Nalp1b*-mediated activation of caspase-1 or alternatively, influence early events which prevent LT-mediated mitochondrial targeting upstream of potassium flux, inflammasome activation, and caspase-1 activity. Bnip3 have been implicated in both apoptosis and a necrosis pathway involving mitochondrial membrane permeabilization, ROS production and sudden loss of plasma membrane integrity

reminiscent of the lysis seen in *Nalp1b^S* macrophages (Vande Velde *et al.*, 2000). The role of these proteins in LT-mediated cell death certainly deserves further study.

3.2.3. Human monocytic cells—While human macrophages thus far tested to date from different donors are resistant to LT-mediated caspase-1 lysis (our unpublished data), human monocytic cell lines (U-937, HL-60 and THP-1) can be sensitized to LT-mediated apoptosis following activation. LT normally induces cell division arrest in these cells over a course of days, without cell death (Kassam *et al.*, 2005), but activation signals make them sensitive to a slow caspase-1-independent apoptosis that is distinctly different than the necrotic death induced by TNF- α mediated “sensitization” of resistant mouse macrophages (Kassam *et al.*, 2005). These findings are not limited to monocytic cell lines, as activated human peripheral blood mononuclear cells undergo apoptosis following inhibition of cell division (Popov *et al.*, 2002a). As will be seen in the next section, dendritic cell (DC) sensitivity to LT is also influenced by activation state (Reig *et al.*, 2008), although maturation signals actually protect in that case.

Human monocytic cell lines, like many cell types, require Mek1 activity for growth. Many reports of “cytotoxicity” by LT in a variety of cell types actually measure the cell arrest induced by LT through inhibition of the Mek1/2 pathway relative to untreated controls. Ha and colleagues have shown that LT induces cell arrest in human monocytic cells through breakdown of cyclin D1/D3, a process which can be reversed by activation of the phosphatidylinositol 3-kinase/Akt pathway. Akt provides protection from cell arrest by inhibition of GSK-3 β (Ha *et al.*, 2007b). One can hypothesize that LT-mediated cell cycle arrest may be the basis for the pathological events linked to LT vascular collapse in species such as mice which succumb over a period of days (Moayeri *et al.*, 2003).

3.3. Dendritic cell apoptosis and necrosis

Reports on LT effects on DC are highly varied. Human DC (Maldonado-Arocho *et al.*, 2006), murine *Nalp1b^R* DC (Tournier *et al.*, 2005) and even murine *Nalp1b^S* DC (Chou *et al.*, 2008) have been reported to be resistant to LT toxicity over treatment periods as long as 48 hours. On the other hand, rapid proteasome and caspase-1 dependent lysis of DC harboring the *Nalp1b^S* allele and slow apoptosis of murine *Nalp1b^R* DC and human DC has also been reported (Alileche *et al.*, 2005; Muehlbauer *et al.*, 2007). Conflicting reports also exist on the role of activation state in DC sensitivity. One group found that activation signals do not affect DC sensitivity, demonstrating that bone-marrow derived DC (immature) and spleen-derived activated DC had equal LT sensitivity (Alileche *et al.*, 2005). Another group showed DC activation protects against apoptosis of *Nalp1b^R* DC (Reig *et al.*, 2008). Furthermore, DC from mice with the *Nalp1b^S* allele in which caspase-1 was knocked out (eliminating the rapid necrosis pathway and allowing the apoptosis pathway to dominate) were also protected against LT by maturation signals. Although Mek1/2 cleavage is not affected by maturation state, inactivation of these kinases by LT is required for DC apoptotic death, since maturation also protected against Mek1/2 inhibitor-mediated cell death (Reig *et al.*, 2008).

It is unclear if the variability in DC literature reports is linked to the different activation states resulting from the isolation method and tissue source of these cells or other confounding factors. Nevertheless, care should be taken in interpretation of LT effects on DC, not only in the context of cytotoxicity effects, but especially as relating to immunomodulatory functions of LT.

3.4. Cytotoxicity in other cell types

As previously mentioned, induction of cell cycle arrest by LT is a common occurrence in any cell type that requires the Mek1/2 pathway or downstream ERK activity for proliferation. While some studies are careful to make the distinction between cell arrest and cell death, reports on

many cell types do not, which may explain the discrepancies in findings for some cell types. The endothelial cell is attractive as a potential relevant *in vivo* target of LT because of the unique vascular collapse induced by this toxin and the association of the two anthrax toxin receptors with vascular function or angiogenesis. However, the initially reported apoptosis of HUVECs (Kirby, 2004) may have reflected cell arrest as numerous laboratories have been unable to induce HUVEC cell death with LT. Of course genetic differences in the human source of these cells may also play a role in cell sensitivity. In the initial study, viable cell numbers were measured relative to untreated cells grown in parallel for the same number of days, such that proliferation arrest would not be distinguishable from apoptotic death. FITC-ZVAD staining, however, accompanied by protection using caspase inhibitors supported an LT-mediated apoptosis in these cells. Some investigators have clearly described LT-mediated proliferation arrest in HUVEC (Huang *et al.*, 2008) and SV40-transformed mouse endothelial cells (Depeille *et al.*, 2007), while others studies on HUVEC and human lung microvascular endothelial cells are unclear as to whether apoptosis or cell arrest is being measured (Paddle *et al.*, 2006; Pandey and Warburton, 2004). A number of laboratories have specifically noted the inability to induce apoptosis in HUVECs (Batty *et al.*, 2006; Warfel *et al.*, 2005), but do report proliferation arrest (Batty *et al.*, 2006) and LT-induced loss of barrier function in the absence of any endothelial cell death (Warfel *et al.*, 2005). Thus it remains unclear whether HUVECs actually undergo apoptosis in response to LT, and even then, these cells may not even be reflective of LT-mediated effects on endothelial cells *in vivo*. LT-induced loss of barrier function, however, would clearly play a very important role in vascular collapse induced by this toxin *in vivo* (Warfel *et al.*, 2005). The rapidity with which LT induces vascular leakage in a modified Miles-assay in mice also seems to support altered endothelial barrier function independent of cell death (Gozes *et al.*, 2006).

LT is not cytotoxic to neutrophils and lymphocytes, but proliferation arrest has been reported in these cells through Mek pathway shutdown over a period of days (Comer *et al.*, 2005a; Fang *et al.*, 2005; Xu *et al.*, 2008). Conflicting results on epithelial cell sensitivity to LT have also been reported (Lehmann *et al.*, 2009; Paddle *et al.*, 2006; Pandey and Warburton, 2004).

3.5. ET is not cytotoxic

With the exception of one report of cytotoxic effects in zebrafish (Voth *et al.*, 2005) and a recent study suggesting that ET can induce cell cycle arrest in macrophages through activation of nuclear GSK-3 β (Larabee *et al.*, 2008), ET has been shown to be nonlethal to all cells on which it has been tested. These include, but are not limited to DC (Chou *et al.*, 2008; Tournier *et al.*, 2005), macrophages (Comer *et al.*, 2006), T-cells (Paccani *et al.*, 2005), neutrophils (Crawford *et al.*, 2006) and human microvascular endothelial cells (Hong *et al.*, 2007). cAMP increases generally do not induce cell death and thus these findings are expected.

4. LT and ET mediated noncytotoxic and immunomodulatory effects

The effects of anthrax toxins on various immune cells are reviewed in by Tournier *et al.* in the current volume and will not be presented in great detail here. A very brief overview is presented for most cell types (SUMMARIZED IN FIGURE 1).

4.1. Macrophages, dendritic cells, neutrophils and lymphocytes

Almost all of LT's immunomodulatory effects are inhibitory for the innate immune response and are a direct result of the shutdown of the Mek1/2-Erk1/2, Mek4/7-SAPK-JNK and Mek3/6-p38 signaling pathways (and thus can be replicated with specific Mek activity inhibitors). In macrophages and dendritic cells these effects include impairment of cytokine responses, antigen presentation function, bactericidal ability and phagocytosis. In neutrophils conflicting reports on altered superoxide responses and NADPH oxidase activity as well as chemotaxis

have been reported. In T-lymphocytes classic cytokine/chemokines pathways and chemotaxis are disrupted. In B cells proliferative responses and IgM production are inhibited. While LT targeting of the adaptive immune response by manifesting inhibitory effects on B-cells or T-cells is unlikely to play a role in an acute disease like inhalational anthrax, it is clearly possible that *B. anthracis* benefits from targeting adaptive immunity in non-acute, cutaneous anthrax infections.

ET manifests its many immunomodulatory effects through increase of cellular cAMP and the resulting activations of the protein kinase A (PKA)/cAMP response element binding protein (CREB) and exchange protein directly activated by cAMP (Epac)/Ras-associated protein-1 (Rap1) pathways. As this important second messenger has both stimulatory and inhibitory effects on an incredible range of cellular functions, it is expected that ET will eventually be found to have the same wide range of effects. It appears that while LT inactivation of Mek pathways is almost invariably suppressive to innate immune response pathways, cAMP activation of the PKA-dependent CREB pathway or the PKA-independent EPAC pathway results in complicated pro- and anti-inflammatory effects in monocytes, DC and lymphocytes and opposing effects on chemotaxis in different cell types.

LT and ET effects in macrophages, DC, neutrophils and lymphocytes are reviewed in detail by other authors in this volume and we refer you to the review by Tournier et. al for the references pertaining to the findings on various immune cells.

4.2. Endothelial cells and vasculature

Although the effects of LT on endothelial cells have not been directly investigated *in vivo*, numerous studies have shown that LT affects angiogenesis and tumor vasculature (Alfano *et al.*, 2008; Depeille *et al.*, 2007; Duesbery *et al.*, 2001; Liu *et al.*, 2008), supporting a targeting of the endothelium in the host. In a study directly linking Mek1/2 inhibition *in vivo* to vascular dysfunction, LT was shown to induce endothelial cell-death independent permeability in zebrafish vasculature with associated pericardial edema in a manner duplicated by Mek1/2 inhibitors (Bolcome, III *et al.*, 2008). It is difficult to understand how Mek cleavage is linked to resistance differences in mice and rats, however, unless cleavage of these kinases in endothelial cells from resistant animals results in very different effects. The role of Mek cleavage in endothelial cell dysfunction deserves further study.

Perhaps the most important *in vitro* finding on LT-mediated effects on endothelial cells is the cell-death independent loss of barrier function induced by toxin (Warfel *et al.*, 2005) (a cell-death independent finding replicated in the previously mentioned zebrafish model (Bolcome, III *et al.*, 2008)). LT can also modulate endothelial cell responses to LPS and cytokine stimuli, examples of which include enhancement of TNF-induced VCAM-1 expression and monocyte adhesion to endothelial cells (Steele *et al.*, 2005; Warfel and D'Agnillo, 2008) and inhibition of IL-8 production through transcript destabilization (Batty *et al.*, 2006).. These types of effects are likely not relevant to LT-mediated lethality or vascular collapse (which are cytokine independent), but play an important role in bacterial infections which are accompanied by strong cytokine production. Classic immune cell-mediated vascular damage is likely responsible for the vasculitis associated with anthrax infections (Fritz *et al.*, 1995; Grinberg *et al.*, 2001; Vasconcelos *et al.*, 2003; Zauha *et al.*, 1998) that is absent in LT treated animals (Cui *et al.*, 2004; Moayeri *et al.*, 2003). Bacterial infection induced vasculitis can be significantly affected by cytokine responses and receptor expression changes (such as that observed with VCAM-1) in endothelial cells. Paradoxically, a number of the thus far *in vitro* reported LT effects on endothelial cells, such as the inhibition of IL-8 (Batty *et al.*, 2006) or other chemokines/chemokines receptors (van Sorge *et al.*, 2008), or tissue factor expression (Rao *et al.*, 2004) would theoretically be inhibitory to classic shock vasculitis but could contribute substantially to immune cell recruitment in the innate immune response. In a manner

opposing LT effects, ET increases transendothelial electrical resistance of endothelial monolayers (Tessier *et al.*, 2007). The toxin is believed to induce vascular leakage in rabbits through the action of neurokinins, prostanoids and histamine rather than alterations in endothelial cell permeability (Tessier *et al.*, 2007).

LT induced platelet aggregation resulting in suppression of human blood clotting, altered P-selectin expression, and endothelial cell interaction have been reported in one study (Kau *et al.*, 2005). The absence of fibrin deposits in mice treated with LT makes it less likely that platelets play a role in the vascular insufficiency induced by LT alone, despite a toxin-induced thrombocytopenic effect (Moayeri *et al.*, 2003). Thus it remains to be seen if LT effects on platelets occur in mice. LT-mediated platelet dysfunction, however, would have great implications for LT's alteration of the host response to bacterial infection. ET has been reported to suppress thrombin-induced platelet aggregation and clotting function (Alam *et al.*, 2005), likely contributing to the hemorrhaging associated with ET in animal models (Firoved *et al.*, 2005).

4.3. Epithelial cells

A recent study of LT effects in human lung epithelial cells found LT-mediated decreases in transepithelial electrical resistance and loss of barrier function associated with altered cellular tight junctions and focal adhesions over days of toxin treatment (Lehmann *et al.*, 2009). LT-mediated effects were due to increases in F-actin levels in cells, and could be reversed by transfection of cells with uncleavable Mek proteins, implicating these kinases directly in the cytoskeletal changes induced by toxin. If toxin-mediated remodeling of the lung epithelium and functional consequences can be verified *in vivo*, it will certainly have important implications for anthrax infection pathogenesis. However, it is unlikely these effects contribute to LT-mediated lethality, especially in the rapid death induced in the rat model, but they may be suggestive of similar cytoskeletal-mediated changes linked to barrier function alterations in endothelial cells.

4.4. ET/LT combinatorial effects in cells

When reviewing the individual studies on LT, ET, and *B. anthracis* infection effects in various cell types, the curious observations emerge that LT appears to shut down the cytokines induced by *B. anthracis*, and that the numerous ET-induced cAMP-mediated effects theoretically negate LT-mediated effects. As an example, ET activates CREB, which is a macrophage survival factor downstream of p38 (Park *et al.*, 2005). LT in turn inhibits the p38 pathway through Mek3/6 cleavage and many of LT's effects in cells are due to blocking this pathway. ET activation of CREB has been shown to promote survival of LT-treated macrophages that normally would undergo apoptosis (Park *et al.*, 2005).

The difficulty of designing experiments to study the combined effects of LT and ET cannot be underestimated, since it is very challenging to accurately model the complex events that occur *in vivo* during an infection. The relative amounts of each component, timing and site of production, and factors effecting binding and uptake (such as receptor clearance) are unknown for most stages of anthrax infection. Thus, the doses and treatment times of each toxin component used in co-treatment experiments are unlikely to accurately mimic events in an actual infection. Furthermore, interpretation of findings on ET and LT combined effects may be complicated by ET's upregulation of PA receptors on monocytic cell populations (Maldonado-Arocho *et al.*, 2006).

In the limited number of studies that have investigated the combined effects of the toxins in T-cells and DCs, LT and ET were found to both cooperatively synergize in manifesting some inhibitory effects, but also to have inverse effects on select pathways, likely reflective of the

pro- and anti-inflammatory effects of cAMP signaling (Chou *et al.*, 2008;Paccani *et al.*, 2005;Tournier *et al.*, 2005).

5. Toxins, spores, macrophages and early infection

The anthrax toxins are believed to play a role in the early stages of infection and the fate of germinated bacteria, prior to eliciting any systemic toxic effects. Reports suggest that toxin action is required for release of the large numbers of dividing intracellular bacteria from cells (Dixon *et al.*, 2000) and that toxin is necessary for spore survival against macrophage killing (Guidi-Rontani *et al.*, 2001). Because most spore studies are performed in LT-sensitive macrophages, it is difficult to extrapolate from these results to LT-resistant human macrophages. The real situation *in vivo* may be a complicated balance between macrophage killing of spores, germination, and dissemination after macrophage killing. In a study using a toxin-receptor negative variant of the LT-sensitive RAW264.7 macrophage cell line injected in mice, these cells were able to provide better protection against a challenge with Ames spores in animals than their receptor-expressing counterparts, suggesting a protective role for macrophages that bacteria can overcome with toxin (Cote *et al.*, 2008). In a way, these studies provide an indirect comparison of LT-sensitive and LT-resistant macrophages for their ability to protect against spores, and it appears that LT-resistance of macrophages (in this case through absence of receptor) would allow better killing of spores and control of infection.

Paradoxically, however, macrophage sensitivity to LT has been shown to have an inverse correlation to spore sensitivity in animals (Lincoln *et al.*, 1967;Welkos *et al.*, 1986), suggesting LT-mediated macrophage killing may be detrimental to establishing infection in some species. While translocation of LT produced by germinating spores from phagolysosomes into cytosol (through unknown mechanisms in the absence of receptor) has been reported to kill LT-sensitive macrophages and allow the escape of germinated bacteria (Banks *et al.*, 2005), it is still unclear if these findings have relevance to LT-resistant human macrophages. It will be important to decipher LT's role in controlling early infection steps including germination and survival in DC/macrophages in hosts harboring LT-resistant *Nalp1b* (or *Nalp1*) alleles.

6. Revisiting historical studies on anthrax toxins

The volume of literature on anthrax and anthrax toxins between 1953 and 1968 is almost as remarkable as the absence of publications in this field from 1969 through the early 1980s. The research work done in the earlier period on the anthrax toxins can now be recognized as remarkable not only for its intensity, but also for the ability of researchers to deduce many key features of the toxins, in spite of not having modern tools such as recombinant DNA technology and powerful protein purification methods. In a period where animal welfare regulations were far more lax, and prior to the advent of cell biology, almost all early studies on anthrax toxin function were performed *in vivo*. Interestingly, in a manner that parallels the more recent period, once the lethal activity of LT was discovered, almost all work focused on this toxin, with little attention being directed to ET. In this section we will revisit the toxin studies from this early period and review important observations in the context of the material reviewed above.

The anthrax toxins were discovered in 1954 when Harry Smith and colleagues observed that bacteria-free filtered serum from *B. anthracis* infected guinea pigs was lethal when injected in mice and guinea pigs (Smith *et al.*, 1955b;Smith *et al.*, 1955a;Smith and Keppie, 1954). The filtrates also induced edema in guinea pigs and rabbits when injected intradermally. The recognition that an exotoxin was responsible for the lethal effects of anthrax disease led to purification of two separate toxin activities which required a third common protein (now known as PA) to manifest their *in vivo* functions (Smith and Stanley, 1962;Stanley *et al.*, 1960;Stanley and Smith, 1961). The combinatorial toxins to this day remain named after the early observations made about their *in vivo* effects (lethality and edema).

The initial reports of toxin-induced vascular collapse and shock in mice and guinea pigs were key aspects of the discovery of the toxin (Smith *et al.*, 1955a). The discovery of the rapid death of the Fischer rat and the extensive pleural edema associated with LT-mediated rat death came after numerous improvements in the purification of the toxins and was the basis for much of the early *in vivo* investigations (Beall *et al.*, 1962; Stanley *et al.*, 1960; Stanley and Smith, 1961; Thorne *et al.*, 1960). Early electron microscopy studies in rats using partially purified LT (which likely had some EF present) showed the elevation of cytoplasmic processes of lung endothelial cells away from basement membranes without destruction of endothelial cells, leading to a conclusion that these pathologies were strictly secondary mechanical manifestations of pulmonary edema (Beall and Dalldorf, 1966). Capillary thrombosis in the lungs was likely due to contaminating ET in early preparations (based on our current understanding of these toxins' histopathology in animals). Interestingly light microscopy studies did not show the changes in rat endothelium, much like the LT-induced changes we have failed to observe *in vivo* over years of performing histopathological analyses in rodents. Recently, however, our EM analyses of the hearts of mice treated with LT show striking effects on cardiac capillary endothelium (Moayeri *et al.*, 2009). Even with all the emphasis on the lung, early studies recognized that LT induced vascular permeability in rat skin and peritoneum as well and that this toxin caused a general effect on the vasculature (Beall and Dalldorf, 1966). Thus investigators concluded that "anthrax toxin acts directly on the membranes of the capillary endothelial cells."

Interestingly, one of the first primary uses of the Fischer rat model was as an assay for toxin purification and specific activity. Most of the early papers that followed the 1962 discovery of this rapid death always used the minutes to death of the Fischer rat model to assess purity and potency of purified toxins and to assign units of activity to preparations (including (Beall *et al.*, 1962; Buzzell, 1967; Fish *et al.*, 1968b; Haines *et al.*, 1965)). As discussed in previous sections, our laboratory has now returned to this method to assess toxin preparation quality.

In the 1960s some investigators began doing side-by-side spore infection and toxin-infusion or injection studies in rats, rabbits, guinea pigs, monkeys and chimpanzees in order to definitively implicate toxin in the pathophysiology associated with spore infection. In all these models, investigators using a variety of physiological measurements noted that the effects seen in terminal anthrax disease and with LT treatment of animals were virtually identical, but simply occurred earlier with toxin treatment (Klein *et al.*, 1962; Klein *et al.*, 1966; Lincoln *et al.*, 1967; Walker *et al.*, 1967). Much can be learned from the many simple observations made in these studies: One of the most interesting findings from these studies was the inverse relationship seen among animal species and strains between the ability to establish anthrax infection and susceptibility to LT, first based on striking differences between the NIH black and Fischer rats. Although the NIH black rat was very resistant to toxin treatment, it was highly sensitive to spore infections, with very high numbers of germinated bacteria in circulation at the same times that the Fischer rat had very low numbers. Yet the much lower number of bacteria in the Fischer rat was sufficient for a more rapid death in this highly LT-sensitive animal (Klein *et al.*, 1963; Lincoln *et al.*, 1967). Investigators interpreted this as reflecting a two phase disease process: a first phase of resistance to germination and outgrowth, and a second stage of succumbing to toxins. We can now suggest that the basis for these differences was the sensitivity of the DC and macrophages in Fischer rats, which would not allow the germination in or transport of the bacteria by these cells, resulting in a relative "resistance" to spore infections.

Another repeated findings that fascinated early investigators of the LT-induced shock state, and is often-mentioned by Dr. Smith in talks reviewing toxin effects, was the extreme hyperglycemia associated with LT-mediated shock (Eckert and Bonventre, 1963; Klein *et al.*, 1966; Slein and Logan, Jr., 1960; Walker *et al.*, 1967). This finding may fit with what is now

known about LT's potent inhibitory effects on GR function both *in vitro* and *in vivo* (see sections on LT *in vivo* effects). This receptor is essential for glucogenesis and many cellular functions controlling glucose levels *in vivo*.

While investigators often revisit the early literature on LT-induced shock, one aspect that is often ignored is the striking and controversial work suggesting that LT targeting of the central nervous system (CNS) could explain the induction of pleural edema, vascular dysfunction and respiratory arrest. In these early reports, Rhesus monkeys and chimpanzees challenged with toxin showed electroencephalogram (EEG) abnormalities a few hours before respiratory cessation and myocardial failure. Spore infections showed the same findings in these animals, and antisera against toxin could reverse these effects if administered within 8 hours of toxin challenge. PA alone and LF alone did not cause the same changes. LT preparations injected directly into the spinal fluid of monkeys, caused 6–10 minute deaths, following rapid drops of left ventricular heart function (Vick *et al.*, 1968; Walker *et al.*, 1967). However, histopathological effects in the CNS were not found, indicating the blood-brain barrier was unaffected by the toxins (Bonventre *et al.*, 1967). Interestingly, abnormal EEG activity was also found associated with the rapid 58 minute death in rats (Fish *et al.*, 1968a). In both the monkey and rat studies, EKG, heart rate and blood pressure were normal until minutes before death (Fish *et al.*, 1968a; Vick *et al.*, 1968). Knowing the timeline of LF entry and delivery to the cytosol, it is hard to imagine how a 6–10 minute death would result from the toxin's intracellular actions. The negative controls for these experiments, however, seem extensive and accurate. LT targeting of the CNS is an area that should be revisited, especially when considering the rapidity of death in the rat model.

Early suggestions of LT effects on cardiac function came not only from the monkey studies described above, but from studies where injection of *B. anthracis* culture supernatants in dogs affected multiple cardiovascular functions such as blood pressure, cardiac contractile force, cardiac output and heart rate (Liu and Williams, 1971). The effects of LT on the heart are today an active area of study. Norepinephrine as a cause for the pleural edema was also postulated because culture supernatants from *B. anthracis* cause vascular changes similar to this mediator (Williams *et al.*, 1967) and it was released after LT injections, but at very low levels (Slein and Logan, Jr., 1960).

Investigators in the field of anthrax toxin research would benefit from a regular re-reading of the older studies in this field, if only to gain an appreciation for the number of astute observations on toxin function that were deduced strictly from *in vivo* studies. In revisiting this literature, there are a large number of observations on the *in vivo* effects of toxin which could be important and useful in guiding investigators today.

7. Concluding Remarks

The discovery of the cellular pathways targeted by LT and ET has allowed researchers to predict and provide a better understanding of LT and ET's cytotoxic and immunomodulatory effects on many cell types *in vitro*. In a way, the anthrax toxins can be thought of as highly specific "drugs" with potencies exceeding those of nearly all small molecule compounds. One would have predicted that the *in vivo* action of these toxins would be more focused, limited, and therefore more easily defined than for traditional drugs. However the involvement of the Mek pathways and cAMP in multiple cellular functions in virtually every cell in the body makes it far more difficult to identify the crucial and relevant *in vivo* targets for LT and ET in real infections. It is an easier first step to demonstrate the sequelae of Mek inhibition or cAMP signaling in response to the toxins in a particular cell type *in vitro* and in fact, researchers would be hard pressed to find a cell type unaffected by these toxins *in vitro*. It is an entirely different problem to find relevance for the findings *in vivo*. Thus far, the links between affected cellular

pathways and the roles the toxins play in infected hosts remain mostly unknown. Ironically, the thus far most often studied anthrax toxin effect, the rapid lysis of murine macrophages, likely has little relevance to anthrax disease.

The challenge now facing anthrax toxin researchers is to extend their analyses so as to understand how the toxins contribute to human infection. There are many important questions needing analysis which come to mind, but some include the following. Is MEK cleavage essential or sufficient for LT-induced lethality? If not, what other LF substrates mediate its *in vivo* effects? If essential, in which target cells does MEK cleavage matter? How does LT induce vascular collapse? Is there an evolutionary advantage to harboring a sensitive *Nalp1b* allele, and are studies on the unique LT-mediated mouse macrophage lysis helpful in understanding the disease process in human hosts? Does the *Nalp1b* allele status determine the response in other cell types such as endothelial cells? What are the relevant intersections of the pathways perturbed by LT and ET during infection, especially in the context of the multitude of other pathways altered during bacterial infection? How many of the regularly reported immunomodulatory findings *in vitro* can be correlated to relevant cellular events during anthrax infection?

Like most problems in biology, the problem here lies in the complexity of the human organism, and the difficulty of understanding it from a “systems biology” view. While there is great value in testing toxin effects in many *in vitro* cell systems, these can provide only a certain amount of information that is of value in understanding an infectious process in mammals. Reductionist approaches using toxin treatment in animal models, while useful, will also only go so far. In the end the true role of these toxins in disease must be studied in the context of infections in different animal species, and especially in non-human primates. This would be the only system in which the complex interplay between LT and ET, already shown to act in contradictory directions in many signaling pathways, can be understood in the context of other virulence factors and the sequelae of bacterial infection.

Before the major advances in molecular and cellular biology, anthrax toxin research began in animals, with simple observations of toxin-mediated pathologies relying on crude toxin preparations prepared directly from infected hosts, and a large volume of what is known today about the actual functions of the toxins was initially observed and documented in these animal systems, even if it was not fully understood. Perhaps with the many additional tools now available to us, future studies on anthrax toxin should again focus on *in vivo* models of infection, as frequently pointed out by Harry Smith (Smith, 2002), whose seminal work started us on this journey (Smith *et al.*, 1955a; Smith and Keppie, 1954).

References

- Alam S, Gupta M, Bhatnagar R. Inhibition of platelet aggregation by anthrax edema toxin. *Biochem Biophys Res Commun* 2005;339:107–114. [PubMed: 16293226]
- Alfano RW, Leppla SH, Liu S, Bugge TH, Duesbery NS, Frankel AE. Potent inhibition of tumor angiogenesis by the matrix metalloproteinase-activated anthrax lethal toxin: Implications for broad anti-tumor efficacy. *Cell Cycle* 2008;7:745–749. [PubMed: 18245947]
- Alileche A, Serfass ER, Muehlbauer SM, Porcelli SA, Brojatsch J. Anthrax lethal toxin-mediated killing of human and murine dendritic cells impairs the adaptive immune response. *PLoS Pathog* 2005;1:e19. [PubMed: 16254597]
- Alileche A, Squires RC, Muehlbauer SM, Lisanti MP, Brojatsch J. Mitochondrial impairment is a critical event in anthrax lethal toxin-induced cytolysis of murine macrophages. *Cell Cycle* 2006;5:100–106. [PubMed: 16357526]
- Banks DJ, Barnajian M, Maldonado-Arocho FJ, Sanchez AM, Bradley KA. Anthrax toxin receptor 2 mediates *Bacillus anthracis* killing of macrophages following spore challenge. *Cell Microbiol* 2005;7:1173–1185. [PubMed: 16008584]

- Batty S, Chow EM, Kassam A, Der SD, Mogridge J. Inhibition of mitogen-activated protein kinase signalling by *Bacillus anthracis* lethal toxin causes destabilization of interleukin-8 mRNA. *Cell Microbiol* 2006;8:130–138. [PubMed: 16367872]
- Beall FA, Dalldorf FG. The pathogenesis of the lethal effect of anthrax toxin in the rat. *J Infect Dis* 1966;116:377–389. [PubMed: 4957317]
- Beall FA, Taylor MJ, Thorne CB. Rapid lethal effect in rats of a third component found upon fractionating the toxin of *Bacillus anthracis*. *J Bacteriol* 1962;83:1274–1280. [PubMed: 13866126]
- Bhatnagar R, Ahuja N, Goila R, Batra S, Waheed SM, Gupta P. Activation of phospholipase C and protein kinase C is required for expression of anthrax lethal toxin cytotoxicity in J774A.1 cells. *Cell Signal* 1999;11:111–116. [PubMed: 10048788]
- Bhatnagar R, Singh Y, Leppla SH, Friedlander AM. Calcium is required for the expression of anthrax lethal toxin activity in the macrophagelike cell line J774A.1. *Infect Immun* 1989;57:2107–2114. [PubMed: 2499545]
- Bolcome RE III, Sullivan SE, Zeller R, Barker AP, Collier RJ, Chan J. Anthrax lethal toxin induces cell death-independent permeability in zebrafish vasculature. *Proc Natl Acad Sci U S A* 2008;105:2439–2444. [PubMed: 18268319]
- Bonventre PF, Sueoka W, True CW, Klein F, Lincoln R. Attempts to implicate the central nervous system as a primary site of action for *Bacillus anthracis* lethal toxin. *Fed Proc* 1967;26:1549–1553. [PubMed: 4963770]
- Boyden ED, Dietrich WF. Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat Genet* 2006;38:240–244. [PubMed: 16429160]
- Buzzell A. Structural model for the lethal components of anthrax toxin based on ultracentrifuge studies. *Fed Proc* 1967;26:1522–1526. [PubMed: 6051331]
- Chandra H, Gupta PK, Sharma K, Mattoo AR, Garg SK, Gade WN, et al. Proteome analysis of mouse macrophages treated with anthrax lethal toxin. *Biochim Biophys Acta* 2005;1747:151–159. [PubMed: 15698949]
- Chou PJ, Newton CA, Perkins I, Friedman H, Klein TW. Suppression of dendritic cell activation by anthrax lethal toxin and edema toxin depends on multiple factors including cell source, stimulus used, and function tested. *DNA Cell Biol* 2008;27:637–648. [PubMed: 18821847]
- Comer JE, Chopra AK, Peterson JW, Konig R. Direct inhibition of T-lymphocyte activation by anthrax toxins in vivo. *Infect Immun* 2005a;73:8275–8281. [PubMed: 16299324]
- Comer JE, Galindo CL, Chopra AK, Peterson JW. GeneChip analyses of global transcriptional responses of murine macrophages to the lethal toxin of *Bacillus anthracis*. *Infect Immun* 2005b;73:1879–1885. [PubMed: 15731093]
- Comer JE, Galindo CL, Zhang F, Wenglikowski AM, Bush KL, Garner HR, et al. Murine macrophage transcriptional and functional responses to *Bacillus anthracis* edema toxin. *Microb Pathog* 2006;41:96–110. [PubMed: 16846716]
- Cordoba-Rodriguez R, Fang H, Lankford CS, Frucht DM. Anthrax lethal toxin rapidly activates caspase-1/ICE and induces extracellular release of interleukin (IL)-1 β and IL-18. *J Biol Chem* 2004;279:20563–20566. [PubMed: 15010463]
- Cote CK, DiMezzo TL, Banks DJ, France B, Bradley KA, Welkos SL. Early interactions between fully virulent *Bacillus anthracis* and macrophages that influence the balance between spore clearance and development of a lethal infection. *Microbes Infect* 2008;10:613–619. [PubMed: 18467145]
- Crawford MA, Aylott CV, Bourdeau RW, Bokoch GM. *Bacillus anthracis* toxins inhibit human neutrophil NADPH oxidase activity. *J Immunol* 2006;176:7557–7565. [PubMed: 16751402]
- Cruz CM, Rinna A, Forman HJ, Ventura AL, Persechini PM, Ojcius DM. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *J Biol Chem* 2007;282:2871–2879. [PubMed: 17132626]
- Cui X, Li Y, Li X, Haley M, Moayeri M, Fitz Y, et al. Sublethal doses of *Bacillus anthracis* lethal toxin inhibit inflammation with lipopolysaccharide and *Escherichia coli* challenge but have opposite effects on survival. *J Infect Dis* 2006;193:829–840. [PubMed: 16479518]
- Cui X, Li Y, Li X, Laird MW, Subramanian M, Moayeri M, et al. *Bacillus anthracis* edema and lethal toxin have different hemodynamic effects but function together to worsen shock and outcome in a rat model. *J Infect Dis* 2007;195:572–580. [PubMed: 17230417]

- Cui X, Moayeri M, Li Y, Li X, Haley M, Fitz Y, et al. Lethality during continuous anthrax lethal toxin infusion is associated with circulatory shock but not inflammatory cytokine or nitric oxide release in rats. *Am J Physiol Regul Integr Comp Physiol* 2004;286:R699–R709. [PubMed: 14715494]
- Dal Molin F, Fasanella A, Simonato M, Garofolo G, Montecucco C, Tonello F. Ratio of lethal and edema factors in rabbit systemic anthrax. *Toxicon* 2008;52:824–828. [PubMed: 18812184]
- Depeille PE, Ding Y, Bromberg-White JL, Duesbery NS. MKK signaling and vascularization. *Oncogene* 2007;26:1290–1296. [PubMed: 17322914]
- Dixon TC, Fadl AA, Koehler TM, Swanson JA, Hanna PC. Early *Bacillus anthracis*-macrophage interactions: intracellular survival and escape. *Cell Microbiol* 2000;2:453–463. [PubMed: 11207600]
- Duesbery NS, Resau J, Webb CP, Koochekpour S, Koo HM, Leppla SH, et al. Suppression of ras-mediated transformation and inhibition of tumor growth and angiogenesis by anthrax lethal factor, a proteolytic inhibitor of multiple MEK pathways. *Proc Natl Acad Sci U S A* 2001;98:4089–4094. [PubMed: 11259649]
- Duesbery NS, Webb CP, Leppla SH, Gordon VM, Klimpel KR, Copeland TD, et al. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. *Science* 1998;280:734–737. [PubMed: 9563949]
- Eckert NJ, Bonventre PF. In vivo effects of *Bacillus anthracis* culture filtrates. *Journal of Infectious Diseases* 1963;112:226–232.
- Ref Type: Abstract Ezzell JW, Ivins BE, Leppla SH. Immunoelectrophoretic analysis, toxicity, and kinetics of in vitro production of the protective antigen and lethal factor components of *Bacillus anthracis* toxin. *Infect Immun* 1984;45:761–767. [PubMed: 6432700]
- Fang H, Cordoba-Rodriguez R, Lankford CS, Frucht DM. Anthrax lethal toxin blocks MAPK kinase-dependent IL-2 production in CD4+ T cells. *J Immunol* 2005;174:4966–4971. [PubMed: 15814725]
- Fink SL, Bergsbaken T, Cookson BT. Anthrax lethal toxin and *Salmonella* elicit the common cell death pathway of caspase-1-dependent pyroptosis via distinct mechanisms. *Proc Natl Acad Sci U S A* 2008;105:4312–4317. [PubMed: 18337499]
- Firoved AM, Miller GF, Moayeri M, Kakkar R, Shen Y, Wiggins JF, et al. *Bacillus anthracis* edema toxin causes extensive tissue lesions and rapid lethality in mice. *Am J Pathol* 2005;167:1309–1320. [PubMed: 16251415]
- Firoved AM, Moayeri M, Wiggins JF, Shen Y, Tang WJ, Leppla SH. Anthrax edema toxin sensitizes DBA/2J mice to lethal toxin. *Infect Immun* 2007;75:2120–2125. [PubMed: 17339348]
- Fish DC, Klein F, Lincoln RE, Walker JS, Dobbs JP. Pathophysiological changes in the rat associated with anthrax toxin. *J Infect Dis* 1968a;118:114–124. [PubMed: 5640983]
- Fish DC, Mahlandt BG, Dobbs JP, Lincoln RE. Purification and properties of in vitro-produced anthrax toxin components. *J Bacteriol* 1968b;95:907–918. [PubMed: 4966833]
- Friedlander AM. Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. *J Biol Chem* 1986;261:7123–7126. [PubMed: 3711080]
- Friedlander AM, Bhatnagar R, Leppla SH, Johnson L, Singh Y. Characterization of macrophage sensitivity and resistance to anthrax lethal toxin. *Infect Immun* 1993;61:245–252. [PubMed: 8380282]
- Fritz DL, Jaax NK, Lawrence WB, Davis KJ, Pitt ML, Ezzell JW, et al. Pathology of experimental inhalation anthrax in the rhesus monkey. *Lab Invest* 1995;73:691–702. [PubMed: 7474943]
- Gozes Y, Moayeri M, Wiggins JF, Leppla SH. Anthrax lethal toxin induces ketotifen-sensitive intradermal vascular leakage in certain inbred mice. *Infect Immun* 2006;74:1266–1272. [PubMed: 16428776]
- Grinberg LM, Abramova FA, Yampolskaya OV, Walker DH, Smith JH. Quantitative pathology of inhalational anthrax I: quantitative microscopic findings. *Mod Pathol* 2001;14:482–495. [PubMed: 11353060]
- Guidi-Rontani C, Levy M, Ohayon H, Mock M. Fate of germinated *Bacillus anthracis* spores in primary murine macrophages. *Mol Microbiol* 2001;42:931–938. [PubMed: 11737637]
- Gupta PK, Moayeri M, Crown D, Fattah RJ, Leppla SH. Role of N-terminal amino acids in the potency of anthrax lethal factor. *PLoS ONE* 2008;3:e3130. [PubMed: 18769623]

- Ha SD, Ng D, Lamothe J, Valvano MA, Han J, Kim SO. Mitochondrial proteins Bnip3 and Bnip3L are involved in anthrax lethal toxin-induced macrophage cell death. *J Biol Chem* 2007a;282:26275–26283. [PubMed: 17623653]
- Ha SD, Ng D, Pelech SL, Kim SO. Critical role of PI3-K/Akt/GSK-3 β signaling pathway in recovery from anthrax lethal toxin-induced cell cycle arrest and MEK cleavage in macrophages. *J Biol Chem* 2007b;282:36230–36239. [PubMed: 17951252]
- Haines BW, Klein F, Lincoln RE. Quantitative assay for crude anthrax toxins. *J Bacteriol* 1965;89:74–83. [PubMed: 14255684]
- Hanna PC, Acosta D, Collier RJ. On the role of macrophages in anthrax. *Proc Natl Acad Sci U S A* 1993;90:10198–10201. [PubMed: 8234277]
- Hanna PC, Kruskal BA, Ezekowitz RA, Bloom BR, Collier RJ. Role of macrophage oxidative burst in the action of anthrax lethal toxin. *Mol Med* 1994;1:7–18. [PubMed: 8790597]
- Heninger S, Drysdale M, Lovchik J, Hutt J, Lipscomb MF, Koehler TM, et al. Toxin-deficient mutants of *Bacillus anthracis* are lethal in a murine model for pulmonary anthrax. *Infect Immun* 2006;74:6067–6074. [PubMed: 16923785]
- Hong J, Doebele RC, Lingen MW, Quilliam LA, Tang WJ, Rosner MR. Anthrax edema toxin inhibits endothelial cell chemotaxis via Epac and Rap1. *J Biol Chem* 2007;282:19781–19787. [PubMed: 17491018]
- Hsu LC, Ali SR, McGillivray S, Tseng PH, Mariathasan S, Humke EW, et al. A NOD2-NALP1 complex mediates caspase-1-dependent IL-1 β secretion in response to *Bacillus anthracis* infection and muramyl dipeptide. *Proc Natl Acad Sci U S A* 2008;105:7803–7808. [PubMed: 18511561]
- Huang D, Ding Y, Luo WM, Bender S, Qian CN, Kort E, et al. Inhibition of MAPK kinase signaling pathways suppressed renal cell carcinoma growth and angiogenesis in vivo. *Cancer Res* 2008;68:81–88. [PubMed: 18172299]
- Kassam A, Der SD, Mogridge J. Differentiation of human monocytic cell lines confers susceptibility to *Bacillus anthracis* lethal toxin. *Cell Microbiol* 2005;7:281–292. [PubMed: 15659071]
- Kau JH, Lin CG, Huang HH, Hsu HL, Chen KC, Wu YP, et al. Calyculin A sensitive protein phosphatase is required for *Bacillus anthracis* lethal toxin induced cytotoxicity. *Curr Microbiol* 2002;44:106–111. [PubMed: 11815854]
- Kau JH, Sun DS, Tsai WJ, Shyu HF, Huang HH, Lin HC, et al. Antiplatelet activities of anthrax lethal toxin are associated with suppressed p42/44 and p38 mitogen-activated protein kinase pathways in the platelets. *J Infect Dis* 2005;192:1465–1474. [PubMed: 16170766]
- Kim SO, Jing Q, Hoebe K, Beutler B, Duesbery NS, Han J. Sensitizing anthrax lethal toxin-resistant macrophages to lethal toxin-induced killing by tumor necrosis factor- α . *J Biol Chem* 2002;278:7413–7421. [PubMed: 12488448]
- Kirby JE. Anthrax lethal toxin induces human endothelial cell apoptosis. *Infect Immun* 2004;72:430–439. [PubMed: 14688124]
- Klein F, Haines BW, Mahlandt BG, Dearmon IA Jr, Lincoln RE. Dual nature of resistance mechanisms as revealed by studies of anthrax septicemia. *J Bacteriol* 1963;85:1032–1038. [PubMed: 14043991]
- Klein F, Hodges DR, Mahlandt BG, Jones WI, Haines BW, Lincoln RE. Anthrax toxin: causative agent in the death of rhesus monkeys. *Science* 1962;138:1331–1333. [PubMed: 14033353]
- Klein F, Walker JS, Fitzpatrick DF, Lincoln RE, Mahlandt BG, Jones WI Jr, et al. Pathophysiology of anthrax. *J Infect Dis* 1966;116:123–138. [PubMed: 4956203]
- Klimpel KR, Arora N, Leppla SH. Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity. *Mol Microbiol* 1994;13:1093–1100. [PubMed: 7854123]
- Kuhn JF, Hoerth P, Hoehn ST, Preckel T, Tomer KB. Proteomics study of anthrax lethal toxin-treated murine macrophages. *Electrophoresis* 2006;27:1584–1597. [PubMed: 16609935]
- Kuo SR, Willingham MC, Bour SH, Andreas EA, Park SK, Jackson C, et al. Anthrax toxin-induced shock in rats is associated with pulmonary edema and hemorrhage. *Microb Pathog* 2008;44:467–472. [PubMed: 18222626]
- Larabee JL, Degiusti K, Regens JL, Ballard JD. *Bacillus anthracis* edema toxin activates nuclear glycogen synthase kinase-3 β . *Infect Immun* 2008;76:4895–4904. [PubMed: 18765729]

- Lehmann M, Noack D, Wood M, Perego M, Knaus UG. Lung epithelial injury by *B. anthracis* lethal toxin is caused by MKK-dependent loss of cytoskeletal integrity. *PLoS ONE* 2009;4:e4755. [PubMed: 19270742]
- Leppla SH. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. *Proc Natl Acad Sci U S A* 1982;79:3162–3166. [PubMed: 6285339]
- Levin TC, Wickliffe KE, Leppla SH, Moayeri M. Heat shock inhibits caspase-1 activity while also preventing its inflammasome-mediated activation by anthrax lethal toxin. *Cell Microbiol* 2008;10:2434–2446. [PubMed: 18671821]
- Li Y, Cui X, Su J, Haley M, Macarthur H, Sherer K, et al. Norepinephrine increases blood pressure but not survival with anthrax lethal toxin in rats. *Crit Care Med* 2009;37:1348–1354. [PubMed: 19242337]
- Lincoln RE, Walker JS, Klein F, Rosenwald AJ, Jones WI Jr. Value of field data for extrapolation in anthrax. *Fed Proc* 1967;26:1558–1562. [PubMed: 4963771]
- Liu CT, Williams RP. Presence of cardioactive substances in cultures of *Bacillus anthracis*. *Arch Int Pharmacodyn Ther* 1971;189:336–347. [PubMed: 4994159]
- Liu S, Wang H, Currie BM, Molinolo A, Leung HJ, Moayeri M, et al. Matrix metalloproteinase-activated anthrax lethal toxin demonstrates high potency in targeting tumor vasculature. *J Biol Chem* 2008;283:529–540. [PubMed: 17974567]
- Mabry R, Brasky K, Geiger R, Carrion R Jr, Hubbard GB, Leppla S, et al. Detection of anthrax toxin in the serum of animals infected with *Bacillus anthracis* by using engineered immunoassays. *Clin Vaccine Immunol* 2006;13:671–677. [PubMed: 16760326]
- Maldonado-Arocho FJ, Fulcher JA, Lee B, Bradley KA. Anthrax oedema toxin induces anthrax toxin receptor expression in monocyte-derived cells. *Mol Microbiol* 2006;61:324–337. [PubMed: 16856939]
- Martinon F, Tschopp J. Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell Death Differ* 2007;14:10–22. [PubMed: 16977329]
- McAllister RD, Singh Y, Du Bois WD, Potter M, Boehm T, Meeker ND, et al. Susceptibility to anthrax lethal toxin is controlled by three linked quantitative trait loci. *Am J Pathol* 2003;163:1735–1741. [PubMed: 14578173]
- Meissner F, Molawi K, Zychlinsky A. Superoxide dismutase 1 regulates caspase-1 and endotoxic shock. *Nat Immunol*. 2008
- Moayeri M, Crown D, Dorward DW, Gardner D, Ward JM, Li Y, et al. The heart is an early target of anthrax lethal toxin in mice: a protective role for neuronal nitric oxide synthase (nNOS). *PLoS Pathog* 2009;4:e1000456. [PubMed: 19478875]
- Moayeri M, Haines D, Young HA, Leppla SH. *Bacillus anthracis* lethal toxin induces TNF- α -independent hypoxia-mediated toxicity in mice. *J Clin Invest* 2003;112:670–682. [PubMed: 12952916]
- Moayeri M, Martinez NW, Wiggins J, Young HA, Leppla SH. Mouse susceptibility to anthrax lethal toxin is influenced by genetic factors in addition to those controlling macrophage sensitivity. *Infect Immun* 2004;72:4439–4447. [PubMed: 15271901]
- Moayeri M, Webster JI, Wiggins JF, Leppla SH, Sternberg EM. Endocrine perturbation increases susceptibility of mice to anthrax lethal toxin. *Infect Immun* 2005;73:4238–4244. [PubMed: 15972515]
- Muehlbauer SM, Evering TH, Bonuccelli G, Squires RC, Ashton AW, Porcelli SA, et al. Anthrax lethal toxin kills macrophages in a strain-specific manner by apoptosis or caspase-1-mediated necrosis. *Cell Cycle* 2007;6:758–766. [PubMed: 17374996]
- Nour AM, Yeung YG, Santambrogio L, Boyden ED, Stanley ER, Brojatsch J. Anthrax lethal toxin triggers the formation of a membrane-associated inflammasome complex in murine macrophages. *Infect Immun* 2009;77:1262–1271. [PubMed: 19124602]
- Nye SH, Wittenburg AL, Evans DL, O'connor JA, Roman RJ, Jacob HJ. Rat survival to anthrax lethal toxin is likely controlled by a single gene. *Pharmacogenomics J* 2007;8:16–22. [PubMed: 17440430]
- Paccani SR, Tonello F, Ghittoni R, Natale M, Muraro L, D'Elia MM, et al. Anthrax toxins suppress T lymphocyte activation by disrupting antigen receptor signaling. *J Exp Med* 2005;201:325–331. [PubMed: 15699068]

- Paddle BM, Wong VK, Muller BD. The cytotoxic effect of anthrax lethal toxin on human lung cells in vitro and the protective action of bovine antibodies to PA and LF. *J Appl Toxicol* 2006;26:162–168. [PubMed: 16278807]
- Panchal RG, Ruthel G, Brittingham KC, Lane D, Kenny TA, Gussio R, et al. Chemical genetic screening identifies critical pathways in anthrax lethal toxin-induced pathogenesis. *Chem Biol* 2007;14:245–255. [PubMed: 17379140]
- Pandey J, Warburton D. Knock-on effect of anthrax lethal toxin on macrophages potentiates cytotoxicity to endothelial cells. *Microbes Infect* 2004;6:835–843. [PubMed: 15374005]
- Park JM, Greten FR, Li ZW, Karin M. Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase Inhibition. *Science* 2002;297:2048–2051. [PubMed: 12202685]
- Park JM, Greten FR, Wong A, Westrick RJ, Arthur JS, Otsu K, et al. Signaling pathways and genes that inhibit pathogen-induced macrophage apoptosis--CREB and NF-kappaB as key regulators. *Immunity* 2005;23:319–329. [PubMed: 16169504]
- Pellizzari R, Guidi-Rontani C, Vitale G, Mock M, Montecucco C. Anthrax lethal factor cleaves MKK3 in macrophages and inhibits the LPS/IFNgamma-induced release of NO and TNFalpha. *FEBS Lett* 1999;462:199–204. [PubMed: 10580119]
- Pellizzari R, Guidi-Rontani C, Vitale G, Mock M, Montecucco C. Lethal factor of *Bacillus anthracis* cleaves the N-terminus of MAPKKs: analysis of the intracellular consequences in macrophages. *Int J Med Microbiol* 2000;290:421–427. [PubMed: 11111921]
- Petrilli V, Papin S, Dostert C, Mayor A, Martinon F, Tschopp J. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ* 2007;14:1583–1589. [PubMed: 17599094]
- Pezard C, Berche P, Mock M. Contribution of individual toxin components to virulence of *Bacillus anthracis*. *Infect Immun* 1991;59:3472–3477. [PubMed: 1910002]
- Popov SG, Villasmil R, Bernardi J, Grene E, Cardwell J, Popova T, et al. Effect of *Bacillus anthracis* lethal toxin on human peripheral blood mononuclear cells. *FEBS Lett* 2002a;527:211. [PubMed: 12220662]
- Popov SG, Villasmil R, Bernardi J, Grene E, Cardwell J, Wu A, et al. Lethal toxin of *Bacillus anthracis* causes apoptosis of macrophages. *Biochem Biophys Res Commun* 2002b;293:349–355. [PubMed: 12054607]
- Rao LV, Ngyuen M, Pendurthi UR. Lethal toxin of *Bacillus anthracis* inhibits tissue factor expression in vascular cells. *J Thromb Haemost* 2004;2:530–532. [PubMed: 15009481]
- Reig N, Jiang A, Couture R, Sutterwala FS, Ogura Y, Flavell RA, et al. Maturation modulates caspase-1-independent responses of dendritic cells to anthrax lethal toxin. *Cell Microbiol* 2008;10:1190–1207. [PubMed: 18194483]
- Roberts JE, Watters JW, Ballard JD, Dietrich WF. *Ltx1*, a mouse locus that influences the susceptibility of macrophages to cytolysis caused by intoxication with *Bacillus anthracis* lethal factor, maps to chromosome 11. *Mol Microbiol* 1998;29:581–591. [PubMed: 9720874]
- Salles II, Tucker AE, Voth DE, Ballard JD. Toxin-induced resistance in *Bacillus anthracis* lethal toxin-treated macrophages. *Proc Natl Acad Sci U S A* 2003;100:12426–12431. [PubMed: 14519843]
- Sapra R, Gaucher SP, Lachmann JS, Buffleben GM, Chirica GS, Comer JE, et al. Proteomic analyses of murine macrophages treated with *Bacillus anthracis* lethal toxin. *Microb Pathog* 2006;41:157–167. [PubMed: 16950595]
- Sherer K, Li Y, Cui X, Li X, Subramanian M, Laird MW, et al. Fluid support worsens outcome and negates the benefit of protective antigen-directed monoclonal antibody in a lethal toxin-infused rat *Bacillus anthracis* shock model. *Crit Care Med* 2007;35:1560–1567. [PubMed: 17452924]
- Shin S, Hur GH, Kim YB, Park KJ, Park YM, Lee WS. Intracellular calcium antagonist protects cultured peritoneal macrophages against anthrax lethal toxin-induced cytotoxicity. *Cell Biol Toxicol* 2000;16:137–144. [PubMed: 10917569]
- Shin S, Kim YB, Hur GH. Involvement of phospholipase A2 activation in anthrax lethal toxin-induced cytotoxicity. *Cell Biol Toxicol* 1999;15:19–29. [PubMed: 10195347]
- Sirard JC, Mock M, Fouet A. The three *Bacillus anthracis* toxin genes are coordinately regulated by bicarbonate and temperature. *J Bacteriol* 1994;176:5188–5192. [PubMed: 8051039]

- Slein MW, Logan GF Jr. Mechanism of action of the toxin of *Bacillus anthracis*. I. Effect in vivo on some blood serum components. *J Bacteriol* 1960;80:77–85. [PubMed: 13831659]
- Smith H. Discovery of the anthrax toxin: the beginning of studies of virulence determinants regulated in vivo. *Int J Med Microbiol* 2002;291:411–417. [PubMed: 11890538]
- Smith H, Keppie J. Observations on experimental anthrax: demonstration of a specific lethal factor produced *in vivo* by *Bacillus anthracis*. *Nature* 1954;173:869–870. [PubMed: 13165673]
- Smith H, Keppie J, Stanley JL. The chemical basis of the virulence of *Bacillus anthracis*. V. the specific toxin produced by *B. anthracis in vivo*. *Br J Exp Pathol* 1955a;36:460–472. [PubMed: 13269658]
- Smith H, Keppie J, Stanley JL, Harris-Smith PW. The chemical basis of the virulence of *Bacillus anthracis*. IV. Secondary shock as the major factor in death of guinea-pigs from anthrax. *Br J Exp Pathol* 1955b;36:323–335. [PubMed: 13239989]
- Smith H, Stanley JL. Purification of the third factor of anthrax toxin. *J Gen Microbiol* 1962;29:517–521. [PubMed: 13989434]
- Squires RC, Muehlbauer SM, Brojatsch J. Proteasomes control caspase-1 activation in anthrax lethal toxin-mediated cell killing. *J Biol Chem* 2007;282:34260–34267. [PubMed: 17878154]
- Stanley JL, Sargeant K, Smith H. Purification of factors I and II of the anthrax toxin produced *in vivo*. *J Gen Microbiol* 1960;22:206–218. [PubMed: 13833788]
- Stanley JL, Smith H. Purification of factor I and recognition of a third factor of anthrax toxin. *J Gen Microbiol* 1961;26:49–66. [PubMed: 13916257]
- Steele AD, Warfel JM, D'Agnillo F. Anthrax lethal toxin enhances cytokine-induced VCAM-1 expression on human endothelial cells. *Biochem Biophys Res Commun* 2005;337:1249–1256. [PubMed: 16242117]
- Tang G, Leppla SH. Proteasome activity is required for anthrax lethal toxin to kill macrophages. *Infect Immun* 1999;67:3055–3060. [PubMed: 10338520]
- Tessier J, Green C, Padgett D, Zhao W, Schwartz L, Hughes M, et al. Contributions of histamine, prostanooids, and neurokinins to edema elicited by edema toxin from *Bacillus anthracis*. *Infect Immun* 2007;75:1895–1903. [PubMed: 17261611]
- Thorne CB, Molnar DM, Strange RE. Production of toxin in vitro by *Bacillus anthracis* and its separation into two components. *J Bacteriol* 1960;79:450–455. [PubMed: 13838209]
- Tournier JN, Quesnel-Hellmann A, Mathieu J, Montecucco C, Tang WJ, Mock M, et al. Anthrax edema toxin cooperates with lethal toxin to impair cytokine secretion during infection of dendritic cells. *J Immunol* 2005;174:4934–4941. [PubMed: 15814721]
- Tschopp J, Martinon F, Burns K. NALPs: a novel protein family involved in inflammation. *Nat Rev Mol Cell Biol* 2003;4:95–104. [PubMed: 12563287]
- Tucker AE, Salles II, Voth DE, Ortiz-Leduc W, Wang H, Dozmorov I, et al. Decreased glycogen synthase kinase 3-beta levels and related physiological changes in *Bacillus anthracis* lethal toxin-treated macrophages. *Cell Microbiol* 2003;5:523–532. [PubMed: 12864812]
- Turnbull PCB. Terminal bacterial and toxin levels in the blood of guinea pigs dying of anthrax. *Salisbury Med Bull* 1990;68:53–55.
- van Sorge NM, Ebrahimi CM, McGillivray SM, Quach D, Sabet M, Guiney DG, et al. Anthrax toxins inhibit neutrophil signaling pathways in brain endothelium and contribute to the pathogenesis of meningitis. *PLoS ONE* 2008;3:e2964. [PubMed: 18698416]
- Vande Velde C, Cizeau J, Dubik D, Alimonti J, Brown T, Israels S, et al. BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. *Mol Cell Biol* 2000;20:5454–5468. [PubMed: 10891486]
- Vasconcelos D, Barnewall R, Babin M, Hunt R, Estep J, Nielsen C, et al. Pathology of inhalation anthrax in cynomolgus monkeys (*Macaca fascicularis*). *Lab Invest* 2003;83:1201–1209. [PubMed: 12920249]
- Vick JA, Lincoln RE, Klein F, Mahlandt BG, Walker JS, Fish DC. Neurological and physiological responses of the primate to anthrax toxin. *J Infect Dis* 1968;118:85–96. [PubMed: 4966665]
- Vitale G, Bernardi L, Napolitani G, Mock M, Montecucco C. Susceptibility of mitogen-activated protein kinase family members to proteolysis by anthrax lethal factor. *Biochem J* 2000;352(Pt 3): 739–745. [PubMed: 11104681]

- Vitale G, Pellizzari R, Recchi C, Napolitani G, Mock M, Montecucco C. Anthrax lethal factor cleaves the N-terminus of MAPKKs and induces tyrosine/threonine phosphorylation of MAPKs in cultured macrophages. *Biochem Biophys Res Commun* 1998;248:706–711. [PubMed: 9703991]
- Voth DE, Hamm EE, Nguyen LG, Tucker AE, Salles II, Ortiz-Leduc W, et al. *Bacillus anthracis* oedema toxin as a cause of tissue necrosis and cell type-specific cytotoxicity. *Cell Microbiol* 2005;7:1139–1149. [PubMed: 16008581]
- Walker JS, Lincoln RE, Klein F. Pathophysiological and biochemical changes in anthrax. *Fed Proc* 1967;26:1539–1544. [PubMed: 4963768]
- Warfel JM, D'Agnillo F. Anthrax lethal toxin enhances TNF-induced endothelial VCAM-1 expression via an IFN regulatory factor-1-dependent mechanism. *J Immunol* 2008;180:7516–7524. [PubMed: 18490752]
- Warfel JM, Steele AD, D'Agnillo F. Anthrax lethal toxin induces endothelial barrier dysfunction. *Am J Pathol* 2005;166:1871–1881. [PubMed: 15920171]
- Watson LE, Kuo SR, Katki K, Dang T, Park SK, Dostal DE, et al. Anthrax toxins induce shock in rats by depressed cardiac ventricular function. *PLoS ONE* 2007a;2:e466. [PubMed: 17520025]
- Watson LE, Mock J, Lal H, Lu G, Bourdeau RW, Tang WJ, et al. Lethal and edema toxins of anthrax induce distinct hemodynamic dysfunction. *Front Biosci* 2007b;12:4670–4675. [PubMed: 17485403]
- Watters JW, Dewar K, Lehoczy J, Boyartchuk V, Dietrich WF. Kif1C, a kinesin-like motor protein, mediates mouse macrophage resistance to anthrax lethal factor. *Curr Biol* 2001;11:1503–1511. [PubMed: 11591317]
- Webster JI, Sternberg EM. Anthrax lethal toxin represses glucocorticoid receptor (GR) transactivation by inhibiting GR-DNA binding in vivo. *Mol Cell Endocrinol* 2005;241:21–31. [PubMed: 15964137]
- Webster JI, Tonelli LH, Moayeri M, Simons SS Jr, Leppla SH, Sternberg EM. Anthrax lethal factor represses glucocorticoid and progesterone receptor activity. *Proc Natl Acad Sci U S A* 2003;100:5706–5711. [PubMed: 12724519]
- Welkos SL, Keener TJ, Gibbs PH. Differences in susceptibility of inbred mice to *Bacillus anthracis*. *Infect Immun* 1986;51:795–800. [PubMed: 3081444]
- Wickliffe KE, Leppla SH, Moayeri M. Killing of macrophages by anthrax lethal toxin: involvement of the N-end rule pathway. *Cell Microbiol* 2008a;10:1352–1362. [PubMed: 18266992]
- Wickliffe KE, Leppla SH, Moayeri M. Anthrax lethal toxin-induced inflammasome formation and caspase-1 activation are late events dependent on ion fluxes and the proteasome. *Cell Microbiol* 2008b;10:332–343. [PubMed: 17850338]
- Williams RP, Hill HR, Hawkins D Jr, Chao KC, Neuenschwander J, Lipscomb HS. Epinephrinelike activity of culture filtrate from *Bacillus anthracis*. *Fed Proc* 1967;26:1545–1548. [PubMed: 4963769]
- Xu L, Fang H, Frucht DM. Anthrax lethal toxin increases superoxide production in murine neutrophils via differential effects on MAPK signaling pathways. *J Immunol* 2008;180:4139–4147. [PubMed: 18322225]
- Yu HB, Finlay BB. The caspase-1 inflammasome: a pilot of innate immune responses. *Cell Host Microbe* 2008;4:198–208. [PubMed: 18779046]
- Zaucha GM, Pitt ML, Estep J, Ivins BE, Friedlander AM. The pathology of experimental anthrax in rabbits exposed by inhalation and subcutaneous inoculation. *Arch Pathol Lab Med* 1998;122:982–992. [PubMed: 9822127]

Fig. 1.

Table 1

Susceptibility of inbred mice to LT doses administered by the IP route.

	25 µg	30 µg	40 µg	50 µg	100 µg
NOD/shiLtJ					95
DBA/2J				100	95
AKR/J				100	78
C3H/HeJ				100	78
C3H/HeOuJ				100	75
CBA/J				80	50
A/J				80	45
C57BL/6J	100		80	60	38
Balb/c	100	100	80	68	5
Cast/Ei	100	85	28	20	0

* Doses represent equal amounts of PA and LF (i.e. LT 25 µg = PA (25 µg)+ LF (25 µg)
** Tables shown percent survival, n values for each group range from n=10 to n>200

Table 2

Susceptibility of inbred mice to LT doses administered by the IV route.

	6 µg	12 µg	25 µg	35 µg	50 µg	100 µg
DBA/2J						
AKR/J		100	100	100	100	72
NOD		100	100	100	100	28
C3H/HeJ		100	68	0	100	25
C3H/HeOu		100	80	0		
CBA/J		100	40	0	0	
A/J		100	38	0	0	
FVB/N		100	33	25	0	
Balb/c		90	20	0	0	
SWR/J	100	20	0			
Cast/EJ	80	0	0			

* Doses represent equal amounts of PA and LF (i.e. LT 25 µg = PA (25 µg)+ LF (25 µg)
** Tables shown percent survival, n values for each group range from n=5 to n=45

Table 3

Susceptibility of inbred mice to ET doses administered by the IV route.

	5 µg	7.5 µg	10 µg	15 µg	20 µg	25 µg	30 µg	35 µg	50 µg	75 µg
AKR/J					100			100	75	0
CBA/J			100		100	100	80	0		
NOD					100	100	0	0		
SWR/J					100	100	0			
C3H/HeJ			100	100	100	80		0		
Balb/c			100		100	70		0		
DBA/2J			100	95	82	50		0		
FVB/N			100		100	0		0		
C3H/HeOuJ			100	100		0	0	0		
A/J			100	33	0		0			
C57BL/6J		100	100	18	0	12	0			
Cast/EJ	100	100	0							

* Doses represent equal amounts of PA and EF (i.e. ET 25 µg = PA (25 µg)+ EF (25 µg)
** Tables shown percent survival, n values for each group range from n=10 to n=40