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Bacillus anthracis Lethal Toxin Attenuates the Inflammatory Response Induced by Interferon gamma and Muramyl Dipeptide in Epidermal Keratinocytes

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Abstract of the Dissertation

Bacillus anthracis Lethal Toxin Attenuates the Inflammatory Response Induced by Interferon gamma and Muramyl Dipeptide in Epidermal Keratinocytes

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We have investigated the effects of anthrax lethal toxin on human epidermal keratinocytes in the presence of factors associated with infection by gram-positive bacteria. Similar to previous results reported with alveolar macrophages, lethal toxin cleaved members of the mitogen activated kinase kinase (MEK) family of proteins, including MEK2 and MEK4, but not MEK5, in keratinocytes. Cleavage of MEK proteins was accompanied by inhibition of cell growth in keratinocyte cultures, but no biomarkers of apparent apoptosis or necrosis could be detected. When keratinocytes were incubated with lethal toxin alone, increases in secreted levels of both RANTES and MCP-1 could be detected by protein microarray analysis of cellular supernatants. When keratinocytes were incubated in the presence of a mixture of the pro-inflammatory mediator interferon gamma (IFNγ) and the NOD2 ligand, muramyl dipeptide (MDP), a broad range of inflammatory biomarkers were released into the surrounding medium. Addition of lethal toxin in the presence of IFNγ and MDP resulted in attenuation of the levels of a number of these biomarkers, *e.g.* IL-6, TNF-α, GM-CSF and MIP-1β, released into the medium. Analysis of transcription factors known to be activated at least in part via the mitogen activated protein kinase (MAPK) pathway indicated that intracellular levels of activated forms of c-Myc and c-Jun were decreased while levels of activated Stat-1α and the p65 subunit of activated members of the NF-κB family were increased after incubation of keratinocytes with lethal toxin. These data reveal the complexity of the response of keratinocytes to pro-inflammatory signals associated with infection by *Bacillus anthracis* and the modulation of that response by the actions of lethal toxin.

Dedications

I dedicate this work to my wonderful wife Michelle who has always been able to bring out the best in me, and to my three lovely children Vincent, Marco and Alexa who have not only brought me such great joy, but whose existence has been an inspiration for me to achieve more out of my life. I am lucky to have all of you.

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More recently in my life, the support of my wife and children deserves a special mention of gratitude. They are collectively my inspiration. Further thanks to my wife’s parents, Alice and Nick Tenneriello, who have endlessly and selflessly helped raise our children, and have made all of our accomplishments possible.

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how anyone can achieve their goals and dreams if the desire lies within their hearts.

I. Introduction

Anthrax has gained widespread attention as a potential weapon in our current war on bioterrorism. Anthrax is caused by exposure to the highly infectious endospores of *Bacillus anthracis*, a gram positive, spore forming, rod shaped bacterium (Friedlander, 1999; Turnbull, 2002). Infection occurs via three separate pathways: gastrointestinal, inhalational and cutaneous. Although gastrointestinal anthrax is uncommon, outbreaks are continually reported in Africa and Asia following ingestion of insufficiently cooked contaminated meat (Sirisanthana *et al.*, 1988). Inhalational anthrax is expected to account for the most serious morbidity and the highest mortality following the use of *B. anthracis* as an aerosolized biological weapon, but is extremely rare in nature (Bush *et al.*, 2001). Following inhalation of the spores, macrophages and dendritic cells in the lungs engulf pathogenic spores and migrate to the lymph nodes, where subsequently the infected cells either enter an apoptotic pathway of cell death or, in the case of the dendritic cells, fail to mature into effective antigen-presenting cells, thereby leading to downregulation of the innate and adaptive arms of the host immune response (Brittingham *et al.*, 2005). Subsequent pathology is assumed to develop from the unopposed dissemination and proliferation of the bacterium, in a process that is beginning to be understood.

Cutaneous anthrax is the most common naturally occurring form of the disease, with an estimated 2000 cases reported annually worldwide (Friedlander, 1999). Because of the prevalence of the disease in parts of the world where healthcare is very limited, however, this is surely an underestimate. Untreated

cutaneous anthrax is fatal in 5-20% of cases, but mortality is rare after antibiotic therapy (Lew, 2000). Infection is caused when spores enter the skin via abrasions; however, the bacteria generally remain at the site of entry.

THE VIRULENCE PLASMIDS

The lethality associated with *B. anthracis* is attributed to two major virulence factors, a toxin (Smith, 2000; Smith and Stoner, 1967) and an anti-phagocytic poly-γ-D-glutamic acid capsule (Green *et al.*, 1985). Genes encoded on two large plasmids, pXO1 and pXO2, contain the information for the proteins that comprise the toxins (Okinaka *et al.*, 1999) and the enzymes for capsule synthesis, respectively (Green *et al.*, 1985). Strains of *B. anthracis* carrying either plasmid alone are non-virulent, implicating a necessary synergy between these two virulence factors.

Analysis of a pXO1 plasmid isolated from the Sterne strain (AF065404) has revealed a sequence of 181,654 nucleotides containing 143 open reading frames (ORF's) (Okinaka *et al.*, 1999). The structural toxin genes, *pagA*, *lef* and *cya*, which encode for the toxin components Protective Antigen (PA), Lethal Factor (LF) and Edema Factor (EF) respectively, can be mapped within a 44.8-kbp region on pXO1, along with regulatory elements, a resolvase, a transposase and a three-gene germination operon, *gerX* (Guidi-Rontani *et al.*, 1999). Also

included in this plasmid is a DNA topoisomerase gene required for relaxation of DNA supercoils (Fouet *et al.*, 1994).

Located on the pXO2 plasmid are the genes involved in capsule synthesis and degradation, namely *capB*, *capC*, *capA* and *dep,* as well as a regulatory gene, *acpA* (Thorne, 1993). However, the function of the majority of the proteins encoded in pXO2 is not clear at this time.

B. anthracis SURFACE STRUCTURE

Anthrax is caused by initial exposure to the highly infectious spores of *B. anthracis*, making the spore surface the first interaction with the host. The presence of an exosporium on the outside of the spore coat of *B. anthracis* has been observed (Hachisuka *et al.*, 1984), and although the function of the exosporium is unknown, it is not believed to be important for either dormancy or germination of the spores (Gerhardt, 1967). Some have suggested that the exosporium may act as a protective barrier blocking enzymatic attack at either the spore coat, or cortex layers (Nishihara *et al.*, 1989).

The cell surface of *B. anthracis* differs from that of other bacteria in part due to the presence of both a capsule and an S-layer. The capsule of *B. anthracis* is currently considered to be one of its two major virulence factors (Thorne, 1993) owing to its ability to evade the host immune response and promote septicemia. The capsule is not only very weakly immunogenic (Goodman and Nitecki, 1967),

but also capable of inhibiting phagocytosis (Makino *et al.*, 1989; Zwartouw and Smith, 1956). Comprised of a polymer of γ-D-glutamic acid (Zwartouw and Smith, 1956), the capsule has an *in vitro* molecular weight of approximately 20 to 55 kilodaltons (kDa), although estimates of the molecular weight *in vivo* have been as high as 215 kDa (Record and Wallis, 1956). The mode of attachment of the capsule to the cell surface of *B. anthracis* is not currently known and the synthesis of the capsule has not been worked out yet; however, similarities in both capsule structure and precursor amino acid (L-glutamic acid) in *B. anthracis* and *B. licheniformis* suggest a possible biosynthetic pathway. In *B. licheniformis,* the biosynthesis of the poly-γ-D-glutamic acid is catalyzed by a membranous enzymatic complex via a mechanism featuring extension of polyglutamyl chains by the sequential addition of D-glutamyl residues to the N-terminal group on the existing chain.

Subjacent to the capsule is the S-layer, comprised of proteinaceous paracrystalline sheets that cover the entire cell surface. Capsule formation is not dependent on the presence of an S-layer; however, such a layer may play a role in the fine structure of the capsule (Mesnage *et al.*, 1998). Although its role is unclear at this time, various proposed functions include shape maintenance, molecular sieving or a role as a virulence factor (Sara and Sleytr, 2000); however, the S-layer does not affect the 50% lethal dose in animal models. A role in evasion of the complement pathway has been suggested by Ray *et al.* (1998), who observed a cumulative effect of the S-layer and the capsule in increasing the resistance to the complement pathway.

In capsulated strains, two major 94 kDa proteins are the sole components of the S-layer, Sap (surface array protein) and EA1 (extractable antigen 1) (Ezzell and Abshire, 1988), encoded by the *sap* and *eag* genes, respectively (Etienne-Toumelin *et al.*, 1995; Mesnage *et al.*, 1997). Both proteins contain a standard gram-positive signal peptide followed by two domains, an N-terminal anchoring domain consisting of three SLH (S-layer homology) motifs fused to a C-terminal domain starting at residue 211 that is thought to be the crystallization domain (Mesnage *et al.*, 1999). The SLH motifs are involved in cell wall binding, as has been demonstrated in *in vitro* binding studies using polypeptides composed of the three SLH motifs of either Sap or EA1 that effectively bound *B. anthracis* purified cell walls (Chauvaux *et al.*, 1999; Mesnage *et al.*, 1999). Sap and EA1 appear to be expressed sequentially at the cell surface, and only co-localize during an intermediate stage when EA1 synthesis commences and progressively replaces Sap. Thus, *B. anthracis* cells are surrounded by a Sap S-layer during the exponential phase, which is replaced by an EA1 S-layer as the cells enter the stationary phase (Mignot *et al.*, 2002).

ANTHRAX TOXINS

The pathology of anthrax is due to a tripartite toxin which is secreted by the bacterium. Anthrax toxin consists of three proteins: Lethal Factor (LF; 90 kDa), Protective Antigen (PA; 83 kDa) and Edema Factor (EF; 89 kDa) (Smith and Stoner, 1967), which collectively bring about the pathologic changes in

infected hosts. A mixture of PA and LF, called Lethal Toxin (LeTx), can cause fatal hemorrhagic necrosis and vascular collapse in experimental animals, while a mixture of PA and EF, called Edema Toxin (EdTx), induces edema at the site of infection. EdTx, once believed to trigger non-lethal toxicity, has recently been shown to be lethal when injected into mice (Firoved *et al.*, 2005). Some groups have reported an activation of pro-apoptotic caspase pathways in cells exposed to anthrax toxin, both *in vitro* and *in vivo* (Moayeri et al., 2003; Popov et al., 2002).

PROTECTIVE ANTIGEN

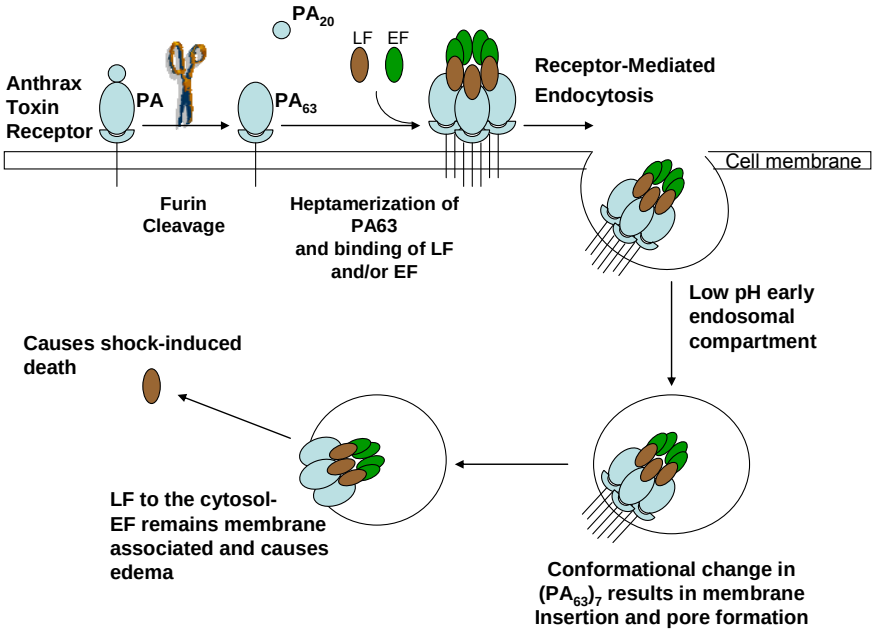
The mature 83 kDa protein (735 amino acids in length) comprises four functional domains, each of which is required for a particular step in the intoxication process. Domain 1, spanning amino acid residues 1 to 249, contains the proteolytic activation site (RKKR; residues 164-167) responsible for the liberation of the N-terminal 20 kDa fragment, yielding a 63 kDa protein capable of oligomerizing into ring shaped heptamers (Milne *et al.*, 1994).

Domain 2, spanning residues 250 to 487, comprises a β-barrel core containing a large flexible loop (between residues 302 and 325) that is believed to be involved in pore formation (Petosa *et al.*, 1997). Under acidic conditions, the heptamer undergoes a conformational change from a prepore complex to a pore.

Domain 3, spanning residues 499 to 594, contains a hydrophobic region believed to be involved in protein-protein interactions that functions primarily in self association of PA_{63} (Mogridge *et al.*, 2001; Petosa *et al.*, 1997).

Mutations in domain 4, spanning residues 595-735, clearly demonstrate that it is involved in receptor binding. Moreover, blockage of receptor binding of PA has been reported using monoclonal antibodies directed to this C-terminal region of the protein (Leppla, 1995). Whereas domains 1 to 3 are intimately associated with each other, domain 4 has little contact with the other domains.

Initially, PA binds to its cellular receptor and, following cleavage by cell surface furin (or furin-like) proteases, forms a heptameric prepore (Milne *et al.*, 1994) capable of binding both LF and EF (see diagram below). Association of LF and/or EF with PA results in formation of complexes containing 1 to 3 bound molecules of EF and/or LF per PA_{63} heptamer (Mogridge *et al.*, 2002). Subsequent endocytosis and trafficking of the complexes to an acidic compartment (Beauregard *et al.*, 2000) triggers a transition in the PA heptamer from a prepore to an integral transmembrane pore (Blaustein *et al.*, 1989), allowing transfer of LF and EF to the cytosol. Blockage of this step via inhibitors of endosomal acidification or of endocytosis prevents toxicity (Leppla, 1995). LF and EF can enter a wide variety of cells in the presence of PA, as the receptors for PA are virtually ubiquitous (Bonuccelli *et al.*, 2005).



Cellular models illustrate that cleavage of PA occurs at the cell surface; however, some evidence exists indicating that PA can be proteolyzed in the serum of infected animals (Brossier *et al.*, 2000; Ezzell and Abshire, 1992). It is likely that cleavage occurs at both locations *in vivo*. This conclusion is supported by the fact that both the native and cleaved forms of PA are able to bind to cell receptors, resulting in similar outcomes.

As described, the proteolytic removal of the PA_{20} fragment exposes the binding sites for EF and LF on the PA_{63} receptor bound fragment. This step has been shown to be crucial for intoxication, as a deletion mutant lacking the proteolytic site was not toxic to macrophages when incubated with LF (Leppla, 1995). Following proteolytic removal of the PA_{20} fragment, LF and EF bind

competitively and with similar affinities to the heptamerized prepore, regardless of whether PA is free or bound to its receptor (Elliott *et al.*, 2000).

EDEMA FACTOR

Edema factor is a 767 amino acid containing protein that functions as an adenylate cyclase, converting intracellular ATP (adenosine triphosphate) to cAMP (cyclic adenosine monophosphate) in a process dependent upon the presence of eukaryotic calmodulin (Leppla, 1982). Unlike LF, EF remains associated with vesicular membranes following translocation to the cytosol (Guidi-Rontani *et al.*, 2000), and likely resembles the membrane associated eukaryotic adenylate cyclase.

X-ray crystallographic investigations of the C-terminal catalytic portion of EF (residues 291-800), either alone, complexed with calmodulin or complexed with both calmodulin and 3`-deoxy-ATP (3`dATP- a non-cyclic ATP analog), have been undertaken (Drum *et al.*, 2002). Upon calmodulin binding, a 15 kDa helical region of EF undergoes a 15 Å (Angstrom) translation and a 30° rotation away from the catalytic region, thereby resulting in stabilization of a disordered loop and leading to EF activation. In the active site, 3`dATP and Mg^{2+} (magnesium^{2+}) along with the imidazole side chain of Histidine 351 (His 351), participate in the catalytic mechanism. Physiological calcium concentrations have been shown to regulate both calmodulin binding and catalysis (Shen *et al.*, 2002).

Until recently it was believed that EdTx alone does not cause tissue damage. Rather, similar to other toxins that elevate cAMP concentration, its main role had been supposed to be impairment of host phagocyte function (Leppla, 2000). This postulated mode of action would be consistent with the studies of Paccani *et al.* (2005), who have shown that elevated cAMP in T lymphocytes disturbs antigen receptor mediated signaling and represses T lymphocyte activation. However, more recent investigations using highly purified EdTx have clearly shown that the toxin can contribute directly to morbidity and mortality in mice: EdTx caused death of BALB/cJ mice more rapidly and at lower doses than LeTx (Firoved *et al.*, 2005). EdTx-induced lethality was accompanied by intraluminal fluid accumulation in the intestine, followed by focal hemorrhaging of the ileum and adrenal glands. At the histological level, EdTx-induced lesions could be observed in several tissues including adrenal glands, lymphoid organs, bone, bone marrow, gastrointestinal mucosa, heart and kidneys. Analysis of clinical chemistry markers in the blood was consistent with the induction of tissue damage and the elevation of several inflammatory cytokines. Physiological measurements revealed a concurrent hypotension and bradycardia.

LETHAL FACTOR

Lethal factor is a 4 domain, 90 kDa (776 residues) Zn^{2+} ($Zinc^{2+}$) metalloproteinase that targets members of the family of mitogen-activated protein

kinase kinases (MAPKKs or MEKs), namely MEKs 1 through 4, 6 and 7. Recognition and binding to the MEKs is mediated by domains II and III of lethal factor, while proteolytic cleavage of the N-terminal regions of the MEKs is accomplished by domain IV (Duesbery *et al.*, 1998; Rainey and Young, 2004). Cleavage of the MEKs disrupts transcriptional activation, compromising a variety of cellular processes, including activation of immune and stress responses. However, the consequences of these events are more directly linked to the loss of innate and adaptive immune responses in anthrax than to the pathology of the end-stage disease. For example, recent reports have shown that parenteral administration of the culture medium of strains of *B. anthracis* which produce no lethal and edema toxins, but which secrete other metalloproteases of the thermolysin and bacterial collagenase families, induces systemic anthrax-like pathology in experimental animals (Popov *et al.*, 2005).

Domain I (residues 28-263) of lethal factor, the PA_{63} binding region, consists of a 12 helix bundle packed against one face of a mixed 4 stranded β-sheet; this domain displays minimal contact with the remaining protein domains (Pannifer *et al.*, 2001). Residues 1-27 are not visible in the crystal structure, and in fact deletion of this region (or residues 1-36) has no effect either on receptor binding or translocation (Lacy *et al.*, 2002). In addition a polypeptide corresponding to residues 1-254 has been shown to be sufficient to mediate binding and translocation; thus the nine C-terminal residues of domain I are also not required for this process (Novoa *et al.*, 1996). Residues on both LF and EF responsible for recognition of the ligand site on PA_{63} have been determined by

Ala-substitution mutagenesis (Lacy *et al.*, 2002). Defective binding of LF could be observed following mutation of residues D182, D187, L188, Y223, H229, L235, and Y236. Homologous mutations in EF yielded similar defects. These essential seven amino acid residues form a convoluted patch on the surface of LF that displays both hydrophobic and hydrophilic properties.

Domains II-IV are intimately connected with each other and may represent a single folding unit. Domain II has an ADP-ribosyltransferase fold, similar to a related toxin from *B. cereus*; however, the active site has been mutated to increase substrate recognition. Domain III is a small α-helical bundle inserted into domain II and contains tandem repeats of a structural element of domain II. Domain IV is distantly related to the thermolysin family of metalloproteases and contains the HexxH Zn^{2+} binding motif associated with both prokaryotic and eukaryotic metalloproteases.

It is unclear at this time how LeTx induces death, either in macrophages or in an infected host. Suppression of proinflammatory cytokine production in murine macrophages has been reported (Erwin *et al.*, 2001) while Pellizari *et al.* (1999) showed that cleavage of MEKs resulted in reduction in the levels of nitric oxide (NO) and tumor necrosis factor alpha (TNFα) induced by lipopolysaccharide (LPS) and interferon gamma (IFNγ). These reports are representative of the multiple ways in which the host innate immune response becomes compromised after exposure to LeTx. In addition, LeTx has been reported to cause an increase in ion permeability of J774 macrophage-like cells, causing colloid osmotic lysis (Hanna *et al.*, 1992), while another report has

suggested a role for reactive oxygen intermediates in cytotoxicity (Hanna *et al.*, 1994). It has also been proposed that inhibition of MEKs by LeTx blocks induction of nuclear factor κB (NF-κB) target genes, thereby allowing apoptosis of activated macrophages (Park *et al.*, 2002). Other groups report that protein synthesis and proteasome activity are required for expression of LeTx cytotoxicity (Bhatnagar and Friedlander, 1994; Tang and Leppla, 1999) and that Kif1C, a kinesin-like motor protein, mediates resistance to LeTx (Watters *et al.*, 2001; Watters and Dietrich, 2001).

ANTHRAX TOXIN RECEPTORS

Intoxication begins with binding of PA to cell surface receptors and subsequent internalization of the pre-pore complex. PA binds to one of the two identified cell surface exposed anthrax toxin receptors (ANTXRs) on mammalian cells. The gene encoding ANTXR-1 was originally identified as tumor endothelial marker 8, (ATR/TEM8) whereas that for ANTXR-2 was identified as capillary morphogenesis gene 2 (CMG2) (Bell *et al.*, 2001; St Croix *et al.*, 2000). *In vitro* studies have demonstrated that intoxication can occur as long as either of these receptors is present alone (Banks *et al.*, 2005); however, it is currently unclear which receptor plays a more relevant role *in vivo*. The toxin binding domains of both ATR/TEM8 and CMG2 share 60% sequence homology, although PA binds to CMG2 with much greater affinity. *In vitro* studies have

established that each receptor displays distinct binding properties at low pH (Rainey *et al.*, 2005; Scobie *et al.*, 2005; Scobie and Young, 2005), and such differences may influence target cell type or affect toxin activity. For example, TEM8 promotes pore formation at pH 6.5 whereas CMG2-bound PA forms pores only below pH 5.5 (Lacy *et al.*, 2004; Rainey *et al.*, 2005; Scobie *et al.*, 2005; Scobie and Young, 2005). Furthermore, although CMG2 is expressed in normal adult tissues (Banks *et al.*, 2005; Scobie *et al.*, 2003), conflicting reports on the expression profile of TEM8 exist (Banks *et al.*, 2005; Bonuccelli *et al.*, 2005; Carson-Walter *et al.*, 2001; Hotchkiss *et al.*, 2005; Rmali *et al.*, 2004a; Rmali *et al.*, 2005a, b; Rmali *et al.*, 2004b; St Croix *et al.*, 2000). However, it should be noted that almost all cells examined express at least one type of receptor, thereby supporting toxin entry (Abi-Habib *et al.*, 2005; Leppla, 1999).

ATR/TEM8

Currently, three different protein isoforms of ATR/TEM8 (variants 1-3) that are generated by alternatively spliced mRNA transcripts are known to exist. TEM8 variant 1 contains 564 amino acids with a signal peptide, an extracellular region, a membrane spanning domain and a 221 amino acid long cytoplasmic tail, which in variant 2 is only 25 amino acids in length. ATR/TEM8 variant 3 (333 residues) lacks both the membrane spanning regions as well as the C-terminal

domains found in variants 1 and 2; thus it has been predicted to be a secreted protein and does not function as an anthrax receptor (Liu and Leppla, 2003).

Confirmation that ATR/TEM8 is a bona fide anthrax toxin receptor was accomplished by Bradley *et al.* (2001) using various experimental methods. First, expression of ATR/TEM8 (variant 2) at the cell surface of PA receptor deficient chinese hamster ovary (CHO) cells (CHO-R1.1 cells) restored PA binding. Second, the expression of ATR/TEM8 restored the sensitivity of the CHO-R-1.1 cells to killing by PA and LF (LeTx). Lastly, Bradley *et al.* demonstrated that ATR/TEM8 was able to bind to wild type PA but not to a mutant (Asp682Ser) form of PA that is unable to bind to cell surfaces. Subsequent studies reported by Liu and Leppla (2003) supported the result that TEM8 variant 1 can function as an anthrax toxin receptor, whereas TEM8 variant 3, lacking a transmembrane domain, cannot.

A region of the extracellular domain located between residues 44 and 218 in all three variants of ATR/TEM8 is highly related to von Willebrand Factor type A domains (VWA), or integrin-inserted domains (I domains) (Bradley *et al.*, 2001). These domains are generally located in cell adhesion proteins (e.g. α-integrins) or in extracellular matrix components where they coordinate protein-protein interactions (Whittaker and Hynes, 2002). A 5 amino acid sub-region within this domain, referred to as the MIDAS (metal ion-dependent adhesion site) motif, is involved in ligand interactions. The MIDAS motif is defined by the amino acid sequence DXSXS…T..D, where X is any amino acid which functions to promote ligand binding through coordination of a divalent metal ion (Shimaoka

et al., 2002). The role of the ATR/TEM8 MIDAS motif in binding to PA was verified by loss of binding in the presence of EDTA (EthyleneDiamineTetraAcetic Acid) (Bradley *et al.*, 2001). The binding of PA to the MIDAS motif was shown to be dependent upon a specific divalent cation that was either Mg^{2+} or Mn^{2+} (Manganese^{2+}) but not Ca^{2+} (Calcium^{2+}) (Bradley *et al.*, 2003). Further evidence supporting the role of the MIDAS motif was confirmed by mutagenesis studies, in which a D50A mutant form of the receptor, expressed in CHO-R1.1 cells, was unable to support both PA binding and subsequent intoxication by PA and LF (Bradley *et al.*, 2003).

A short stretch of acidic amino acids in the cytoplasmic tails of variants 1 and 2, in residues 360-368 and 360-364, respectively, resembles an acidic cluster found in the cytoplasmic tail of furin, which is thought to be involved in cell surface localization. It was theorized that these residues in ATR/TEM8 increase the efficiency of intoxication by allowing the co-localization of both the receptor and the furin enzyme that processes PA (Bradley *et al.*, 2001). However, the cytoplasmic tails are not required for PA binding, uptake and intoxication, as receptors that either lack a cytoplasmic tail altogether or are linked via a glycosylphosphatidylinositol-linkage to the outer leaflet of the cell membrane are not negatively affected (Liu and Leppla, 2003).

CAPILLARY MORPHOGENESIS PROTEIN 2 (CMG2)

Capillary morphogenesis protein 2 (CMG2) was originally identified by Bell *et al.* (2001), who observed increased expression in human umbilical vein endothelial cells (HUVECs) undergoing capillary formation *in vitro*. More recently, CMG2 has been shown to be an anthrax toxin receptor.

The CMG2 cDNA described by Bell *et al.* encodes a 386 amino acid protein ($CMG2^{386}$) that shares features with ATR/TEM8, including a signal peptide, an extracellular VWA/I domain with an intact MIDAS motif (D50, S52, S54, T118, D148) that has 60% homology to the VWA/I domain in ATR/TEM8, a membrane spanning region, and a cytoplasmic tail. Another CMG2 isoform containing 488 residues ($CMG2^{488}$) more closely resembles ATR/TEM8 variants 1 and 2 due to the presence of an approximate 100 residue spacer region located between the VWA/I domain and the membrane spanning region. Two additional isoforms of CMG2 are predicted ($CMG2^{322}$ and $CMG2^{489}$) to be encoded by four other alternatively spliced mRNA transcripts. $CMG2^{322}$ contains 322 amino acid residues and is predicted to be a secreted protein. The cytoplasmic tails of the three predicted transmembrane forms of CMG2 are similar to ATR/TEM8, with respect to the presence of acidic amino acid residue repeats (EEEEE) (residues 259-263 in $CMG2^{386}$ and residues 362-366 in $CMG2^{488}$ and $CMG2^{489}$). The VWA/I domain of CMG2 binds selectively to both collagen and laminin, which are likely natural ligands for the receptor *in vivo* (Bell *et al.*, 2001).

CMG2 was shown to act as an anthrax toxin receptor by employing PA deficient CHO-R1.1 cells that recombinantly expressed $CMG2^{489}$ protein. This protein was detected at the cell surface and supported PA binding as well as intoxication with PA and LF (Scobie *et al.*, 2003). Furthermore, *in vitro* binding experiments revealed a divalent cation dependent direct interaction between PA and a soluble version of CMG-2 (Scobie *et al.*, 2003). These data strongly suggest a role for CMG-2 as a bona fide receptor for PA; however, the importance of this receptor relative to ATR/TEM8 remains to be elucidated.

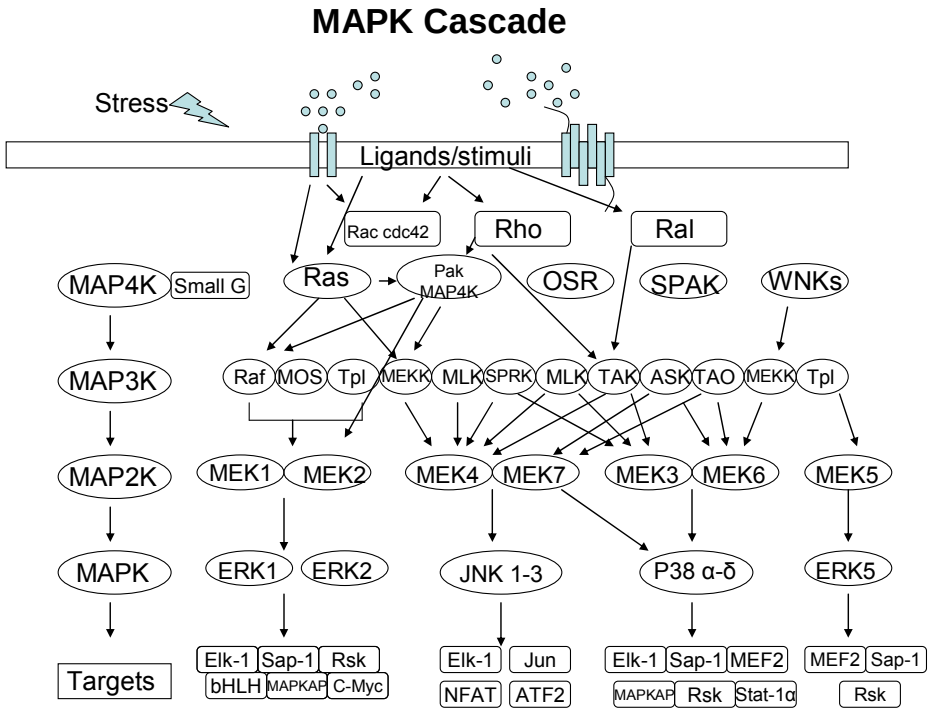
LDL RECEPTOR RELATED PROTEIN 6 (LRP6)

Recently Wei *et al.* (2006) discovered that low density lipoprotein receptor related protein 6 (LRP6) displayed an unexpected capacity to deliver anthrax toxins into cells. LRP6 is a member of the gene family encoding low density lipoprotein receptors that participate in endocytosis of their ligands (Howell and Herz, 2001). Additionally, LRP6, acting as a key component of a larger complex, functions to mediate the transduction of signals from secreted Wnt proteins to β-catenin, in a process that is involved in embryogenesis and cell proliferation (Tamai *et al.*, 2000). The involvement of LRP6 in delivery of anthrax toxins was supported by evidence generated through multiple experimental approaches described in Wei *et al.* (2006). For example, results obtained from fluorescent microscopy as well as immunoprecipitation

demonstrated that LRP6 protein interacts with both TEM8 and CMG2 and controls PA triggered internalization of these receptors. Furthermore, use of siRNAs directed against lrp6 mRNA yielded reductions in the levels of internalized PA, while antibodies against LRP6 extracellular domain protected cells from anthrax toxicity. Lastly, expression of a dominant negative truncated LRP6 mutant protein lacking a cytoplasmic domain conferred toxin resistance which could also be achieved following expression of antisense mRNA for lrp6 in naïve cells.

MITOGEN ACTIVATED PROTEIN KINASES (MAPKs)

Mitogen activated protein kinases (MAPKs) are a family of serine/threonine kinases that regulate a diverse group of cellular programs including proliferation, embryogenesis, differentiation and apoptosis in virtually all cell types (reviewed in Raman *et al.*, 2007; Turjanski *et al.*, 2007). Signaling through the MAPK pathway involves a three kinase system, in which the terminal MAPKs are phosphorylated and activated by mitogen activated protein kinase kinase (MAPKKs), which themselves are phosphorylated and activated by mitogen activated protein kinase kinase kinase (MAPKKKs). In vertebrates, five families of MAPKs exist, including extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK1/2/3), p38α/β/δ/γ, ERK5 and ERK7 (Uhlik *et al.*, 2004) (see illustration below).



Activation of MAPKs and the subsequent cellular response has been attributed to a diverse group of stimuli, including growth factors, cytokines, and stresses such as toxins, numerous drugs, changes in cell adherence, osmolarity, oxygen radicals, ultraviolet light and temperature (Pearson *et al.*, 2001). Dysregulated MAPK activity is therefore associated with a variety of pathological states, including those arising from inflammation, such as arthritis and inflammatory bowel disease (Hollenbach *et al.*, 2004; Johnson and Lapadat, 2002), as well as diseases accompanied by uncontrolled cellular proliferation, such as cancer (Gollob *et al.*, 2006).

EPIDERMAL BIOLOGY

The role of the barrier function of the skin lies not only in the prevention of systemic water loss, but also in the protection of the organism from external pathogens, thereby acting as the body's first major line of defense. Formation of the cutaneous barrier begins in the innermost proliferative layer of the epidermis, known as the basal layer (Fuchs and Byrne, 1994). Daughter cells migrate outward toward the cell surface in a process that is marked by the loss of the proliferative capacity of these cells with concomitant induction of cellular differentiation (Eckert *et al.*, 1997; Fuchs and Byrne, 1994). The second epidermal layer, known as the spinous layer, is characterized by cells containing an increased number of desmosomal connections that contribute to intercellular strength. As cells progress up toward the third layer, the granular layer, they are marked with many granules containing protein and lipids (Lavker, 1976; Lavker and Matoltsy, 1971; Matoltsy, 1966), many of which are ultimately deposited onto the inner surface of the developing cornified envelope (Steven *et al.*, 1990). Above the granular layer lies a zone of transition between living and dead epidermis, referred to as the stratum corneum. It is in this zone that intracellular organelles are destroyed by proteases and nucleases, while the assembly of the cornified envelope and the stabilization of the keratin filaments is occurring. The formation of these stratum corneum "corneocytes" is characterized by two major structures: a network of disulfide bond stabilized keratin filament bundles surrounded by an envelope of covalently cross-linked protein (Nemes and

Steinert, 1999) and associated lipids (Segre, 2003). As mentioned above, a 15 nm thick cornified envelope forms beneath the plasma membrane in differentiating keratinocytes (Nemes and Steinert, 1999), and is comprised of two functional parts; a 10 nm thick backbone of cross-linked protein and a 5 nm thick layer of covalently linked lipids that coat the exterior (Elias and Friend, 1975; Grayson and Elias, 1982; Matoltsy and Matoltsy, 1966; Nemes *et al.*, 1999; Nemes and Steinert, 1999; Robinson *et al.*, 1997; Steinert and Marekov, 1999; Wertz *et al.*, 1989). Various proteins comprise the cornified envelope including soluble (e.g. involucrin and small proline-rich proteins (SPR's)) and insoluble (e.g. loricrin, periplakin and envoplakin) proteins, with other proteins completing envelope formation (cystatin A, elafin, filaggrin, desmoplakin, type II keratins, S100 proteins (A10 and A11), annexin I and cornifelin) (Eckert *et al.*, 1993; Kalinin *et al.*, 2002; Nemes and Steinert, 1999). The important step of envelope protein crosslinking is accomplished by the enzyme transglutaminase (Hennings *et al.*, 1981; Robinson *et al.*, 1996; Steinert *et al.*, 1998; Steinert and Marekov, 1995, 1997; Steven and Steinert, 1994), which catalyzes interprotein bond formation by forming a thiolester acyl-enzyme intermediate and subsequently transferring the acyl residue to a primary amine (Folk, 1980; Folk and Finlayson, 1977). The amine acceptor is generally the ε-amino group of a protein-bound lysine culminating in the formation of an N^6-(γ-glutamyl)lysine isopeptide bond.

CHEMOKINE GENE SUPERFAMILY

The recruitment of circulating leukocytes toward inflammatory regions, beginning with extravasation from the capillary network, is a critical event for the generation of inflammation and immune responses. While cytokines such as Interleukiin-1 (IL-1) and Tumor Necrosis Factor-α (TNF-α) are important contributors to inflammation, release of chemotactic factors at inflammatory sites is believed to be essential for the extravasation of leukocytes to occur. Three major types of chemotactic factors are known to exist: membrane phospholipid derived mediators such as leukotriene B4 (LTB4) (Samuelsson *et al.*, 1980); the complement component C5a; and the various members of the chemokine gene superfamily (Baggiolini *et al.*, 1994; Oppenheimer-Marks and Ziff, 1988; Schall and Bacon, 1994).

Members of the chemokine gene superfamily of cytokines that are induced in a number of pathophysiological processes (Conti *et al.*, 1995; Meurer *et al.*, 1993) are small proteins (8-10 kDa) with homologous sequences including a highly conserved cysteine motif in their primary amino acid structure. Four branches of chemokines are known to exist; these subgroups are designated by the sequence containing the first two cysteine residues; CXC, CC, C or CX_3C (Bazan *et al.*, 1997; Pan *et al.*, 1997). With respect to their chemotactic effects, the cellular targets of each family member can be divided into major categories. The CXC subfamily members (e.g. human growth related oncogene-α, or Groα, macrophage inhibitory protein-2 or MIP-2) are chemotactic for neutrophils but

not mononuclear cells (Baggiolini *et al.*, 1994; Oppenheim *et al.*, 1991; Schall and Bacon, 1994). Members of the CC subfamily including monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) are primarily chemotactic for monocytes, basophils and eosinophils, but not neutrophils. (Baggiolini *et al.*, 1994; Oppenheim *et al.*, 1991; Schall and Bacon, 1994). Lymphotactin, representative of the C subfamily of chemokines, is principally chemotactic for $CD8^+$ T lymphocytes and does not appear to act on other myeloid cells (Kelner *et al.*, 1994), while the chemokine neurotactin has been reported to function as a chemoattractant for T cells, monocytes and neutrophils (Bazan *et al.*, 1997; Pan *et al.*, 1997).

The development of an acute local inflammatory response is characterized first by infiltration of a large number of neutrophils followed by mononuclear cells. Interactions between neutrophils and chemokines are integral to this process, which is mediated by the cell surface chemokine receptors CXCR2 (Cacalano *et al.*, 1994; Lee *et al.*, 1995) and CCR1 (Zhang *et al.*, 1999). Blockade of CXCR2 or its ligands, KC and MIP-2 (murine homologs of Groα), has been shown to inhibit neutrophil migration in murine models of infection (Hall *et al.*, 2001; Kernacki *et al.*, 2000; Mehrad *et al.*, 1999; Moore *et al.*, 2000) and inflammation (Terkeltaub *et al.*, 1998; Tessier *et al.*, 1997). In addition, blockade of CCR1 binding by MIP-1α has been reported to inhibit neutrophil recruitment in certain models (Khan *et al.*, 2001; Standiford *et al.*, 1995; Tessier *et al.*, 1997).

PATHOLOGY

Experimental infection with both anthrax toxins provokes the two main characteristics of anthrax: edema and shock-like death. *In vivo* experiments using mutated variants of the toxins have been useful in delineating the mechanism of action of *B. anthracis* in animal models. Genetic deletions or point mutations rendering both EF and LF inactive are sufficient to abolish edema and lethality, respectively (Pezard *et al.*, 1991). Additionally, EF or LF deficient strains of *B. anthracis* are less effective at causing death and edema when compared to their parental EF- and LF-expressing strains, confirming the synergy between EdTx and LeTx. Mutants expressing PA molecules that lack the furin cleavage site and the chymotrypsin cleavage site in domain 2 are nonpathogenic, while deletions of the receptor-binding domain on PA (or the loop involved in binding) abolished or reduced virulence (Brossier *et al.*, 2000).

The effect that LeTx mediated cleavage of MEKs has on cell function currently remains unclear. One study investigating effects of addition of LeTx to dendritic cells demonstrated inhibition of secretion of all cytokines (Agrawal *et al.*, 2003), while a separate study observed a more selective effect which correlated with a balance between p38 and ERK inhibition (Tournier *et al.*, 2005). Macrophage cell death induced by LeTx has been observed in several mouse strains (Friedlander *et al.*, 1993), with the susceptibility of these cells to toxin being recently linked to the polymorphic locus Nalp1b (Boyden and Dietrich, 2006). NALP1, or NACHT-LRR-PYD-containing protein-1, is a key component

of the inflammasome, a multiprotein complex involved in the activation of caspases 1 and 5 which are in turn responsible for the processing and secretion of interleukin-1β (IL-1β) and interleukin-18 (IL-18) (Petrilli *et al.*, 2005). While MEK cleavage occurs in both resistant and susceptible mouse cells (Alileche *et al.*, 2005), NALP1 activation occurs only in susceptible macrophages (Boyden and Dietrich, 2006), suggesting that LeTx-associated cell death is not dependent on MEK cleavage alone. It has been independently shown that the induction of apoptosis by LeTx in activated macrophages occurs via a p38 dependent pathway (Park *et al.*, 2002). Another apoptotic pathway has been identified downstream of toll-like receptor 4 (TLR4) following recognition of *B. anthracis* motifs leading to subsequent activation of PKR (protein kinase interferon-inducible double stranded RNA dependent) (Hsu *et al.*, 2004). Further work established that rescue of macrophages from LeTx-induced TLR4-dependent cell death involves activation of the transcription factor CREB (Park *et al.*, 2005). As expected, EdTx induction of adenylate cyclase also rescues cells from TLR4-triggered apoptosis following the activation of PKA and CREB. These reports indicate that the processes leading to murine macrophage cell death following exposure to the components of anthrax toxin are indeed complex, and extension of these pathways to human macrophages remains largely uninvestigated.

EARLY PHASE OF INHALATIONAL ANTHRAX

The early phase of inhalational anthrax involves recognition of the spores by the resident pulmonary phagocytes, mainly the alveolar macrophages, although dendritic cells are involved in this process as well (Cleret *et al.*, 2006). Motifs on the spore surface are recognized by pattern recognition receptors via a MyD88 dependent mechanism (Glomski *et al.*, 2007), while those of vegetative bacilli signal through TLR2 (Hughes *et al.*, 2005) and anthrolysin O through TLR4 (Park *et al.*, 2004). Discrimination of the roles of alveolar macrophages and lung dendritic cells remains somewhat uncertain, but phagocytosis is most efficient in lung dendritic cells (Cleret *et al.*, 2006), while sporocidal activity of alveolar macrophages has been shown *in vitro* (Hu *et al.*, 2006). In studies designed to resolve the ambiguities associated with the early phase of infection, the role of lung dendritic cells has been highlighted, mainly due to the ability of anthrax spores to block the transcription of chemokine receptors that retain these cells in the lung (CCR2 and CCR5 (chemokine (C-C motif) receptor)), while inducing transcription of lymph node homing receptors (CCR7) in these cells (Brittingham *et al.*, 2005). These effects on gene transcription for the chemokine receptors appear to account for the process by which *B. anthracis* becomes systemic and localizes to the lymph nodes. The fact that lung dendritic cells are especially efficient at both phagocytosis and migration implicates this cell type as the most suitable vehicle for the spreading of germinating spores across the alveolar-capillary barrier (Cleret *et al.*, 2006).

As discussed above, LeTx has been shown to abolish the release of proinflammatory cytokines from macrophages (Bergman *et al.*, 2005; Erwin *et al.*, 2001; Pellizzari *et al.*, 1999; Ribot *et al.*, 2006) and from dendritic cells in both human and murine models (Agrawal *et al.*, 2003; Alileche *et al.*, 2005; Brittingham *et al.*, 2005; Tournier *et al.*, 2005). In addition, Tournier *et al.* (2005), using a spore infection model, have shown how EdTx cooperates with LeTx by inhibiting release of proinflammatory cytokines as well. Furthermore, the production of sporocidal phospholipase A2 type IIA by pulmonary phagocytes is inhibited by LeTx (Gimenez *et al.*, 2004). Ribot *et al.* (2006) have described inhibition of the bactericidal activity of non-human primate alveolar macrophages by anthrax toxin components. Collectively, these reports clearly show how LeTx and EdTx inhibit the early secretion of proinflammatory cytokines, thereby preventing the activation and/or recruitment of immune cells such as polymorphonuclear neutrophils (PMN's) and monocytes that might otherwise control the progression of infection by *B. anthracis*.

Inhibition of PMN priming (O'Brien *et al.*, 1985) and of bactericidal superoxide generation (Crawford *et al.*, 2006) by LeTx and EdTx has been reported. Additionally, inhibition of PMN chemotaxis by LeTx, in a process likely involving the inhibition of actin polymerization, has been shown (During *et al.*, 2005). In inhalational infections, LeTx functions to block the recruitment of PMNs via two separate strategies: by blocking the production of chemokines by dendritic cells (Brittingham *et al.*, 2005) and by blocking the chemotaxis of responding cells (During *et al.*, 2005). Monocytes are the remaining cell type that

could respond and clear *B. anthracis* infection; however, LeTx inhibits their differentiation into pathogen destroying macrophages (Kassam *et al.*, 2005). In a recent study using a mouse model of infection, macrophages were found to be critical for host survival, while PMNs played a secondary but necessary role in a fully functional immune response to spores (Cote *et al.*, 2006).

In summary, LeTx and EdTx initially silence both macrophages and dendritic cells encountered in the lung alveoli. Next germinating spores appear to use macrophages and/or dendritic cells as "Trojan horses" to carry them systemically to the lymph nodes while preventing the recruitment of monocytes and PMNs.

THE SECOND PHASE OF INHALATIONAL ANTHRAX

Upon arrival to the mediastinal lymph node in host phagocytes, some spores have germinated into bacilli and proliferated, in a process previously reported in studies using macrophages *in vitro* (Ruthel *et al.*, 2004); however, the method by which bacilli escape from the phagosome leading to their extracellular proliferation is currently unknown. As bacilli proliferate in the lymph nodes, their secreted toxins compromise the process of antigen presentation and subsequently blunt the adaptive immune response. Due to the relatively quick time to death in both humans and animals with inhalational anthrax, the adaptive arm of the

immune system does not play any significant role (Starnbach and Collier, 2003). In fact, studies of the five patients who succumbed to the 2001 anthrax bioterrorist attack indicated that none of the patients had any detectable levels of anti-PA antibodies in their initial blood samples (Quinn *et al.*, 2004). However, results of an animal vaccination study indicated that the functions of memory B and T cells effectively counteracted inhalational infection with *B. anthracis* (Fellows *et al.*, 2001). In any event, several studies have shown impairment of antigen presentation by LeTx through its ability to inhibit the expression of co-stimulatory molecules by dendritic cells (Agrawal *et al.*, 2003; Cleret *et al.*, 2006). Interestingly, while LeTx inhibits the humoral response, EdTx induces a type 2 helper T cell (Th2) shift thereby enhancing the humoral response, as observed in studies in which EdTx was administered either subcutaneously or via the mucosal route (Duverger *et al.*, 2006; Quesnel-Hellmann *et al.*, 2006).

The process of immune silencing encompasses the effect LeTx has on cell death of both macrophages and dendritic cells. Recently it was suggested that apoptosis of antigen presenting cells may act as a negative signal for the immune system (Tournier and Quesnel-Hellmann, 2006); apoptotic blebs were observed in macrophages and dendritic cells in which activation appeared to be blocked.

In studies of T lymphocytes, LeTx and EdTx abrogated T cell activation by interfering with antigen receptor signaling that was a consequence of the inhibition of NFAT (nuclear factor of activated T cells) and AP-1 (activator protein 1), two transcription factors essential for cytokine secretion (Paccani *et al.*, 2005). Additionally LeTx inhibits IL-2 production by CD4 positive T cells

(Fang *et al.*, 2005) and inhibits the activation of mouse lymphocytes *in vivo* (Comer *et al.*, 2005). Lastly, MEK-dependent B cell proliferation and secretion of IgM were shown to be blocked by LeTx (Fang *et al.*, 2006), thereby impairing the humoral response.

In summary, the adaptive arm of the immune response is effectively crippled by both LeTx and EdTx. The affected cells include dendritic cells, T helper cells and B cells. The resulting effect in systemic anthrax, as observed in the patients of the terrorist attacks of 2001, includes septicemia associated with mediastinal lymphadenopathies and pleural effusion. Clinically, these patients experience chest pain, dyspnea, coughing and vomiting. Without the use of antibiotic therapy, the excessive proliferation of bacilli and their resulting toxins ultimately progresses to the final stages of the disease and death.

THE TERMINAL PHASE OF INHALATIONAL ANTHRAX

The terminal phase of systemic infection is marked by proliferation of long chains of bacilli in the bloodstream and other sites such as the brain, where they cause meningitis. Consequently, large amounts of both LeTx and EdTx are found in the blood, where LeTx damages the endothelial barrier by increasing the number of central actin fibers and by modifying endothelial distribution of VE-cadherin, a major constituent of the alveolo-capillary wall (Warfel *et al.*, 2005). This, coupled with LeTx induced apoptosis (Kirby, 2004), likely accounts for the

increase in permeability of the capillary wall that leads to the cardiovascular distress observed in terminal phase patients with systemic disease.

The *in vivo* effect of each toxin has been extensively studied in mouse models following intravenous injection. Disruption of the endothelial barrier by LeTx and subsequent vascular leakage was observed following intraperitoneal injection (Gozes *et al.*, 2006), confirming previous reports of hypoxemia mediated toxicity in a context of vascular collapse in mice and rats (Cui *et al.*, 2004; Moayeri *et al.*, 2003).

Recent studies that have focused on the *in vivo* effect of EdTx have shed some light on its role in infection. Injections of EdTx kill mice rapidly, and, notably, at lower doses than LeTx (Firoved *et al.*, 2005). These mice rapidly develop hypotension and bradycardia while macroscopic and pathological analyses reveal intestinal intraluminal fluid accumulation, followed by necrosis in many tissues, including the adrenal glands, lymphoid organs, bone, bone marrow, heart and kidneys.

A major feature observed upon autopsy has been extensive hemorrhaging of the lymphoid organs, namely the lymph nodes and the spleen (Guarner *et al.*, 2003). Administration of LeTx to mice has been found to induce thrombopenia (Moayeri *et al.*, 2003), associated with slower clotting time, a decrease in fibrinogenemia and the accumulation of fibrin deposits in tissue sections (Culley *et al.*, 2005). These findings are consistent with reports showing how both LeTx and EdTx extensively disrupt platelet function and aggregation (Alam *et al.*, 2006; Kau *et al.*, 2005).

CUTANEOUS ANTHRAX

As discussed above, the high morbidity and mortality associated with inhalational anthrax, which invariably proceeds to systemic disease unless aggressively treated in the early stages, can be contrasted with the relatively benign course of cutaneous anthrax, which generally is restricted to a focal lesion and often resolves without intervention. In the United States, cutaneous anthrax accounts for 95% of all naturally occurring *B. anthracis* infections (Lew, 2000), with most cases occurring in patients who have occupational contact with animals or animal products contaminated with *B. anthracis* spores (Smith, 1973; Steele, 1968). Pathogenic spores enter through cutaneous cuts or abrasions, with an anatomical prevalence observed in the head, neck and extremities. Unlike the progression of untreated inhalational anthrax to systemic disease, bacteremia and toxemia following cutaneous infection are uncommon sequelae; however, approximately 20-25% of untreated cases can progress to a fatal outcome (Gold, 1955; LaForce, 1994; McSwiggan *et al.*, 1974). Clinically, cutaneous anthrax is characterized by the formation of a black eschar surrounded by edema and vesicles; however, the similarity in appearance to lesions associated with the bite of the brown recluse spider has often led to misdiagnosis (Elston *et al.*, 2000; Freedman *et al.*, 2002; Mallon and McKee, 1997). Interestingly, although an intact skin barrier is resistant to infection, a compromised barrier requires fewer spores than the 8,000 to 10,000 believed to be required to cause inhalational anthrax (Meselson *et al.*, 1994).

The incubation period following introduction of spores to a compromised area is about 7 days but has been reported to be as brief as 1 day or as long as 12 days (CDC, 2001). The cases of cutaneous anthrax that were linked to the 2001 bioterrorism episode had an average incubation period of 5 days (CDC, 2001). Following the incubation period, visible signs of infection are manifested in the form of painless red macules that may itch, and within 48 to 72 hours, develop into papules. Within the next 2 days following papule formation, edema is observed and papules begin to be surrounded by weeping vesicles. Sampling of the vesicle exudates reveals viable bacilli in patients who have not received antibiotic treatment (LaForce, 1994; WHO, 1998). One to three days after the appearance of vesicles, the papule itself will rupture and ulcerate, culminating in the characteristic necrotic brown and black eschar that can measure between 1 and 5 cm in diameter. The process of healing occurs over a period of up to 6 weeks and can resolve itself without therapeutic intervention, although lack of treatment can increase the risk of systemic infection. Interestingly, early initiation of antibiotic treatment has been observed to limit the size of anthrax lesions, but later use of antibiotics does not affect the length of time for such lesions to heal (Kobuch E, 1990).

The ultimate formation of the necrotic eschar is indicative of an inflammatory event mediated by the invasion of PMN's. Immunohistochemical analysis of the bioterrorism-related cases of cutaneous anthrax confirmed the presence of neutrophils in tissue lesions (Shieh *et al.*, 2003). These analyses revealed that perivascular infiltration by mononuclear cells (lymphocytes and

monocytes) predominated in cases of longer duration, while PMNs were observed in cases of shorter duration. Additionally, the role of neutrophils in the engulfment of spores and in the killing of *B. anthracis* was reported by Mayer-Scholl *et al.* (2005), who describe a reactive oxygen species-independent process that utilizes α-defensins to kill *B. anthracis*.

Our goals in this study were to describe the course of cellular events associated with exposure of human keratinocytes to the components of infection with *B. anthracis*. In the experiments reported here, we investigated the role lethal toxin plays following infection of epidermal keratinocytes. Initially we observed that, unlike the responses of macrophages and dendritic cells, incubation of epidermal keratinocytes with lethal toxin did not result in cell death, but rather stimulated the secretion of a subset of specific inflammatory mediators such as RANTES (regulated upon activation normal T cell expressed and secreted) and MCP-1 (monocyte chemotactic protein 1). Nevertheless, even in the absence of overt cytotoxicity of anthrax toxin to keratinocytes *in vitro*, cases of cutaneous anthrax are associated with a characteristic necrotic lesion that appears to be caused by factors unrelated to the direct effects of lethal toxin. We thus set out to investigate the effects of lethal toxin on the early inflammatory phase of cutaneous anthrax to understand how keratinocytes signal to initiate an innate immune response that could result in necrotic tissue injury. To perform these studies, monolayer cultures of human keratinocytes pre-incubated with an inflammatory cocktail of components likely to be present during a focal infection with a gram positive bacterium such as *B. anthracis* were exposed to lethal toxin.

Addition of lethal toxin to these "activated" keratinocytes resulted in suppression of a number of recognized mediators; however, levels of secreted RANTES and MCP-1, two chemokines in the CC subclass, were elevated. These data are consistent with a model of neutrophil and monocyte recruitment toward the site of a cutaneous anthrax lesion stimulated by the chemokines RANTES and MCP-1, respectively. Suppression of several inflammatory cytokines and chemokines after exposure of monocytes to lethal toxin has been described by other groups as well as our laboratory (Bergman *et al.*, 2005; Erwin *et al.*, 2001; Pellizzari *et al.*, 1999; Ribot *et al.*, 2006). The especially marked induction of CC chemokines after exposure of keratinocytes to lethal toxin, however, appears to be distinctive, if not unique, to this cell type. Neutrophils, which have been classically reported to respond to chemotactic gradients established by the CXC family of chemokines, have more recently been reported to respond as well to the CC family of chemokines, specifically RANTES, in a murine model of peritoneal chemotaxis (Ferrandi *et al.*, 2007). Levels of RANTES in synovial fluid samples from patients with rheumatoid arthritis have also been positively correlated with the number of neutrophils in the synovium (Stanczyk *et al.*, 2005). Our observations on the possible role of neutrophil-recruiting CC chemokines and consequent neutrophil activation predicted from our *in vitro* model of cutaneous anthrax are therefore consistent with previous observations that cutaneous anthrax presents as a focal inflammatory response, marked by the necrotic eschar, whereas inhalational anthrax progresses to a condition marked by systemic immunosuppression. The ability of cutaneous anthrax to resolve without

intervention, in comparison to the high morbidity and mortality associated with inhalational anthrax, may reflect the distinctive profiles of acute inflammatory activation in cutaneous anthrax vs. suppression of the innate and adaptive immune responses in systemic anthrax, which may be correlated with the roles of neutrophils in the cutaneous disease vs. monocytes and macrophages in the systemic disease.

II. Materials and Methods

Materials

Normal human epidermal keratinocytes (HEKn), maintained in EPI-Life medium containing human keratinocyte growth supplements (HKGS), were purchased from Cascade Biologics, Inc., (Portland, OR). Lethal Factor, Protective Antigen and *B. anthracis* cell wall extract were purchased from List Biological Laboratories, Inc., (Campbell, CA). Muramyl dipeptide (MDP), lipopolysaccharide (LPS) and lipoteichoic acid (LTA) were purchased from InvivoGen™ (San Diego, CA). Interferon-γ was purchased from Sigma-Aldrich® Inc. (St. Louis, MO). RayBio® Human Inflammation Antibody Arrays were purchased from RayBiotech, Inc. (Norcross, GA) and used according to the manufacturer's instructions. ELISA kits for IL-6, GM-CSF (granulocyte macrophage colony stimulating factor), MIP-1β (macrophage inflammatory protein 1β), TNF-α, RANTES and MCP-1 were purchased from BioSource International, Inc. (Camarillo, CA) and used according to the manufacturer's instructions. TransAM™ assays for activated MAPK and NF-κB transcription factor components were purchased from Active Motif® (Carlsbad, CA) and used according to the manufacturer's instructions. Novex 10% Bis-Tris gels and associated supplies for SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and Western blotting, were all purchased from Invitrogen™ Life Technologies (Carlsbad, CA). Blots were visualized with Amersham ECL Plus Western Blotting reagents purchased from GE Healthcare (Piscataway, NJ).

Antibodies against MEK 2, MEK4, MEK 5, MEK6, involucrin, anti-goat HRP and anti-rabbit HRP were all purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

The CellTiter 96® AQueous One Solution Cell Proliferation Assay was purchased from the Promega Corporation (Madison, WI), and Brefeldin A (BA) was purchased from Biomol International LP (Plymouth Meeting, PA). The Sensolyte AFC™ Caspase Profiling Kit was purchased from Anaspec Inc. (San Jose, CA) and the Pierce Micro BCA protein assay kit was purchased from ThermoFisher Scientific, Inc. (Rockford, IL). All assay kits were used according to the manufacturers' instructions.

Cell Culture

Normal human epidermal keratinocytes (HEKn) were cultured at 37°C/5% CO_2 in EpiLife media supplemented with HKGS. Cells were allowed to incubate 16-24 hours prior to incubation with experimental reagents. For Western blotting, HEKn were grown in 100 mm dishes until the cells covered approximately 75% of the surface area. For viability experiments, 12 well plates containing 1.7×10^5 HEKn/well were maintained overnight prior to addition of experimental reagents. Cells to be employed in experiments on caspase activity, microarrays, ELISAs, and transcription factor assays were all cultured in 6 well culture dishes at a density of 3×10^5 cells/well. Lethal Factor (LF) and Protective Antigen (PA)

were each diluted in EpiLife media to a final concentration of 1 μg/ml, in a total volume of 2 ml per well. Together, LF and PA, referred to as Lethal Toxin (LeTx), confer the toxicity associated with anthrax. As a control, we added either LF or PA alone in early experiments investigating the effect of LeTx on cellular MEKs. Additionally, we set out to address the effect LeTx has on cells that were induced with a pro-inflammatory cocktail. For studies in which co-incubations of pro-inflammatory mediators with LeTx were carried out, 10 ng/ml IFN-γ and 100 μg/ml MDP were added to cells either in combination or separately, one hour prior to the addition of LeTx, or concurrently. Viability was assessed by conversion of the tetrazolium salt MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) to its blue formazan according to the manufacturer's protocol (Promega Corporation, Madison, WI).

Cell Extracts

At time points listed, the supernatant medium was collected from appropriate wells and stored at -20°C. Prior to assay, supernatant medium was cleared of cell debris by centrifugation at 5000g for 5 minutes at 4°C. To obtain cell lysates, the keratinocytes were washed twice with room temperature PBS, detached by trypsinization, and lysed by vigorous pipeting with ice cold lysis buffer containing 40 mM Tris, pH 7.2, 150 mM NaCl, 0.1% NP-40, 5 mM NaF, 1 mM Na pyrophosphate, 1 mM Na orthovanadate and COMPLETE™ protease

inhibitor cocktail (Roche, Mannheim, Germany). In assays investigating caspase activity, protease inhibitor-free lysis buffer supplied by the manufacturer was used. Lysates were stored frozen at -20°C and were thawed on ice prior to clarification by centrifugation at 20,000 g for 10 minutes at 4°C. The total protein content of the supernatants was determined using the Pierce BCA protein assay.

Western blotting for MEKs and Involuctrin

Aliquots of cell lysate total protein were resolved on 10% SDS-Polyacrylamide Bis-Tris gels, and the protein bands were transferred to nitrocellulose membranes using an XCell II Blot Module (Invitrogen™ Life Technologies (Carlsbad, CA)). Immunoblotting with individual anti-MEK or involucrin antibodies and subsequent chemiluminescent detection were performed according to the manufacturer's guidelines (ECL, Amersham). The optimal protein concentration in cell lysates to be employed for immunodetection of MEKs by gel electrophoresis and Western blotting was determined to lie within a range of 5 to 20 μg/ml. Additionally, we investigated the effect of exposure of HEKn to LeTx on the expression of involucrin, a highly crosslinked cornified envelope protein that has been established as a biomarker for early stages of keratinocyte differentiation.

Evaluation of apoptosis

It has been reported that LeTx induces apoptosis in a number of cell types, including macrophages. To investigate whether exposure of keratinocytes to LeTx also induces apoptosis, levels of the activated forms of the pro-apoptotic caspases were measured using a caspase profiling kit according to the manufacturer's guidelines (Anaspec). Briefly, cells were incubated with lethal toxin or with a known apoptosis inducer, Brefeldin A, for the time periods listed. Cells were lysed using ice-cold lysis buffer supplied by the manufacturer. Cell lysates were added to wells containing fluorogenic substrates for caspases 3/7, 6, 8 or 9. Caspase activity was determined by measuring fluorescence generated by substrate cleavage.

Assessment by protein microarray of pro-inflammatory mediators secreted by HEKn after incubation with Lethal Toxin

In some animal models of anthrax, an initial inflammatory response has been described which is then reduced once the replicating bacterium begins to release significant quantities of LeTx. To determine the effect of LeTx on the release of proinflammatory mediators from HEKn, a RayBio protein microarray designed specifically to evaluate expression of multiple pro-inflammatory mediators in cell culture supernatants was employed. One milliliter of

supernatant medium from cells incubated with or without LeTx was added to a microarray membrane containing capture antibodies for forty inflammatory cytokines, growth factors, and mediators, following the manufacturer's instructions. Addition of detection antibodies and visualization of immunoreactive biomarkers by chemiluminescence were then carried out according to the manufacturer's protocol. Films of the chemiluminescent spots were analyzed by the use of the ImageJ software package made available by the National Institutes of Health. Use of the protein microarray provided us with a broad, but only roughly semiquantitative, assessment of alterations in the levels of these multiple inflammatory biomarkers resulting from the different treatments employed in our experiments as discussed below.

Assessment by protein microarray of pro-inflammatory mediators released by HEKn incubated with LPS, LTA, IFNγ or MDP

From the outset of engulfment of anthrax spores by alveolar macrophages and dendritic cells, LeTx begins to silence the innate and adaptive arms of the immune response. To investigate how these effects of LeTx might be extended to keratinocytes that had been exposed to proinflammatory stimuli associated with infection by *B. anthracis*, we set out to induce a proinflammatory response in keratinocytes prior to the addition of LeTx. We evaluated the toll-like receptor 2 ligand lipoteichoic acid (LTA, 0.2 or 2 μg/ml), the Nod2 (nucleotide-binding

oligomerization domain 2) ligand muramyl dipeptide (MDP, 100 μg/ml), interferon gamma (IFNγ, 10 ng/ml) and an extract of *Bacillus anthracis* cell walls (1 μg/ml). As a positive control, cells were also incubated with the gram negative bacterial endotoxin, *E. coli* lipopolysaccharide (LPS, 100 ng/ml). HEKn were incubated with 0.2 or 2.0 μg/ml LTA for 6 hours, 1, 2 and 3 days; or with *B. anthracis* cell wall extract for 1, 3 and 5 days; or with IFNγ alone, LPS or IFNγ combined with LTA or MDP for 1 or 4 days. One milliliter of supernatant medium from cells incubated with the above inflammatory mediators was added to a microarray membrane according to manufacturer's instructions.

Effects of LeTx on the release of pro-inflammatory mediators from IFNγ/MDP stimulated HEKn: comparative assessment by protein microarray and ELISA

The robust increases in the release of proinflammatory mediators from HEKn exposed to the combination of IFNγ and MDP allowed us to investigate the effect of LeTx on the release of these biomarkers. One milliliter of supernatant medium from cells incubated with either the inflammatory cocktail alone or in combination with LeTx was added to a microarray membrane according to manufacturer's instructions and detection and visualization of captured proinflammatory biomarkers was carried out as described above. Those inflammatory mediators most affected by exposure of HEKn to LeTx were then

further analyzed and quantitated by ELISA. Volumes of supernatant medium needed for each ELISA (enzyme-linked immunosorbent assay) were determined empirically; all ELISAs were performed according to manufacturer's instructions.

Activation of the MAPK and NFκB transcription factor family of proteins and HIF-1α transcription factor

Our observations of effects of LeTx exposure on MEK cleavage and on the production of inflammatory cytokines/chemokines in HEKn prompted us to investigate effects of LeTx on the MAPK and NFκB family of transcription factors. In addition, our earlier observations of growth inhibition prompted us to investigate the transcription factor HIF-1α that is activated under conditions of tissue hypoxia and necrosis. These assays were performed using the TransAM transcription factor activation assays from Active Motif®. In TransAM assays, transcription factor activation is quantitatively detected by incubating nuclear lysates with multiwell microplates on which different consensus oligonucleotide sequences specific for binding the different transcription factors have been immobilized. The captured transcription factors are then detected on the plates with specific antibodies using methods like those employed in ELISAs. Keratinocytes were incubated for 24 hours with either IFNγ/MDP, LeTx or both agents, at which time we had observed both a loss of immunoreactive MEKs and a change in the profile of secreted inflammatory mediators. For experiments

involving evaluation of activated HIF-1α, keratinocytes were incubated with LeTx alone. Cells were collected and lysed as described above and levels of activated forms of transcription factors known to be activated through the MAPK pathway (ATF-2, c-Jun, c-Myc, Stat1-a, MEF2) and levels of the subunits in activated forms of NFkB (p52, p65, c-Rel, Rel B, p50) transcription factors and levels of activated HIF-1α were determined according to manufacturer's instructions.

Statistical Analysis

Data were analyzed using a one-way analysis of variance (ANOVA) and significant means were tested by Bonferroni multiple comparisons test (GraphPad Instat software).

III. Results

Lethal Toxin (LeTx) induces cleavage of MEK2, MEK4 but not MEK5 in human epidermal keratinocytes

To elucidate the effects of LeTx on normal human epidermal keratinocytes (HEKn), cells were incubated with either lethal factor (LF), protective antigen (PA), or both (lethal toxin, LeTx) for the periods indicated. Cellular lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and subsequently probed with antibodies for MEK2, MEK4 and MEK5. Cleavage of MEK2 and subsequent loss of an immunoreactive band was observed as early as 45 minutes after addition of lethal toxin, while exposure of cells to lethal factor or protective antigen individually did not affect MEK2 immunoreactivity (Figure 1a). Nearly all immunoreactive MEK2 protein was lost by 1.5 hours after incubation of the cells with lethal toxin, with total loss observed 3 hours post addition and persisting as long as 5 days post addition (Figure 1b). Furthermore, MEK2 degradation was observed in HEKn monolayers at 1.5 and 3 hours after treatment of the cells with lethal toxin, even when the toxin was washed away from the surrounding medium after exposure of the cells to the toxin for intervals as short as 20 minutes (Figure 1c). Further analysis of the MAPKK family members by immunoblotting displayed a similar loss of immunoreactive MEK4 in cell lysates (Figure 1d), while MEK5 levels in the lysates were unaffected by

exposure of the cells to LeTx (Figure 1d). These results are consistent with published reports which clearly describe the substrates of LeTx in the MAPK pathway in other cells (Duesbery *et al.*, 1998; Klimpel KR, 1993; Pellizzari *et al.*, 1999; Vitale *et al.*, 1998).

Effect of LeTx on keratinocyte viability does not involve an apoptotic or necrotic process

Cutaneous anthrax lesions ultimately form necrotic lesions *in vivo* if left untreated. To determine if LeTx is sufficient to induce cell death in HEKn, viability was assessed by quantitating the capacity of the keratinocytes to reduce the tetrazolium salt MTS to its formazan. Viability was minimally affected 24 hours after LeTx addition, as evidenced by an 8% diminution in MTS reductase activity (Figure 2). At later time points the MTS reductase activity of HEKn that had been exposed to LeTx was less than that of untreated HEKn, but this effect appeared to be due primarily to growth inhibition. By employing fluorogenic substrates specific for caspases 3 and 7 we could conclude that a classical apoptotic pathway was not activated after 3 days of exposure of HEKn to LeTx, whereas exposure to brefeldin A, a known initiator of apoptosis in keratinocytes, resulted in significant increases in activity of caspases 3 and 7 within 24 hours (Figure 3). Similar assays with substrates specific for caspases 6, 8, and 9 also did not reveal evidence of caspase activation by LeTx (data not shown).

Furthermore, on analysis of HEKn incubated with LeTx for 1, 2, 3, or 5 days, no increased levels of the transcription factor HIF-1α, a known biomarker for a pathway of necrotic cell death could be detected (data not shown).

Investigation of Involucrin levels in Normal Human Epidermal Keratinocytes exposed to LeTx.

Monolayer cultures of HEKn incubated with LeTx morphologically resembled cells committed toward cellular differentiation. To determine whether the addition of LeTx to HEKn induces biomarkers of cellular differentiation, we evaluated the epidermal protein marker involucrin that is expressed not only in intact skin, but also in monolayer cultures of keratinocytes *in vitro*. HEKn were exposed to LeTx for 1, 2, 3 or 5 days and cellular lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and subsequently probed with antibodies for involucrin. Incubation of keratinocytes with LeTx for 24 hours yielded no significant difference in the levels of involucrin protein synthesized by these cells (Figure 4). Interestingly, at 2 days post addition of LeTx, we began to observe lower levels of involucrin protein in the lysates of the toxin-exposed cells relative to the untreated group. This observed difference became more apparent at days 3 and 5, by which point control cells were expressing significantly greater levels of involucrin compared to the LeTx incubated cultures.

LeTx selectively activates inflammatory mediators in human epidermal keratinocytes.

It has been reported that one way in which lethal toxin functions to silence the innate and adaptive arms of the immune response is through induction of apoptosis in alveolar macrophages and dendritic cells. In contrast to these reports, our experiments indicated that LeTx failed to induce apoptosis or necrosis in monolayer cultures of HEKn. We therefore wanted to ascertain the effects of LeTx on the production of inflammatory biomarkers in HEKn. HEKn were incubated either with or without LeTx for 1, 2, 3 and 5 days and the supernatant medium was then analyzed for multiple inflammatory biomarkers using an inflammatory biomarker protein microarray (RayBiotech Inc.). Keratinocytes grown in monolayer cultures were observed to secrete moderate baseline levels of various inflammatory mediators (e.g. GM-CSF, IL-8, MCP-1, sTNFRI (soluble tumor necrosis factor receptor I) and others) (Figure 5a) which would be consistent with the consensus that keratinocytes in monolayer cultures express a "wound-like" phenotype. Interestingly, LeTx induced the increased secretion of MCP-1, RANTES, and IL-6 in these cultures that had not been exposed to additional inflammatory stimuli at all time points investigated. The observation of increased release of these inflammatory biomarkers is consistent with our observations that HEKn exposed to LeTx do not undergo marked downregulation

of metabolic processes consistent with cytotoxicity, but rather may only undergo reduced replication, consistent with a cytostatic effect of the toxin.

Investigation of the secretion of pro-inflammatory mediators from HEKn incubated with various proinflammatory agents.

The ability of LeTx to silence the innate and adaptive arms of the immune response by diminishing the release of inflammatory mediators from macrophages and dendritic cells prompted us to investigate the effects of LeTx on the secretion of inflammatory mediators from keratinocytes stimulated by known pro-inflammatory agents. To achieve this, we screened different pro-inflammatory mediators, including the Toll-like receptor 2 ligand lipoteichoic acid (LTA), a Nod2 ligand muramyl dipeptide (MDP), interferon gamma (IFNγ) and an extract of *Bacillus anthracis* cell walls, to determine which would yield the most robust induction of inflammatory cytokines, chemokines and other proinflammatory biomarkers, as visualized by protein microarray analysis. Incubation of HEKn with 0.2 or 2 μg/ml LTA did not alter the profile of secreted inflammatory mediators as assessed by protein microarray (RayBiotech Inc.) on samples from culture supernatants collected at time intervals as brief as 6 hours or as long as 3 days after introduction of the stimulus (Figure 5b). Similar results revealing minimal alternations in levels of secreted inflammatory biomarkers were noted in experiments in which HEKn were incubated with *B. anthracis* cell wall extract for

1, 3 or 5 days (Figure 5c) or 24 hours with the gram negative bacteria lipopolysaccharide (LPS), known to stimulate pro-inflammatory responses (Figure 5d).

One publication reported on the capacity of IFNγ to synergize with TNFα to induce an inflammatory response in human keratinocytes (Li *et al.*, 2000). To provide a second inflammatory stimulus more relevant to infection by a gram-positive bacterium such as *B. anthracis*, we exposed HEKn to mixtures of IFNγ and either Muramyl Dipeptide (MDP) or Lipoteichoic Acid (LTA), soluble analogs of the cell wall of gram positive bacteria and known ligands for the intracellular Nod2 receptor and the Toll-like receptor 2, respectively. Cells were incubated for 24 hours with IFNγ combined with either MDP or LTA and the supernatant medium was then analyzed for multiple inflammatory biomarkers with an inflammatory biomarker microarray as above. Keratinocytes incubated with IFNγ alone released markedly elevated levels of IL-6, MIP-1β, MCP-1 and RANTES into the culture medium, whereas MDP or IFNγ/LTA were both ineffective at stimulating the secretion of inflammatory biomarkers (Figure 5, d and e). In cultures incubated with IFNγ/MDP, marked increases in the secretion of a number of inflammatory mediators from HEKn were observed that surpassed the levels of these mediators released by the cells in response to treatment with IFNγ alone (Figure 5e). The qualitative results from the protein microarray were further substantiated and quantitated using ELISAs specific for those biomarkers that we observed to be further modulated by LeTx (Figure 6). Consistent with published reports of the ability of IFNγ to synergize with other inflammatory

mediators, incubation of HEKn with both IFNγ and MDP resulted in reproducible increases in the levels of secreted TNFα, IL-6, MIP-1β, and RANTES, and, to a lesser extent, GM-CSF that were greater than the sum of those detected in the medium of cells treated with IFNγ or MDP alone (Figure 6, a-e). Significant increases in secreted MCP-1 were also observed after treatment of HEKn with IFNγ and MDP; however, the levels of MCP-1 released after exposure of the cells to IFNγ and MDP were not greater than those observed after exposure to IFNγ alone (Figure 6f).

LeTx attenuates the induction of inflammatory mediators from HEKn by IFNγ/MDP.

To determine the effects of exposure of HEKn to LeTx on induction of inflammatory cytokines by IFNγ/MDP, monolayer cultures of HEKn were incubated with IFNγ/MDP and LeTx for 24 hours prior to collection of media supernatants and analysis by protein microarray. Incubation of HEKn with LeTx alone caused significant increases in both RANTES and MCP-1 when compared to control keratinocytes (Figure 5, a, b and e), consistent with our observations that the toxin does not inhibit metabolic activity in this cell type, and further supporting earlier findings demonstrating that LeTx does not induce cell death in cultured keratinocytes. Concurrent addition of LeTx along with IFNγ/MDP to HEKn in repeat trials yielded significant attenuation of levels of secreted IL-6,

TNF-α and MIP-1β (Figure 5e), while MCP-1 was unchanged from its elevated level. This effect was confirmed and quantitated by ELISA assay (Figure 6, a-c and f). An ELISA also revealed a similar attenuation of GM-CSF in the presence of LeTx that had been less apparent from examination of the protein microarray. Exposure of HEKn to lethal toxin either alone, or in combination with IFNγ/MDP, resulted in reductions in released GM-CSF to levels below those in supernatants from untreated controls (Figure 6d). In contrast, addition of LeTx to the medium augmented induction of RANTES by IFNγ/MDP nearly four fold in these experiments (Figure 6e).

Exposure to lethal toxin leads to reduced levels of activated c-Myc and c-Jun and increased levels of activated Stat-1α and the p65 subunit of activated NF-κB

Exposure of HEKn to lethal toxin resulted in selective and differential alterations in MAPKK family members, with proteolytic cleavages occurring in MEK2 and MEK4, but not MEK5, within hours of treatment with toxin. To elucidate the downstream effects of MEK cleavage as well as any as yet uncharacterized effects of LeTx, activation of multiple transcription factors known to be controlled by MAPK pathways was investigated. At the same time, we monitored the effects of LeTx on activation of the NF-κB family of transcription factors, which also function in controlling expression of

inflammatory biomarkers. Transcription factor activation was detected with a series of TransAM™ transcription factor activation ELISAs (Active Motif), in which consensus oligonucleotide sequences for binding the activated forms of the transcription factors are immobilized on 96 well microplates and, after addition of cell lysates suspected of containing activated transcription factors, the bound activated forms are detected with specific monoclonal antibodies. The oligonucleotide sequences in the MAPK TransAM kit include consensus sequences for binding activated forms of Stat1α, MEF2, c-Jun, c-Myc, and ATF-2; the NF-κB kit includes antibodies for the p50, p52, p65, c-Rel and Rel-B subunits of the dimeric transcription factor family. Analysis of cell lysates for activated transcription factors by these assays revealed decreased levels of the MAPK pathway-activated forms of the transcription factors c-Jun and c-Myc in lysates of cells exposed to LeTx (Figure 7, a and b), while treatment with LeTx, alone or in combination with IFNγ/MDP, resulted in increases in activated Stat1α; the increases in Stat1α levels in the presence of LeTx and IFNγ/MDP exceeded those in the presence of toxin alone or the pro-inflammatory mediator mixture alone, suggesting some additivity in the cell response (Figure 7c). The observed decreases in activated forms of c-Jun and c-Myc are consistent with the known mechanisms of activation of these transcription factors via phosphorylation by MEK4 and MEK2, respectively (Turjanski *et al.*, 2007), and our observation that both of these MEKs are cleaved by LeTx. The observed increase in levels of activated Stat1α, which can be generated through multiple pathways, including phosphorylation via pathways involving MEKs 3 and 6, may reflect yet another

pathway for activation mediated by the Janus kinase family. Transcription factor MEF2, the downstream target of the MAPK ERK5 (Turjanski *et al.*, 2007) which is itself phosphorylated by MEK5 was unaffected by LeTx (Figure 7d), consistent with our observation that LeTx fails to cleave MEK5 in HEKn. Examination of the components of the NF-κB family of transcription factors revealed that treatment of HEKn with IFNγ/MDP resulted in increased levels of the p65 subunit in activated forms of NF-κB, which were augmented further by co-incubating the cells with LeTx (Figure 8a). Slight but statistically significant and reproducible increases in the p52 subunit of activated NF-κB species were detected after incubation of HEKn with IFNγ/MDP, but no further changes in p52 levels in the activated forms of NF-κB resulted from co-incubation of the cells with the inflammatory mediator mixture and LeTx (Figure 8b). The remaining subunits of p50, c-Rel, RelB were unchanged at all experimental conditions investigated (Figure 8 c-e).

IV. Discussion

In this study we evaluated the effects of LeTx on inflammatory signaling cascades in Human Epidermal Keratinocytes (HEKn). The results indicate that, similar to earlier results reported for the effects of LeTx on human mononuclear phagocytes (Bergman *et al.*, 2005; Erwin *et al.*, 2001; Pellizzari *et al.*, 1999; Ribot *et al.*, 2006), LeTx blunts the response of HEKn to an infection by *B. anthracis* by blocking the release of a number of inflammatory cytokines and chemokines that would otherwise contribute to an effective host immune response. Compromise to the immune response can be correlated, if not directly causally linked, to LeTx-mediated cleavage of the MAPKK family of proteins responsible for transcriptional upregulation of various inflammatory mediators. Consistent with studies on murine macrophages, we observed LeTx-mediated cleavage of MEK2 and MEK4 but not MEK5 in HEKn. MEK2 cleavage began as early as 45 minutes after exposure of the cells to the toxin and resulted in virtually complete loss of immunologically detectable MEK2 by 3 hours following LeTx addition. The rapidity of MEK2 cleavage led us to investigate whether this process could be prevented by removal of the toxin by washing the cells shortly after its addition. We observed that a time interval for exposure of HEKn to LeTx in the medium of as brief as 20 minutes was sufficient to irreversibly start the process of MEK2 cleavage in the cytosol of the cells, reflecting the efficiency with which Lethal Factor is delivered to the cytosol in the infectious process. Collectively, these data demonstrate that the same MAPKK

family members that are substrates of LeTx in murine macrophages are also substrates in HEKn (Duesbery *et al.*, 1998; Klimpel KR, 1993; Pellizzari *et al.*, 1999; Vitale *et al.*, 1998). This led us to investigate the consequences of MAPKK cleavage in HEKn and more specifically the effect such cleavage has on the production of inflammatory mediators by these cells.

In inhalational anthrax, the early stage of infection begins with engulfment of spores by macrophages and dendritic cells and is marked by an initial burst in the production of inflammatory mediators (Brittingham *et al.*, 2005). During transport to the lymph nodes, the production of LeTx by germinating bacilli begins the process of "silencing" the immune response by inactivation of the MAPK signaling cascade that is responsible for transcriptional activation of various inflammatory mediators. In the absence of effective innate and adaptive immune responses, the bacterial infection progresses until a systemic bacteremia develops. Subsequent cell death, marked by the expression of biomarkers of tissue hypoxia and necrosis, characterizes the pathology of the multiple organ failure that is clinically observed in human patients and in animal models of the systemic end stage of inhalational anthrax. To better understand the process leading to the formation of the necrotic lesions in cutaneous anthrax, which are almost always limited and focal, we incubated HEKn with LeTx and investigated its effects on cellular viability over time. We did not observe induction of biomarkers of apoptotic or necrotic cell death pathways in HEKn at time intervals significantly beyond those required for complete MAPKK cleavage. Although cells exhibited a modest change in morphology, the overall metabolic activity, as

measured by cellular dehydrogenases, was relatively unchanged by 24 hours following the addition of LeTx. At longer time intervals, LeTx decreased the amount of cellular dehydrogenase activity in HEKn cultures compared to the levels detected in untreated cultures; however, this reduction appears to reflect inhibition of cellular proliferation rather than the consequence of a cytotoxic event. This observation was supported by the inability of LeTx to either induce biomarkers of apoptosis as measured by caspase activity (Figure 2), or necrosis, as measured by expression of hypoxia inducible factor-1 in HEKn. This apparent lack of cell death in cultured keratinocytes contrasts with the necrotic ulceration observed on pathologic examination of cutaneous anthrax lesions excised from patients. These lesions are characterized by infiltration of neutrophils into the epidermis and both neutrophils and mononuclear cells surrounding the microvessels of the dermis (Shieh *et al.*, 2003). Immunohistochemical staining of these lesions generally reveals components of the *B. anthracis* cell wall, but culture of viable bacilli from the lesions is frequently not possible, suggesting that the infiltration of neutrophils has not only contributed to the necrosis of the skin but also has also limited the progression of the bacterial infection. Mayer-Scholl *et al.* (2005) have investigated the interactions of *B. anthracis* with cells of the innate immune response and have concluded that neutrophils, which are recruited to sites of cutaneous anthrax lesions but not to the lung or the gastrointestinal tract, are responsible for killing both spores and germinated bacilli via a mechanism involving the action of α-defensins.

Monolayer cultures of HEKn incubated with LeTx morphologically resembled cells transitioning into a differentiated state. Under normal conditions, epidermal keratinocytes in both intact skin and in monolayer cultures will terminally differentiate in a complex process that culminates in the formation of an epidermal barrier that serves to maintain hydration as well as protection from microbial infection. Our visual observations that HEKn incubated with LeTx undergo a morphological transition similar to that seen during differentiation prompted us to investigate the keratinocyte differentiation marker involucrin by Western blotting. In contrast to the morphological features of keratinocytes exposed to LeTx, exposure of HEKn cultures to the toxin for periods of up to 5 days resulted in significantly lower levels of involucrin protein detected in cell lysates relative to levels seen in untreated control cultures. Although this result may be inconsistent with the morphological changes seen on visual observation, it is consistent with our finding that HEKn incubated with LeTx did not display increased levels of the active forms of caspases, considering that active caspase 14 has been implicated in the process of keratinocyte terminal differentiation (Rendl *et al.*, 2002).

Mitogen-activated protein kinases (MAPKs) regulate diverse cellular programs, including inflammation. We therefore investigated the effects of LeTx mediated MEK cleavage on production of inflammatory mediators by HEKn. Levels of a number of inflammatory biomarkers in a panel which had been initially analyzed qualitatively by protein microarray were quantitated by individual ELISAs. It was noteworthy that after incubation of HEKn with LeTx

alone for 24 hours, the levels of inflammatory biomarkers were not significantly attenuated, but rather increases in levels of both MCP-1 and RANTES were observed. These increases in the levels of RANTES and MCP-1 were apparent in cultures incubated in the presence of LeTx for as long as 5 days. Although informative, the response of HEKn to LeTx alone does not fully represent the conditions to which the epithelium is exposed during cutaneous infection by a Gram-positive bacterium such as *B. anthracis*, including activation of cell surface Toll-like receptors and intracellular Nod receptors (Athman and Philpott, 2004) by products of the bacterial infection and secretion of paracrine factors from neighboring cells in the infected host. The pathology of cutaneous anthrax is accompanied by an initial release of inflammatory mediators at the site of infection, as demonstrated by the formation of a red papule. To mimic the early stages of cutaneous infection, we incubated HEKn with known pro-inflammatory mediators relevant to bacterial infections: lipoteichoic acid, a component found in the cell walls of gram-positive bacteria and a Toll-like receptor 2 ligand; *B. anthracis* cell wall extract, composed mainly of peptidoglycans; IFNγ, a potent mononuclear cell-derived modulator of immune responses during infection; and muramyl dipeptide, a peptidoglycan common to all bacteria and a ligand for the Nod2 receptor. Samples of supernatant medium from HEKn incubated with one or more of the pro-inflammatory mediators were screened using a protein microarray specific for the detection of cytokines and chemokines involved in inflammation. The induction of a robust pro-inflammatory response would create a model system allowing for the investigation of the effects of LeTx during the

early stages of cutaneous anthrax infection. Interestingly, incubation of HEKn with the Toll-like receptor 2 ligand and gram positive bacterial membrane constituent lipoteichoic acid alone did not stimulate the release of pro-inflammatory mediators from these cells; however, consistent with our previous results, the addition of LeTx to HEKn yielded increases in both RANTES and MCP-1. In another experiment, incubation of HEKn with *B. anthracis* cell wall extract alone failed to increase the release of inflammatory mediators from these cells, while co-incubation with LeTx again displayed increases in both RANTES and MCP-1. Next we incubated HEKn for 24 hours with the inflammatory cytokine IFNγ and the Nod2 ligand muramyl dipeptide. Analysis of HEKn supernatants by protein microarray with confirmation by individual ELISAs revealed increased levels of released IL-6, MCP-1 and RANTES in the presence of IFNγ alone; however, the addition of muramyl dipeptide alone was ineffective at stimulating the release of secreted inflammatory mediators from HEKn. Interestingly, incubation with both IFNγ and MDP together led to the apparent synergistic induction of levels of GM-CSF, IL-6, TNF-α, and the three members of the CC family of chemokines we measured, MIP-1β, MCP-1 and RANTES, that were greater in the presence of IFNγ and MDP than the sum of the levels released in the presence of either one of the pro-inflammatory components alone (Figure 6). It was noteworthy that co-addition of LeTx with IFNγ/MDP attenuated the augmented release of GM-CSF, MIP-1β, IL-6 and TNF-α, demonstrating how LeTx effectively diminishes the host immune response toward infection. In contrast, addition of LeTx to the mixture of IFNγ and MDP

enhanced release of RANTES four- to five-fold, further supporting earlier results demonstrating that LeTx is not inducing cell death in these experiments. In addition, the enhanced induction of the monocytic chemokine MCP-1 in HEKn by culture in the presence of IFNγ/MDP was undiminished by addition of LeTx. The augmented levels of RANTES and the undiminished levels of MCP-1 would be expected to stimulate recruitment of both monocytes and macrophages to the site of infection; without the appropriate cytokines present (GM-CSF, MIP-1β, IL-6 and TNF-α), however, further maturation and activation of mononuclear phagocytes would be prevented and the full repertory of components of the immune response could not be displayed.

Our observations that exposure of HEKn to LeTx attenuates levels of the immunoreactive forms of nearly all MEK proteins, as well as levels of inflammatory cytokines and chemokines the production of which is regulated by MAPK pathways, led us to evaluate activation of transcription factors involved in these cascades. Additionally, we investigated the components of activated members of the NF-κB family of transcription factors known to be involved in inflammation. We observed that incubation of HEKn with LeTx led to diminutions in the levels of activated c-Jun and c-Myc, which are converted to their active forms by MEK4 and MEK2, respectively (Figure 7, a and b), clearly indicating that cleavage of MEK4 and MEK2 by LeTx is sufficient to block the activation of these transcription factors. In contrast, levels of activated MEF2 were unaffected by exposure of HEKn to LeTx, consistent with the fact that MEF2 activation is dependent upon upstream phosphorylation by MEK5, the only

MEK that we as well as others have observed to be unaffected by LeTx. It should be mentioned that infection by *B. anthracis* may still impact activation of MEF2 *in vivo*. For example, the presence of Edema toxin (EdTx) at the site of infection induces cAMP activity, which has been reported to inhibit EGF-induced ERK-5 activation (Pearson *et al.*, 2006). Keratinocytes that have been infected by both EdTx and LeTx may shut down virtually the entire MAPK signaling family.

It is possible that redundant pathways exist which could compensate for the loss of MEK proteins. For example, we observed that levels of activated Stat-1α were increased upon incubation of HEKn with a mixture of IFNγ and MDP, consistent with reports of activation of Stat-1α by IFNγ. It is noteworthy that co-addition of LeTx with IFNγ/MDP augmented the IFNγ/MDP-mediated activation of Stat-1α in an additive but not synergistic manner. Wong and Fish (1998) reported on activation of Stat-1α in T cells in response to incubation with RANTES. The 5 fold increase in levels of RANTES we observed following incubation of HEKn with LeTx may contribute to this additive increase in activated Stat-1α levels. Additionally, Janus kinases which are known to activate Stats may be playing a role in modulation of activated Stat-1α levels by LeTx.

Activation of the MAPK family of transcription factors represents one pathway toward cellular activation of inflammatory mediators; however, the NF-κB family of transcription factors plays a prominent role in this process as well. To better understand the process by which LeTx and IFNγ/MDP contribute to regulation of the inflammatory cytokines mentioned above, we investigated the levels of the p50, p52, p65, c-Rel and RelB subunit constituents of the activated

forms of NF-κB. Although LeTx did not affect levels of any of these subunits of activated NF-κB when added to HEKn alone, we observed synergistically augmented levels of the p65 subunit in activated NF-κB species when HEKn were incubated with LeTx in combination with IFNγ/MDP. A modest but statistically significant increase in the p52 subunit in activated NF-κB was also observed after exposure of HEKn to the combination of LeTx and IFNγ/MDP. A number of reports have appeared on increased RANTES expression driven by NF-κB transcription factors (Krensky and Ahn, 2007; Lee *et al.*, 2000; Song *et al.*, 1999). MCP-1 has also been reported to be induced by members of the NF-κB family of transcription factors (Kammanadiminti *et al.*, 2007). We have observed that levels of the CC family of chemokines, reported to be under the control of NF-κB, are increased along with the levels of at least some of the activated forms of the same transcription factor family. Our data are therefore consistent with a model for a signaling network in which LeTx, in concert with signals from IFNγ and MDP through Nod2, initially stimulates activation of NF-κB which then facilitates increased expression of RANTES and related C-C chemokines. This increase in CC chemokine levels may then contribute to the activation of Stat-1α, which can in turn act in a positive feedback loop to further augment chemokine production (Ohmori *et al.*, 1997).

In summary, our data elucidate a series of events following infection of epithelial tissue by *Bacillus anthracis*. Epithelial cells are a target of the toxins produced by the bacterium in all three forms of anthrax infection, along with mononuclear phagocytes. *B. anthracis*, through the actions of LeTx and EdTx,

essentially abrogates the entire complement of the MAPK signaling pathways in targeted cells. Even before the bacterium begins to replicate in the infected host, LeTx which is bound to the surface of spores initiates silencing of the innate and adaptive arms of the immune response by inhibiting cytokine secretion (GM-CSF, IL-6, TNF-α), thereby preventing activation of dendritic cells and macrophages. As bacilli begin to proliferate, they release additional toxin which prolongs and enhances the early immunosuppressed state of mononuclear cells. The synergistic increases in activated forms of NF-κB observed in keratinocytes exposed to LeTx and IFNγ/MDP lead to induction of the C-C chemokines RANTES and MCP-1. As has been described in immune-complex triggered lung injury and rheumatoid arthritis (Stanczyk *et al.*, 2005; Standiford *et al.*, 1995), it appears that significant induction of RANTES and other CC chemokines in keratinocytes exposed to toxins and cell wall components is correlated with recruitment of both neutrophils and monocytes to sites of cutaneous infection by *B. anthracis*. Once recruited to a site where *B. anthracis* spores are present, mononuclear phagocytes can become Trojan horses for spore germination and intracellular proliferation of bacilli with subsequent toxin production, while neutrophils may cause tissue necrosis at the site of infection as a result of released proteinases and ROS, even as they simultaneously limit the extent of bacterial infection through the action of defensins (Mayer-Scholl *et al.*, 2005; Shieh *et al.*, 2003).

The detailed mechanism of neutrophil recruitment and activation in cutaneous anthrax lesions remains somewhat obscure; RANTES is not reported to be an effective chemoattractant or activator of human neutrophils *in vitro* (Schall,

1991). However, the RANTES/CCR5 pathway appears to play a prominent role in a number of acute inflammatory processes, especially in the lung, where the pathway has been proposed to contribute to emphysema and most recently, tumor invasion and progression (Borczuk *et al.*, 2008; Ma *et al.*, 2005) In addition, Ferrandi *et al.* (2007) reported on the induction of neutrophil recruitment by RANTES in a murine model of peritoneal chemotaxis. Further support of the involvement of RANTES in recruitment of neutrophils to the lung was provided by a transgenic mouse model in which RANTES was expressed in an inducible fashion (Pan *et al.*, 2000). Increased production of RANTES alone promoted neutrophilia in the lung, however these authors discussed the concomitant induction of other chemokines, including MCP-1 (a CCR ligand), MIP-2 (the human homologue of Groα) and IP-10 (10 kDa interferon gamma inducible protein), and the latter two of which are members of the CXC chemokine family. It is possible that the observed increases in neutrophils in different models in which RANTES has been shown to be elevated may be a result of the indirect induction of these CXC chemokines. However, a recent report by Hartl *et al.* (2008) elucidates phenotypic changes that neutrophils can undergo depending on the nature of their surrounding microenvironment (e.g. cytokines, TLR ligands, NOD2 ligands and apoptosis) to which they are exposed. Neutrophils from patients with chronic inflammatory disease and rheumatoid arthritis express cell surface receptors CCR1, CCR2, CCR3, CCR5, CXCR3 and CXCR4 that are either absent or only marginally expressed on circulating neutrophils. *In vitro* studies by this group demonstrated that chemokine receptors could be

differentially expressed as a result of cytokine-mediated protein synthesis (CCR1 and CCR3); engagement of toll-like receptors (CXCR3) or NOD2 receptors (CCR5); neutrophil apoptosis (CCR5, CXCR4); and/or mobilization of intracellular CD63+ granules (CXCR3).

The distinctive upregulation of the expression of the CC chemokines in keratinocytes may represent a critical event leading to infiltration of neutrophils into cutaneous sites of *B. anthracis* infection with resultant necrotic ulceration and protection from further bacterial dissemination that would otherwise result in fatal systemic disease. However, further work will be needed to elucidate if this is a direct effect of CC chemokines, or an indirect path upstream of induction of CXC chemokines.

V. Figures and Legends

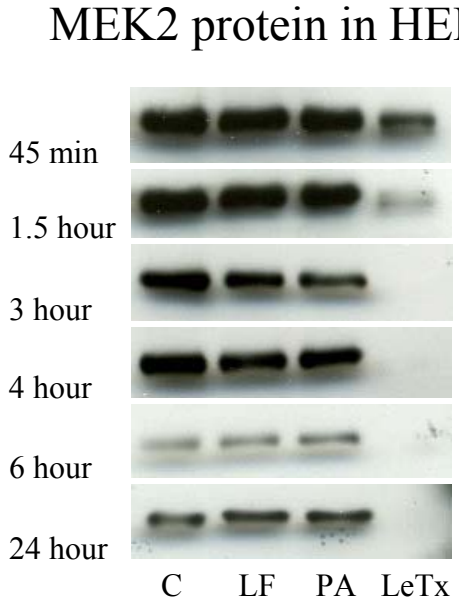


Figure 1a. ***Kinetic analysis of Lethal Toxin degradation of MEK2 in Normal Human Epidermal Keratinocytes.*** Normal Human Epidermal Keratinocytes (HEKn) were incubated with 1 μg/ml Lethal Factor (LF), 1 μg/ml Protective Antigen (PA), or 1 μg/ml of both (LeTx) for the time intervals listed. Cells were collected, lysed, and lysates cleared by centrifugation. Protein supernatant was quantitated using BCA reagent (Pierce), and 10 μg aliquots of total protein were loaded and separated by SDS-PAGE on a 10% Bis-Tris gel prior to transfer to an immunoblot and probing for the N-terminus of MEK2. Losses of immuno-detectable bands were observed in cells incubated with LeTx, but not in LF- or PA- incubated cells. Diminution of signal began as early as 45 minutes post LeTx addition with near complete loss at 1.5 hours. No immunodetectable bands were observed at time intervals of 3 hours or longer.

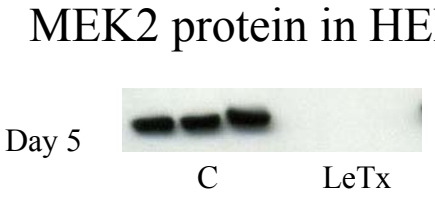


Figure 1b. ***Persistence of Lethal toxin mediated degradation of MEK2 in Normal Human Epidermal Keratinocytes.*** Normal Human Epidermal Keratinocytes (HEKn) were incubated with or without LeTx (1μg/ml of both LF and PA) over a five day period. Cells were collected, lysed, and lysates cleared by centrifugation. Protein supernatant was quantitated using BCA reagent and 10 μg aliquots of total protein from triplicate samples were loaded and separated by SDS-PAGE on a 10% Bis-Tris gel prior to transfer to an immunoblot and probing for the N-terminus of MEK2. Loss of immunodetectable bands persisted as long as 5 days post LeTx addition.

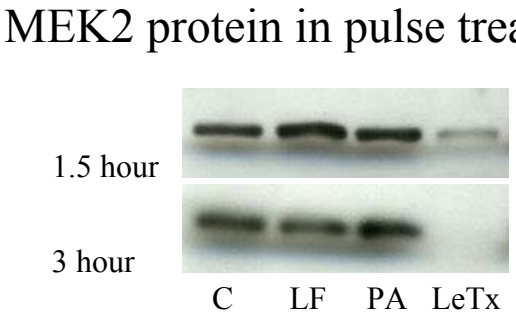


Figure 1c. ***Pulse treatment with Lethal Toxin for 20 minutes was sufficient for MEK2 degradation in Normal Human Epidermal Keratinocytes.*** Normal Human Epidermal Keratinocytes (HEKn) were incubated with 1 μg/ml Lethal Factor (LF), 1 μg/ml Protective Antigen (PA), or 1 μg/ml of both (LeTx) for twenty minutes prior removal with excessive washing with phosphate buffered saline (PBS). Cells were then allowed to incubate for the time intervals listed. Cells were collected, lysed, and lysates were cleared by centrifugation. Protein supernatant was quantitated using BCA reagent, and 10 μg aliquots of total protein were loaded and separated by SDS-PAGE on a 10% Bis-Tris gel prior to transfer to an immunoblot and probing for the N-terminus of MEK2. Addition of LeTx for only 20 minutes was sufficient to allow for the internalization of toxin protein and subsequent MEK2 cleavage. Kinetics of MEK2 cleavage by LeTx followed the same pattern as observed in figure 1a.

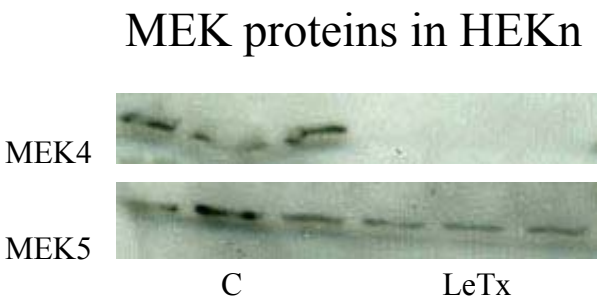


Figure 1d. ***MEK4 but not MEK5 was degraded by Lethal Toxin in Normal Human Epidermal Keratinocytes.*** Normal Human Epidermal Keratinocytes (HEKn) were incubated with or without 1 μg/ml LF and PA (LeTx) for 24 hours. Cells were collected, lysed, and lysates centrifuged. Protein supernatant was quantitated using BCA reagent, and 10 μg aliquots of total protein were loaded from triplicate samples and separated by SDS-PAGE on a 10% Bis-Tris gel prior to transfer to an immunoblot and probing for the N-termini of both MEK4 and MEK5. While LeTx effectively cleaved MEK4 culminating in the loss of immunodetectable bands, MEK5 was not affected.

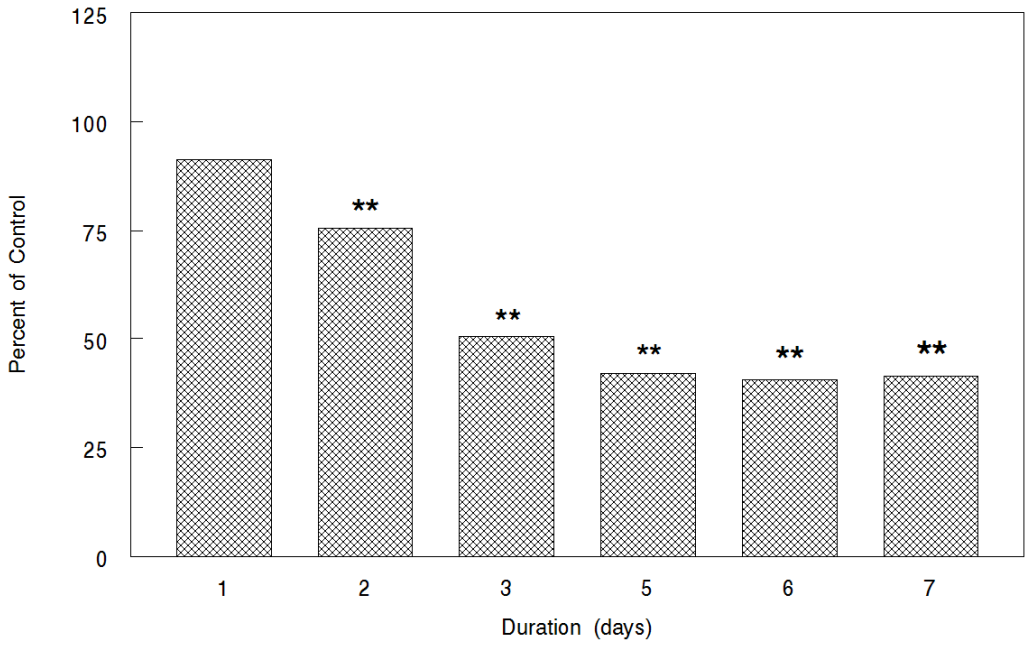


Figure 2. ***Viability kinetics in Normal Human Epidermal Keratinocytes incubated with LeTx.*** HEKn were incubated either with or without 1 μg/ml of LF and PA (LeTx) for the time intervals listed, and viability was then assessed by cellular dehydrogenase-mediated conversion of MTS reagent to a blue formazan product; MTS reduction in cultures of LeTx-exposed cultures was compared to that in control cultures. Viability was virtually unchanged at 24 hours post addition of LeTx. At durations of 3 days or greater, MTS reduction by LeTx-exposed cultures was only 50% of that observed in control cultures. By visual assessment, this appeared to be due to a cytostatic effect of LeTx on HEKn, rather than cytotoxicity. (N=3, **p< .01; ANOVA, Bonferroni post test).

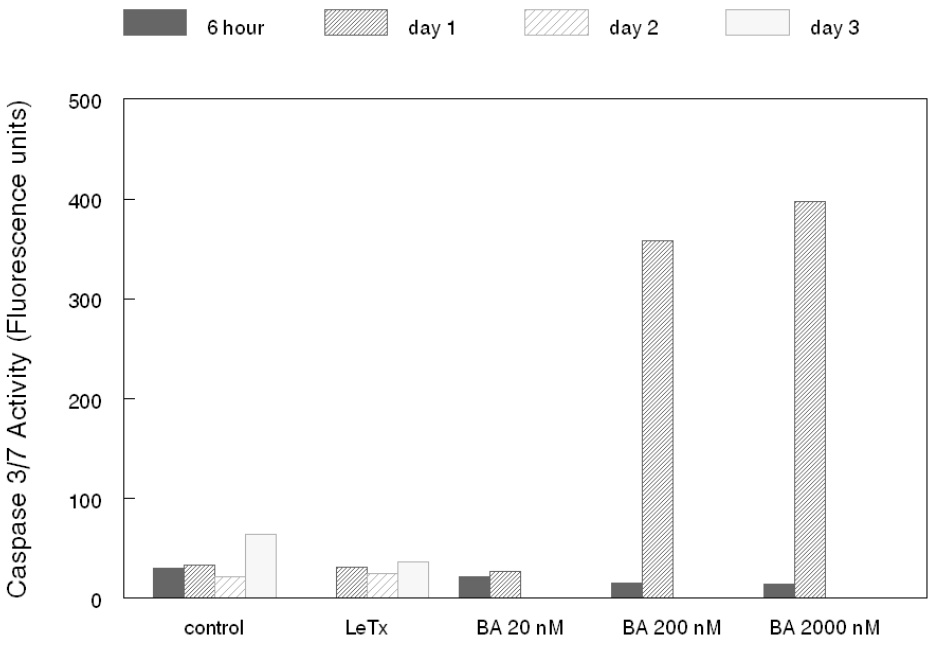


Figure 3. ***Caspase 3/7 activity in Normal Human Epidermal Keratinocytes incubated with LeTx.*** HEKn were incubated either with or without 1 μg/ml of LF and PA (LeTx) or Brefeldin A (BA), for the time intervals listed. Cells were collected, lysed and cleared by centrifugation following the protocol described in Materials and Methods. Caspase activity in cellular lysates was assessed by the relative amounts of enzymatic cleavage of a fluorogenic peptide substrate specific for caspase 3/7. After as brief as one day of incubation with either 200 or 2000 nM concentrations of the known apoptotic agent Brefeldin A, caspase 3/7 activity was induced in the cells. Incubations of keratinocytes with LeTx had no effect on caspase 3/7 levels at durations of up to 3 days.

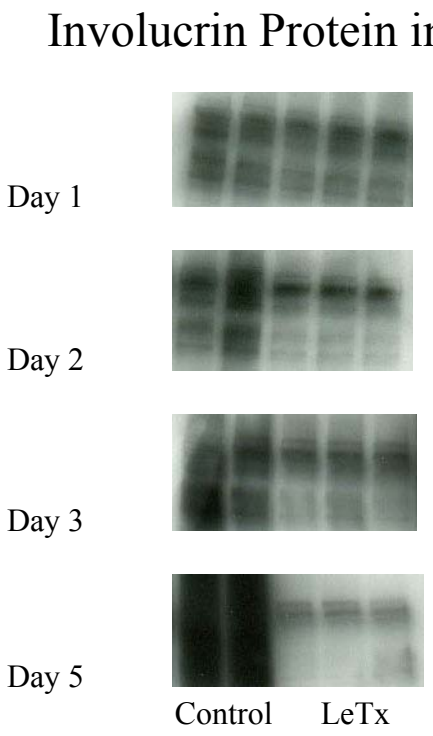


Figure 4. ***Inhibition of involucrin protein by LeTx in Normal Human Epidermal Keratinocytes.*** Normal Human Epidermal Keratinocytes (HEKn) were incubated with or without 1 μg/ml of LF and PA (LeTx) over a five day period. Cells were collected, lysed, and lysates cleared by centrifugation. Protein supernatant was quantitated using BCA reagent (Pierce), and 10 μg aliquots of total protein from duplicate untreated controls and triplicate LeTx incubated cells were loaded and separated by SDS-PAGE on a 10% Bis-Tris gel prior to transfer to an immunoblot and detection using antibodies against the keratinocyte differentiation marker involucrin. Diminished levels of involucrin protein were noted in LeTx incubated HEKn relative to the untreated controls beginning at day 2. This effect was even more dramatic at later time points.

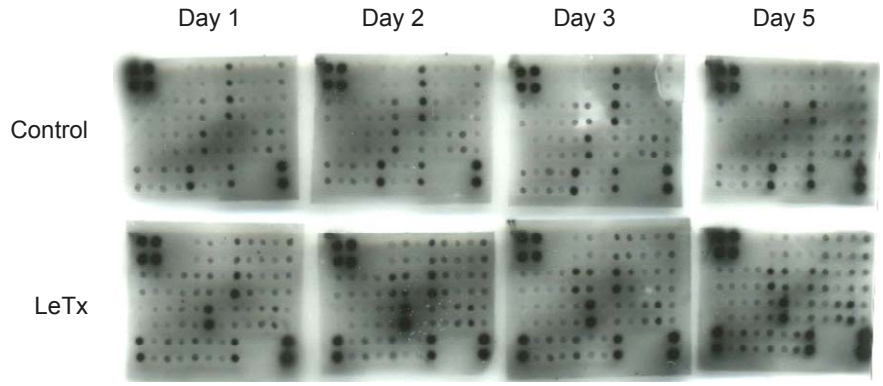


Figure 5a. ***Inflammatory cytokine profiles in Normal Human Epidermal Keratinocytes incubated with LeTx.*** HEKn were exposed to 1 μg/ml of LF and PA (LeTx) for 1, 2, 3 or 5 days at 37°C unless labeled differently. Aliquots of supernatant medium were transferred to an inflammatory protein array (RayBiotech) following manufacturer's protocol. LeTx stimulated the release of RANTES and MCP-1 from HEKn as early as 24 hours; increased release of these chemokines persisted as long as 5 days post-addition. Capture antibody legend listed in figure 5e.

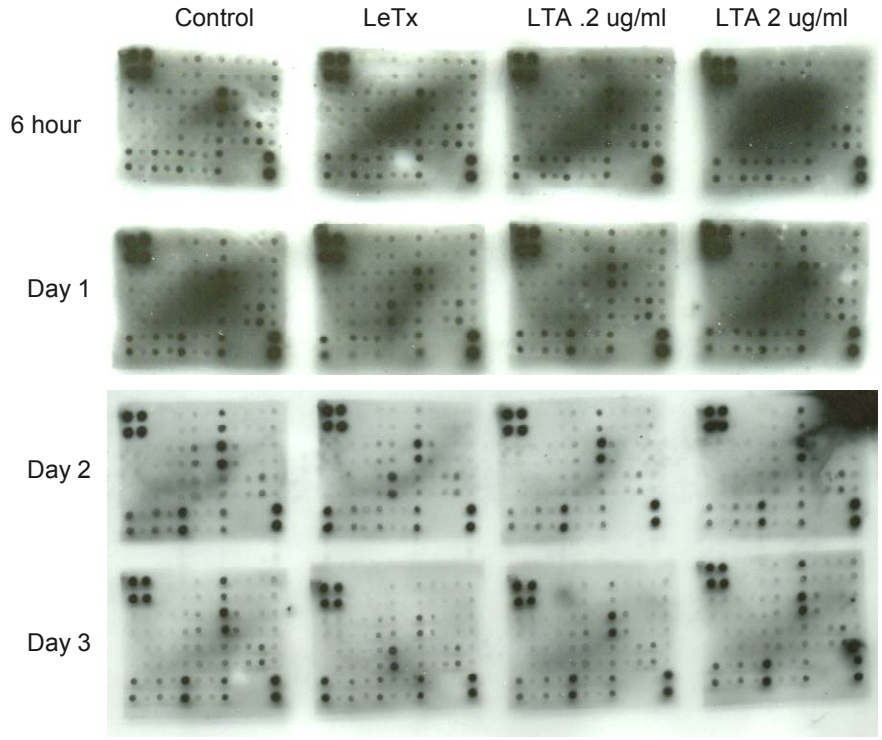


Figure 5b. ***Inflammatory cytokine profiles in Normal Human Epidermal Keratinocytes incubated with Lipoteichoic acid or LeTx.*** HEKn were exposed to 1 μg/ml of LF and PA (LeTx), or LTA (concentrations listed above) at 37°C and aliquots of supernatant media collected at the times listed above were transferred to an inflammatory protein array (RayBiotech) following the manufacturer's protocol. As observed in Figure 5a, incubation of HEKn with LeTx stimulated the release of both RANTES and MCP-1. Addition of lipoteichoic acid to HEKn did not affect the profile of secreted inflammatory mediators. Capture antibody legend listed in figure 5e.

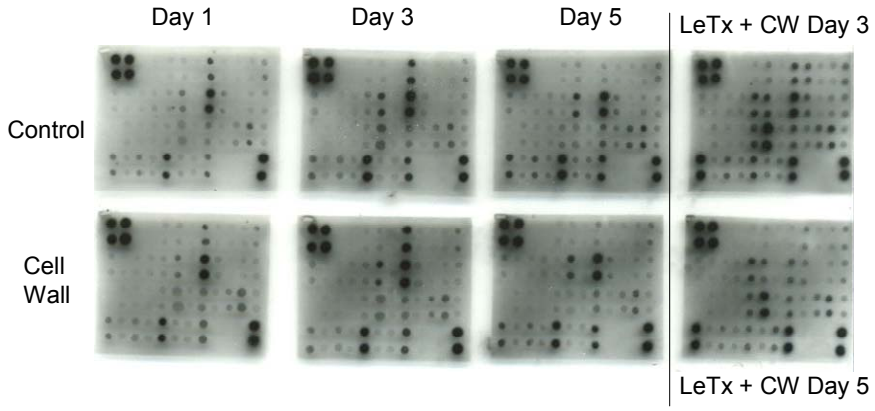


Figure 5c. ***Inflammatory cytokine profiles in Normal Human Epidermal Keratinocytes incubated with B. anthracis cell wall extract and LeTx.*** HEKn were exposed to *B. anthracis* cell walls (1 μg/ml) alone or in combination with 1 μg/ml of LF and PA (LeTx) at 37°C and aliquots of supernatant media collected at the times listed above were transferred to an inflammatory protein array (RayBiotech) following manufacturer's protocol. The addition of an extract of *B. anthracis* cell walls to HEKn did not affect the profile of secreted inflammatory mediators, while LeTx effectively stimulated an increase in RANTES and MCP-1 from HEKn even in the presence of such an extract. Capture antibody legend listed in figure 5e.

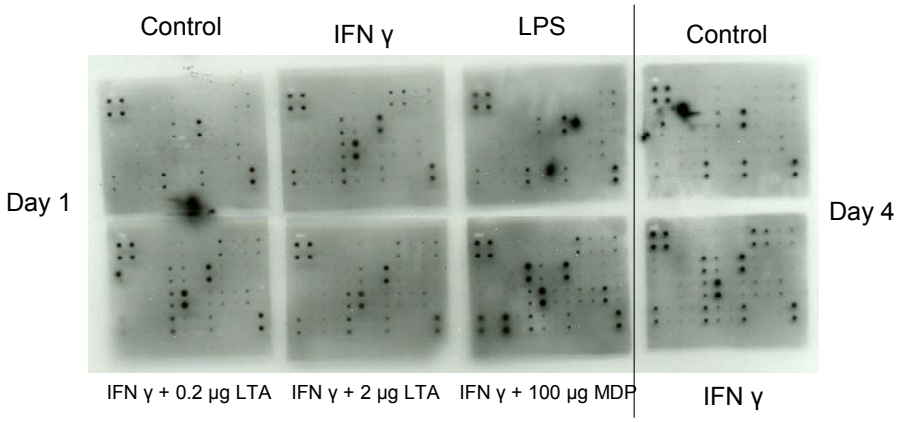
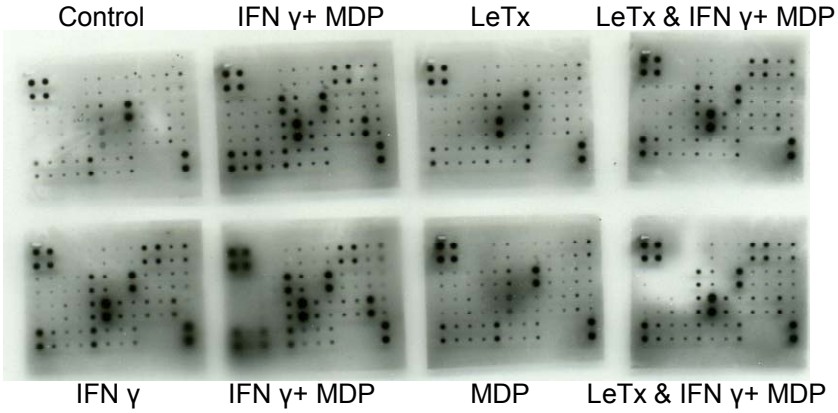


Figure 5d. ***Inflammatory cytokine profiles in Normal Human Epidermal Keratinocytes incubated with pro-inflammatory mediators IFNγ, LPS, LTA and MDP.*** HEKn were exposed to either 10 ng/ml IFNγ, 100 ng/ml LPS or 10ng/ml IFNγ in combination with LTA or MDP (concentrations listed above), at 37°C and aliquots of supernatant media collected at the times listed above were transferred to an inflammatory protein array (RayBiotech) following the manufacturer's protocol. Incubation of HEKn with lipopolysaccharide did not affect the profile of secreted inflammatory mediators (the prominent dark areas of the film are artifacts). Exposure of cells to interferon gamma resulted in subtle increases in MCP-1 and to a lesser extent RANTES and IL-6 at both days 1 and 4. Addition of both interferon gamma and lipoteichoic acid to cells resulted in the same pattern of expression as seen after exposure of the cells to interferon gamma alone. Interestingly, the addition of muramyl dipeptide and interferon gamma to HEKn yielded a robust increase in the profile of a subset of inflammatory mediators, including RANTES, MCP-1, IL-6 and TNF-α, surpassing the induction observed by either component alone. Capture antibody legend listed in figure 5e.



POS	POS	NEG	NEG	Eotaxin	Eotaxin-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
POS	POS	NEG	NEG	Eotaxin	Eotaxin-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 P40	IL-12 P70
IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 P40	IL-12 P70
IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
RANTES	TGF-β1	TNF-α	TNF-β	s TNF R1	s TNF R2	PDGF-BB	TIMP-2	BLANK	BLANK	NEG	POS
RANTES	TGF-β1	TNF-α	TNF-β	s TNF R1	s TNF R2	PDGF-BB	TIMP-2	BLANK	BLANK	NEG	POS

Figure 5e. ***Inflammatory cytokine profiles in Normal Human Epidermal Keratinocytes incubated with IFNγ/MDP and/or LeTx.*** HEKn were exposed to 10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx), IFNγ/MDP in combination or IFNγ/MDP in combination with LeTx at 37°C for 24 hours and aliquots of supernatant media were transferred to an inflammatory protein array (RayBiotech) following manufacturer's protocol. Exposure to IFNγ/MDP resulted in robust induction of inflammatory mediators secreted by HEKn, surpassing the induction observed by either component alone. Co-incubation of cells with IFNγ/MDP and LeTx resulted in attenuated induction of several secreted inflammatory biomarkers; however, levels of both RANTES and MCP-1 were unaffected by LeTx. Duplicates from both IFNγ/MDP and LeTx and IFNγ/MDP were assayed.

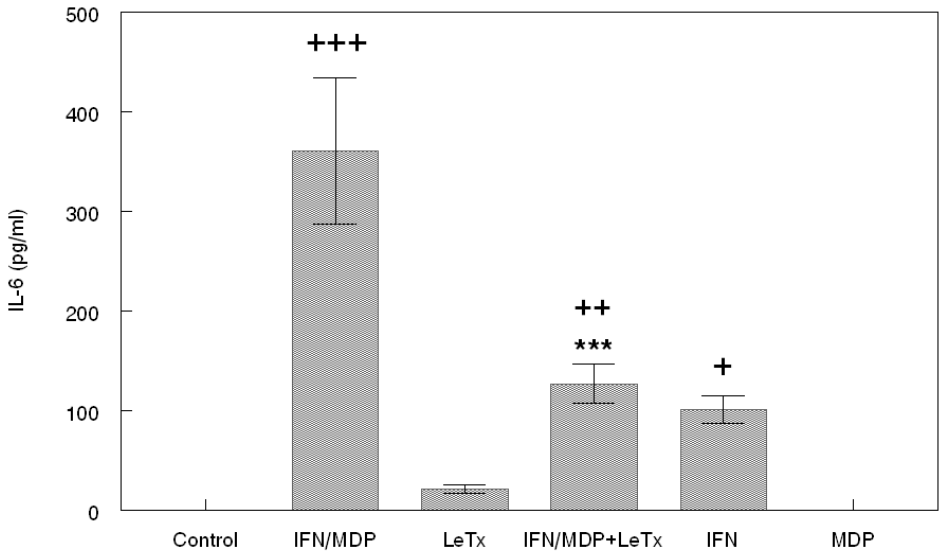


Figure 6a. ***IFNγ/MDP-mediated secretion of IL-6 from Normal Human Epidermal Keratinocytes is modulated by LeTx.*** Normal Human Epidermal Keratinocytes were incubated with materials listed above (10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx)) at 37°C for 24 hours. Levels of IL-6 protein, which we had observed to be modulated by LeTx in microarrays, were further analyzed by ELISA. Exposure of cells to IFNγ/MDP synergistically induce IL-6 protein over control levels. This effect was attenuated by the co-addition of LeTx. Data were analyzed using a one-way analysis of variance (ANOVA) and significant means (n=3) were tested using the Bonferroni multiple comparisons test (***P< .001 from IFNγ/MDP group, +++P< .001, ++P<.01, +P<.05 from control). Experiments were performed twice with similar results.

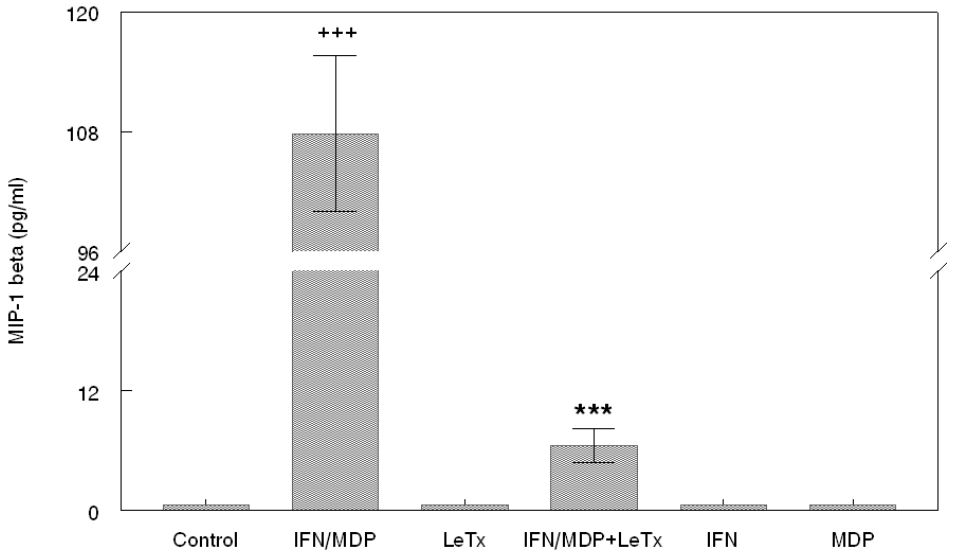


Figure 6b. ***IFNγ/MDP mediated secretion of MIP-1β from Normal Human Epidermal Keratinocytes is modulated by LeTx.*** Normal Human Epidermal Keratinocytes were incubated with materials listed above (10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx)) at 37°C for 24 hours. Levels of MIP-1β, which we had observed to be modulated by LeTx in our microarrays, were further analyzed by ELISA. While protein levels of MIP-1β were not detectable upon incubation with either IFNγ or MDP alone, when added together they resulted in a robust synergistic induction that was attenuated to near control levels with the co-addition of LeTx. Data were analyzed using a one-way analysis of variance (ANOVA) and significant means (n=3) were tested using the Bonferroni multiple comparisons test (***P< .001 from IFNγ/MDP group, +++P< .001 from control). Experiments were performed twice with similar results.

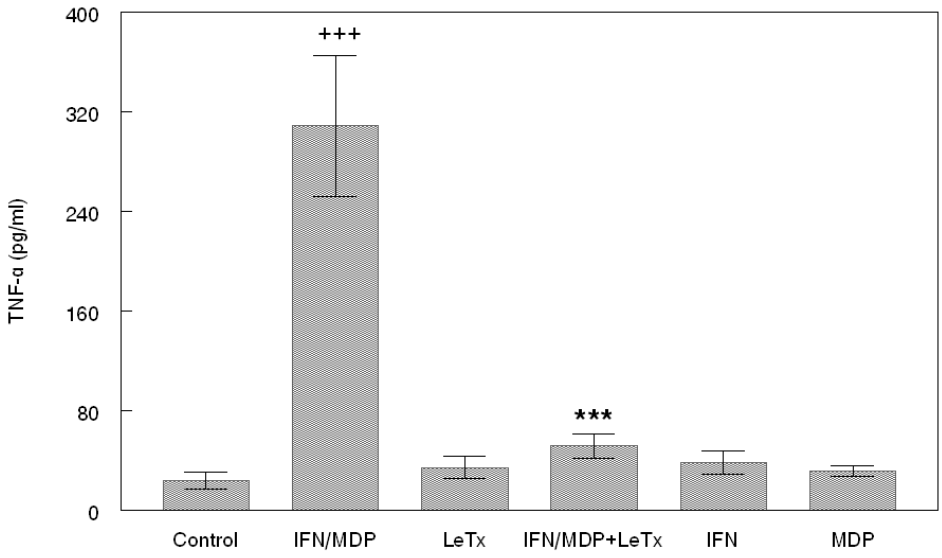


Figure 6c. ***IFNγ/MDP mediated secretion of TNF-α from Normal Human Epidermal Keratinocytes is modulated by LeTx.*** Normal Human Epidermal Keratinocytes were incubated with materials listed above (10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx)) at 37°C for 24 hours. Levels of released TNF-α, an inflammatory cytokine which we observed to be modulated by LeTx in our microarrays, were further analyzed by ELISA. A synergistic induction of TNF-α in HEKn exposed to IFNγ/MDP was attenuated to near control levels by co-addition of LeTx. Data were analyzed using a one-way analysis of variance (ANOVA) and significant means (n=3) were tested using the Bonferroni multiple comparisons test (***P< .001 from IFNγ/MDP group, +++P< .001 from control). Experiments were performed twice with similar results.

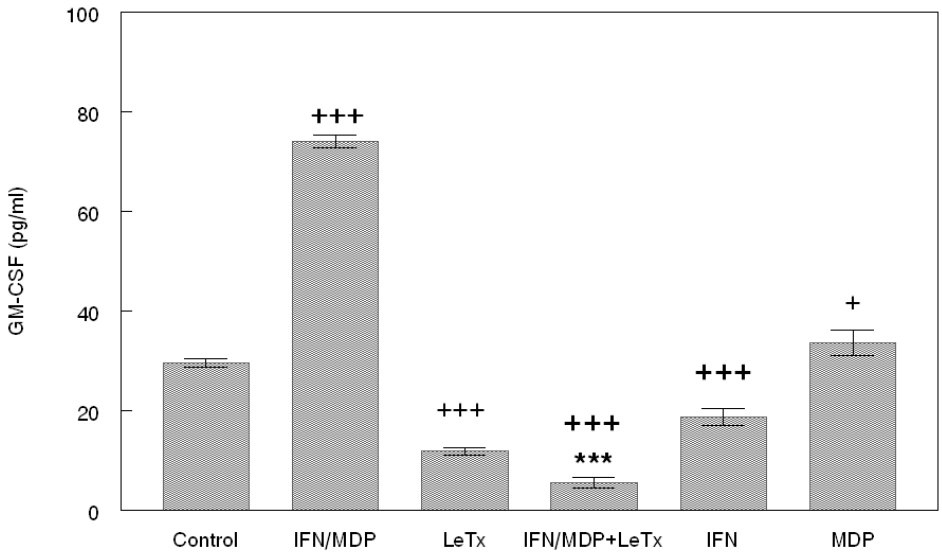


Figure 6d. ***IFNγ/MDP mediated secretion of GM-CSF from Normal Human Epidermal Keratinocytes is modulated by LeTx.*** Normal Human Epidermal Keratinocytes were incubated with materials listed above (10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx)) at 37°C for 24 hours. Levels of GM-CSF, a growth factor which we observed to be modulated by LeTx in our microarrays, were further analyzed by ELISA. Exposure of cells to IFNγ/MDP resulted in a synergistic induction of GM-CSF protein over control levels. Co-addition of LeTx with IFNγ/MDP to the cells attenuated this response, while exposure of cells to LeTx alone resulted in reductions in the baseline protein levels of GM-CSF. Data were analyzed using a one-way analysis of variance (ANOVA) and significant means (n=3) were tested using the Bonferroni multiple comparisons test (***P< .001 from IFNγ/MDP group, +++P< .001, +P< .05 from control). Experiments were performed twice with similar results.

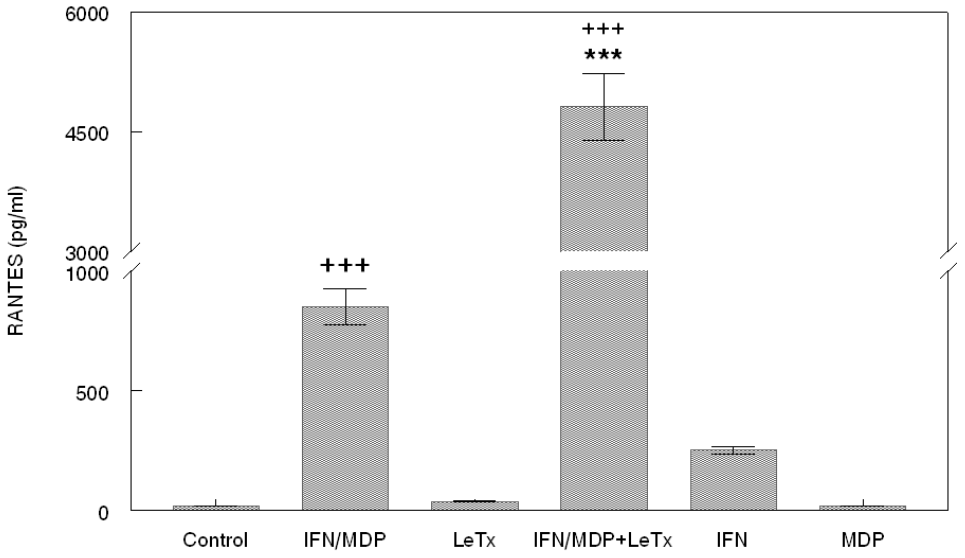


Figure 6e. ***IFNγ/MDP mediated secretion of RANTES from Normal Human Epidermal Keratinocytes is modulated by LeTx.*** Normal Human Epidermal Keratinocytes were incubated with materials listed above (10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx)) at 37°C for 24 hours. Levels of secreted RANTES, a chemokine which we observed to be modulated by LeTx in our microarrays, were further analyzed by ELISA. A synergistic induction of RANTES protein levels in cells exposed to IFNγ/MDP was augmented by the co-addition of LeTx to the incubation mixture. Data were analyzed using a one-way analysis of variance (ANOVA) and significant means (n=3) were tested using the Bonferroni multiple comparisons test (***P< .001 from IFNγ/MDP group, +++P< .001 from control). Experiments were performed twice with similar results.

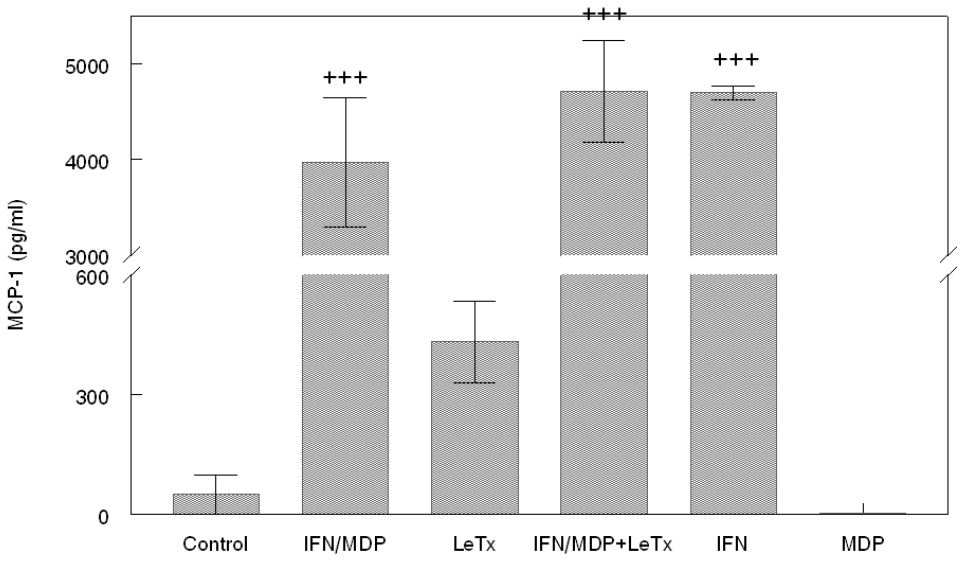


Figure 6f. ***IFNγ/MDP mediated secretion of MCP-1 from Normal Human Epidermal Keratinocytes is unaffected by LeTx.*** Normal Human Epidermal Keratinocytes were incubated with materials listed above (10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx)) at 37°C for 24 hours. Levels of released MCP-1, a chemokine which we observed to be modulated by LeTx in our microarrays, were further analyzed by ELISA. Induction of MCP-1 protein by exposure to IFNγ/MDP were attributable to the effects of IFNγ alone on the cells. Addition of LeTx did not affect IFNγ/MDP induction of MCP-1 in keratinocytes; however, when added alone, LeTx appeared to induce secretion of augmented levels of MCP-1 compared to those detected in control cell supernatants. Data were analyzed using a one-way analysis of variance (ANOVA) and significant means (n=3) were tested using the Bonferroni multiple comparisons test (+++P< .001 from control). Experiments were performed twice with similar results.

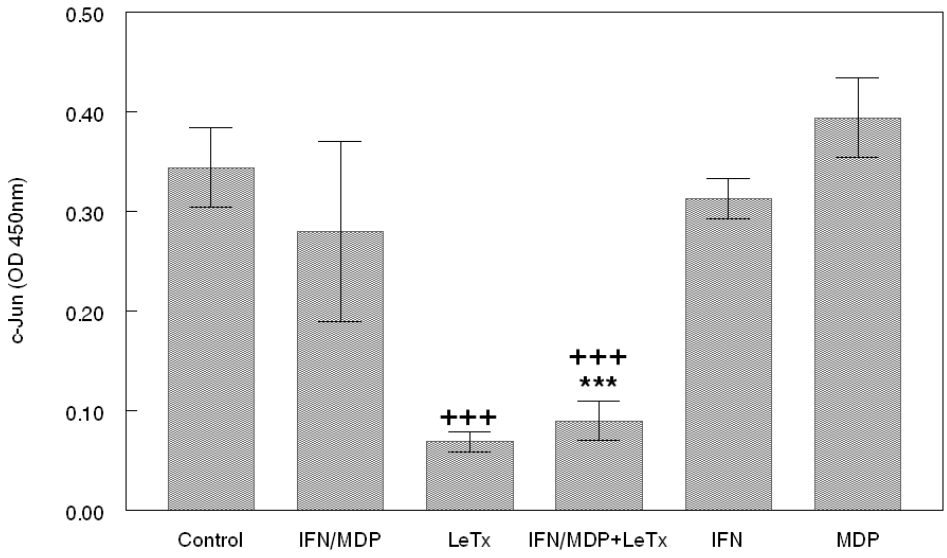


Figure 7a. ***Modulation of the MAPK transcription factor c-Jun in Normal Human Epidermal Keratinocytes exposed to IFNγ/MDP and/or LeTx.*** Normal Human Epidermal Keratinocytes were incubated with materials listed above (10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx)) at 37°C for 24 hours. Collected cells were lysed and cleared as described in Methods, and lysate supernatant was assayed for activation of the MAPK transcription factor c-Jun. LeTx decreased the amount of activated c-Jun when added alone or in combination with IFNγ/MDP, whereas IFNγ and MDP had no effect on c-Jun levels, either alone or in combination. Data were analyzed using a one-way analysis of variance (ANOVA) and significant means (n=3) were tested using the Bonferroni multiple comparisons test (***P< .001 from IFNγ/MDP group; +++P< .001 from control). Experiments were performed twice with similar results.

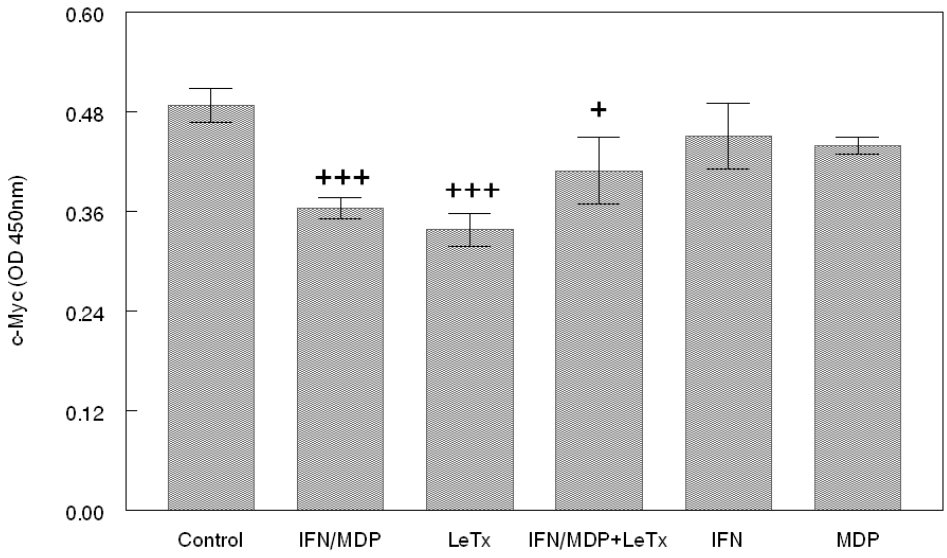


Figure 7b. ***Modulation of the MAPK transcription factor c-Myc in Normal Human Epidermal Keratinocytes exposed to IFNγ/MDP and/or LeTx.*** Normal Human Epidermal Keratinocytes were incubated with materials listed above (10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx)) at 37°C for 24 hours. Collected cells were lysed and cleared as described in the methods, and lysate supernatant was assayed for activation of the MAPK transcription factor c-Myc. Activated levels of c-Myc were mildly decreased by incubation with either LeTx or IFNγ/MDP. Data were analyzed using a one-way analysis of variance (ANOVA) and significant means (n=3) were tested using the Bonferroni multiple comparisons test (+P< .05, +++P< .001 from control). Experiments were performed twice with similar results.

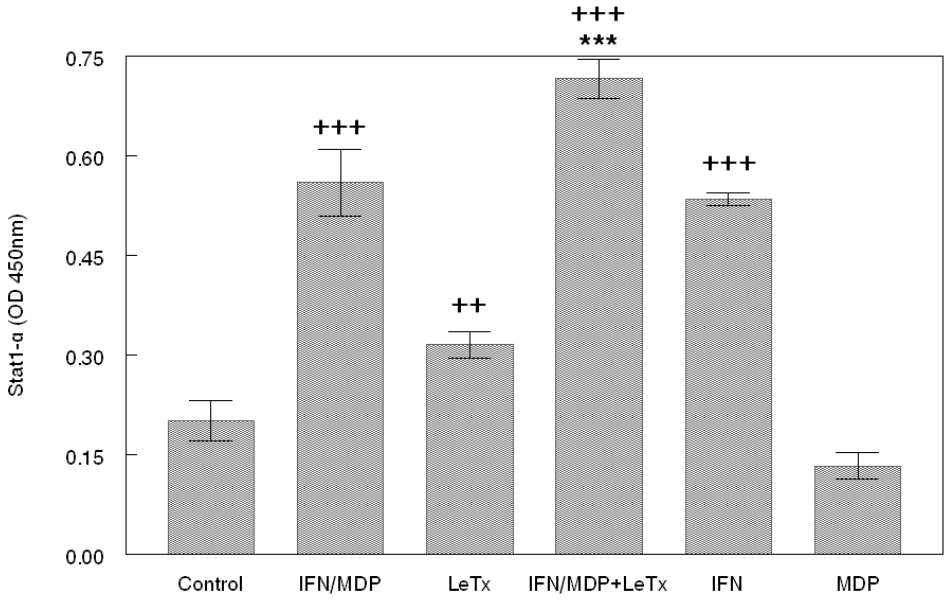


Figure 7c. ***Modulation of the MAPK transcription factor Stat1-α in Normal Human Epidermal Keratinocytes exposed to IFNγ/MDP and/or LeTx.*** Normal Human Epidermal Keratinocytes were incubated with materials listed above (10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx)) at 37°C for 24 hours. Collected cells were lysed and cleared as described in the methods, and lysate supernatant was assayed for activation of the MAPK transcription factor Stat1-α. Protein levels of activated Stat1-α were induced over 2 fold beyond control levels following incubation with IFNγ alone. This induction of Stat1-α was not affected by the combination treatment of IFNγ/MDP. LeTx displayed a slight but significant increase in Stat1-α which upon co-incubation with IFNγ/MDP yielded an additive increase. Data were analyzed using a one-way analysis of variance (ANOVA) and significant means (n=3) were tested using the Bonferroni multiple comparisons test (***P< .001 from IFNγ/MDP group; ++P< .01, +++P< .001 from control). Experiments were performed twice with similar results.

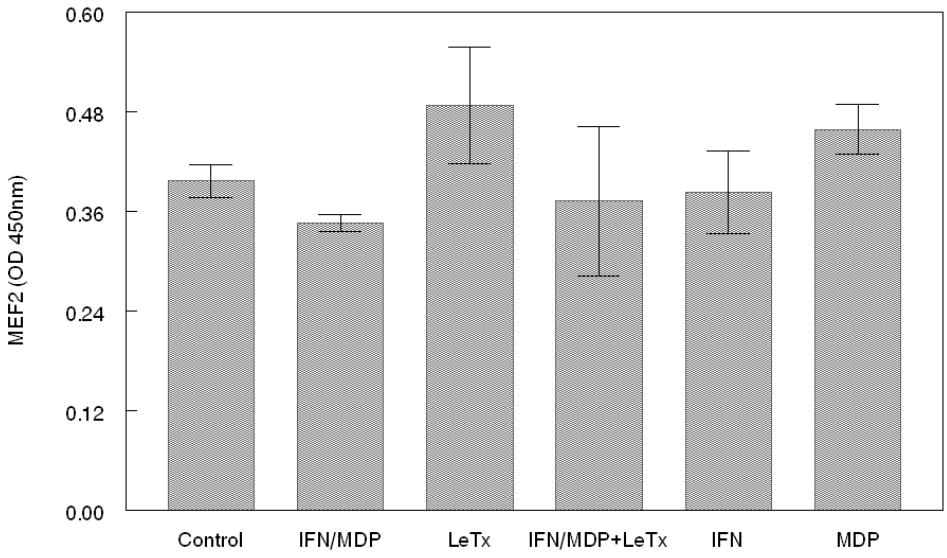


Figure 7d. ***Modulation of the MAPK transcription factor MEF2 in Normal Human Epidermal Keratinocytes exposed to IFNγ/MDP and/or LeTx.*** Normal Human Epidermal Keratinocytes were incubated with materials listed above (10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx)) at 37°C for 24 hours. Collected cells were lysed and cleared as described in the methods, and lysate supernatant was assayed for activation of the MAPK transcription factor MEF2. Transcription factor MEF2 was unaffected by all treatment groups in this assay while levels of levels of the transcription factor ATF-2 (data not shown) was not detected in our studies. Data were analyzed using a one-way analysis of variance (ANOVA) and significant means (n=3) were tested using the Bonferroni multiple comparisons test. Experiments were performed twice with similar results.

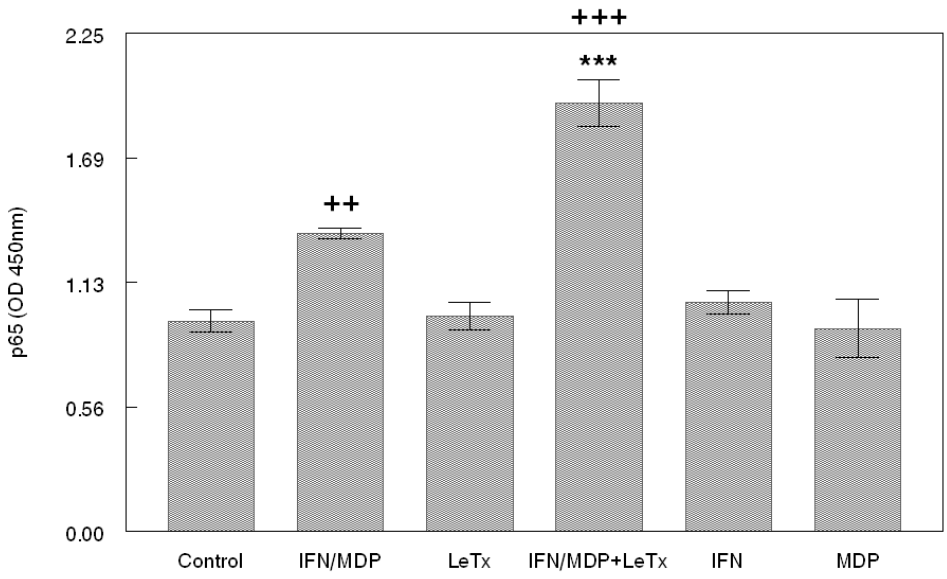


Figure 8a. ***Modulation of the NFκ-B transcription factor p65 in Normal Human Epidermal Keratinocytes exposed to IFNγ/MDP and/or LeTx.*** Normal Human Epidermal Keratinocytes were incubated with materials listed above (10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx)) at 37°C for 24 hours. Collected cells were lysed and cleared as described in the methods, and lysate supernatant was assayed for activation of the NFκ-B transcription factor p65. Induction of p65 by IFNγ/MDP was synergistically induced by the co-incubation with LeTx. Data were analyzed using a one-way analysis of variance (ANOVA) and significant means (n=3) were tested using the Bonferroni multiple comparisons test (***P< .001 from IFNγ/MDP group; ++P< .01, +++P< .001 from control). Experiments were performed twice with similar results.

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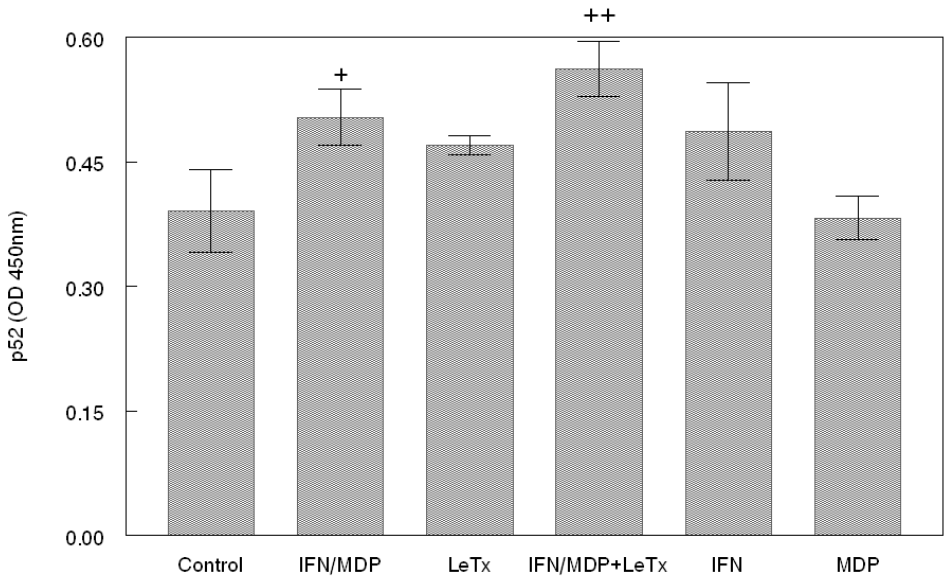

Figure 8b. ***Modulation of the NFκ-B transcription factor p52 in Normal Human Epidermal Keratinocytes exposed to IFNγ/MDP and/or LeTx.*** Normal Human Epidermal Keratinocytes were incubated with materials listed above (10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx)) at 37°C for 24 hours. Collected cells were lysed and cleared as described in the methods, and lysate supernatant was assayed for activation of the NFκ-B transcription factor p52. Levels of p52 were moderately increased by the addition of either LeTx or IFNγ/MDP. Data were analyzed using a one-way analysis of variance (ANOVA) and significant means (n=3) were tested using the Bonferroni multiple comparisons test (+P< .05. ++P< .01 from control). Experiments were performed twice with similar results.

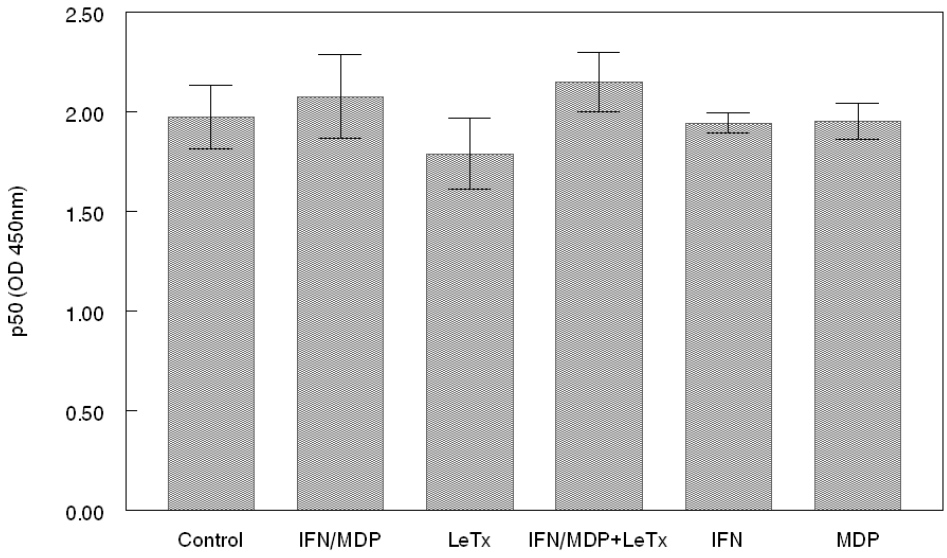

Figure 8c. ***Modulation of the NFκ-B transcription factor p50 in Normal Human Epidermal Keratinocytes exposed to IFNγ/MDP and/or LeTx.*** Normal Human Epidermal Keratinocytes were incubated with materials listed above (10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx)) at 37°C for 24 hours. Collected cells were lysed and cleared as described in the methods, and lysate supernatant was assayed for activation of the NFκ-B transcription factor p50, which was unaffected by our treatments. Data were analyzed using a one-way analysis of variance (ANOVA) and significant means (n=3) were tested using the Bonferroni multiple comparisons test. Experiments were performed twice with similar results.

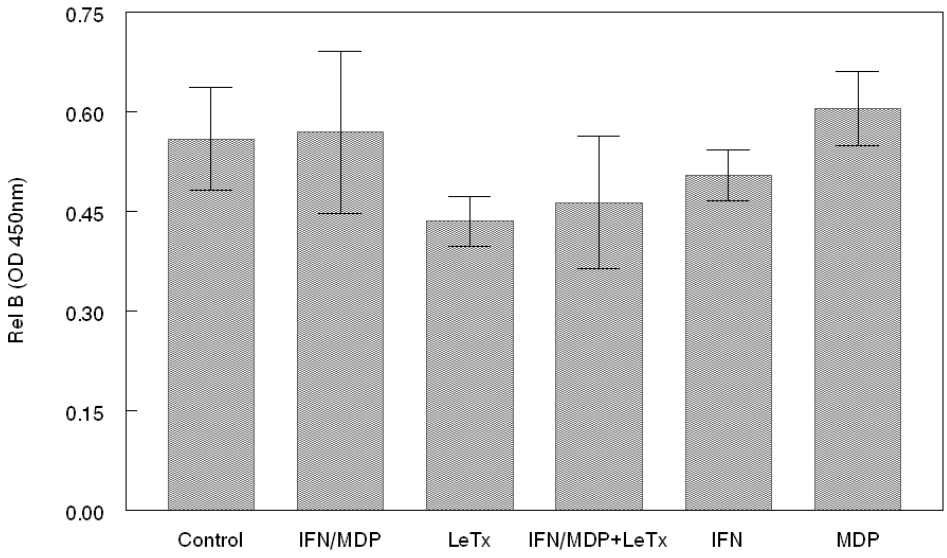

Figure 8d. ***Modulation of the NFκ-B transcription factor RelB in Normal Human Epidermal Keratinocytes exposed to IFNγ/MDP and/or LeTx.*** Normal Human Epidermal Keratinocytes were incubated with materials listed above (10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx)) at 37°C for 24 hours. Collected cells were lysed and cleared as described in the methods, and lysate supernatant was assayed for activation of the NFκ-B transcription factor RelB, which was unaffected by our treatments. Data were analyzed using a one-way analysis of variance (ANOVA) and significant means (n=3) were tested using the Bonferroni multiple comparisons test. Experiments were performed twice with similar results.

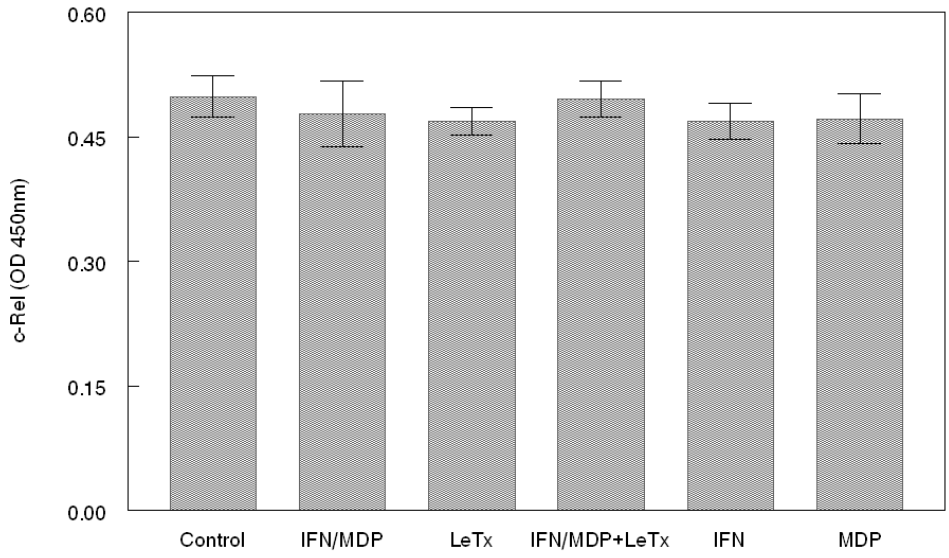

Figure 8e. ***Modulation of the NFκ-B transcription factor c-Rel in Normal Human Epidermal Keratinocytes exposed to IFNγ/MDP and/or LeTx.*** Normal Human Epidermal Keratinocytes were incubated with materials listed above (10 ng/ml IFNγ, 100 μg/ml MDP, 1 μg/ml LF and PA (LeTx)) at 37°C for 24 hours. Collected cells were lysed and cleared as described in the methods, and lysate supernatant was assayed for activation of the NFκ-B transcription factor c-Rel, which was unaffected by our treatments. Data were analyzed using a one-way analysis of variance (ANOVA) and significant means (n=3) were tested using the Bonferroni multiple comparisons test. Experiments were performed twice with similar results.

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