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Review

Anthrax lethal toxin: a weapon of multisystem destruction

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Abstract. Lethal toxin (LT) is a major virulence factor secreted by anthrax bacteria. It is composed of two proteins, PA (protective antigen) and LF (lethal factor). PA transports the LF inside the cell, where LF, a zinc-dependent metalloprotease cleaves the mitogen activated protein kinase kinase (MAPKK) enzymes of the mitogen activated protein kinase (MAPK) signaling pathway, thereby impairing their function. This disruption of the MAPK pathway, which serves essential functions such as proliferation, survival and inflammation in all cell types, results in multisystem dysfunction in the host. The inactivation of the MAPK pathway in both macrophages and dendritic cells leads to inhibition of proinflammatory cytokine secretion, downregulation of costimulatory molecules such as CD80 and CD86, and ineffective T cell priming. The net result is an impaired innate and adaptive immune response. Endothelial cells of the vascular system undergo apoptosis upon LT exposure, also likely due to inactivation of the MAPK pathway. The activity of various hormone receptors such as glucocorticoids, progesterone and estrogen is also blocked, due to inhibition of p38 MAPK phosphorylation, thus affecting the body's response to stress. The present review summarizes the various disarming effects of *Bacillus anthracis* through the use of a single weapon, the lethal toxin.

Key words. Anthrax lethal toxin; Dendritic cells; Macrophages; MAPkinase; innate immunity.

Introduction

Anthrax is caused by a Gram-positive spore-forming bacteria, *Bacillus anthracis* [1]. Primarily a disease of herbivores and cattle, it has gained heightened interest in the aftermath of the events following 9/11, as a potential agent of bioterrorism. The bacterial spores can gain entry into the body either through the skin, or by inhalation or ingestion [2, 3]. The organism has evolved an extraordinarily high degree of virulence, which kills the host rapidly within a few days of infection [2, 3]. The primary factors considered responsible for the severity of the disease are the poly-D-glutamic acid capsule of the bacillus and the toxins secreted by the bacteria [3, 4]. Antibiotics are effective in treatment against anthrax in the initial stages of the infection when the bacillus is multiplying, but once large amounts of the toxins are secreted in the bloodstream they lose their efficacy, and the victim succumbs to the lethal effects of the toxins. Systemic infection is thus usually fatal, since early symptoms are similar to those of flu, and difficult to diagnose. Recent advances are starting to reveal that anthrax toxins exert a plethora of effects on different host systems, including the immune system. In this review, we present an overview of these diverse effects, with a focus on how anthrax lethal toxin disarms the host's immune system by impairing the function of dendritic cells and macrophages.

Anthrax toxin

The production of anthrax toxin is mediated by plasmid pX01. Anthrax toxin is composed of three proteins: PA, the protective antigen; EF, edema factor; and LF, the lethal factor. EF, an adenylate cyclase, can combine with PA to form the edema toxin (ET) while LF, a zinc metallopro-

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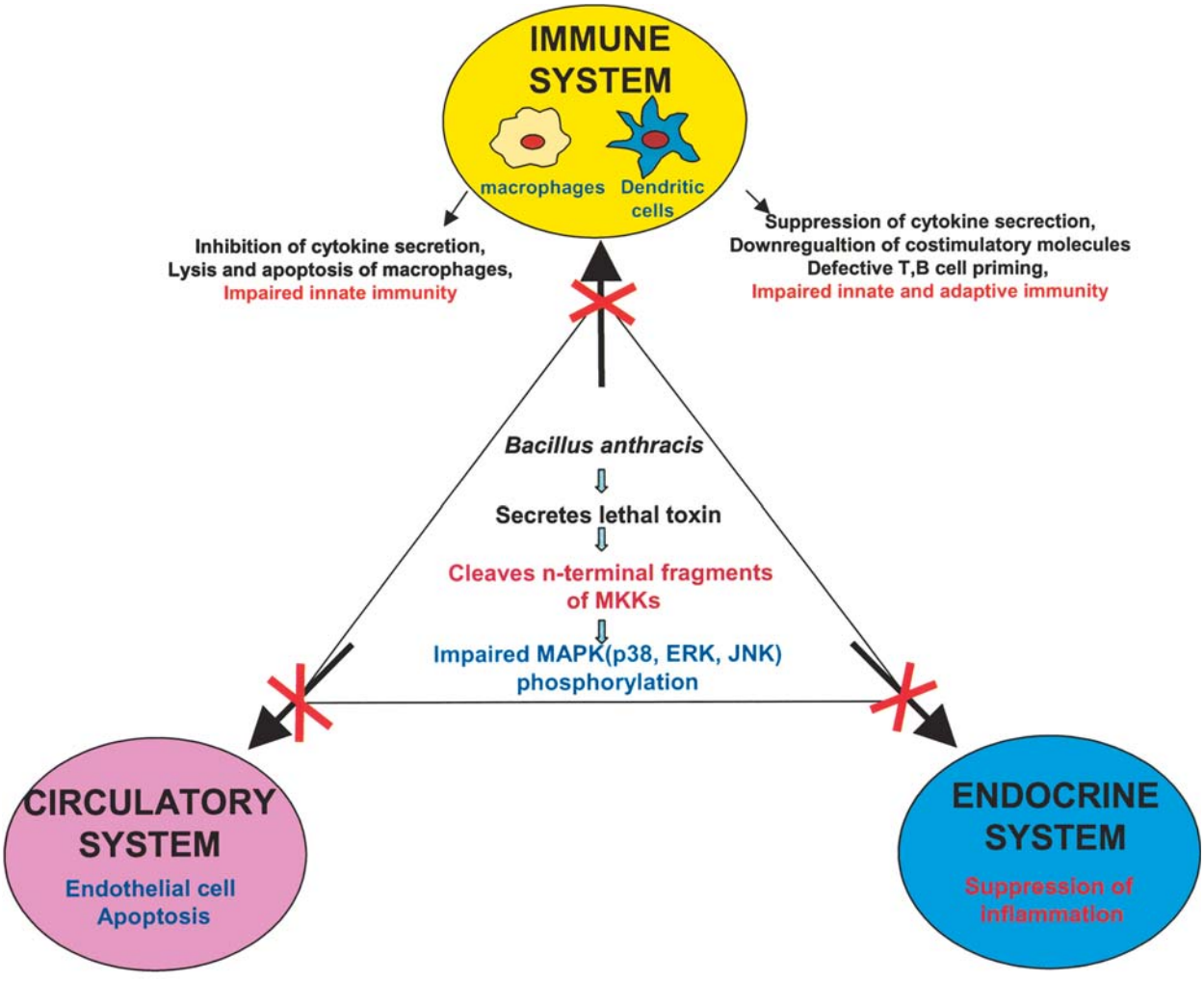
tease, and PA form the lethal toxin (LT) [5]. PA, which is the major component of the anthrax vaccine, transports the edema and lethal factors inside the cell. PA is produced as a protein of 85.8 kDa with 764 amino acids. Mature 83-kDa PA is formed by release of a 29-residue signal sequence [6]. This PA83 binds to recently discovered cell surface receptors where a 20-kDa portion is cleaved off by a host protease, furin, retaining 63-kDa PA on the surface. The PA63 heptamerizes to form a prepore to which EF and LF bind, and the complex is taken up by receptor-mediated endocytosis [6, 7]. The acidified endosomal compartment causes a conformational change, converting the prepore into a pore to translocate EF and LF in the cytosol, where they exert their toxic actions [6, 7].

The structure of the anthrax toxin receptor (ATR) has recently been elucidated. Two different but highly related proteins have thus far been identified as receptors for anthrax thus far toxin [8, 9]. Encoded by two different genes – TEM8, tumor endothelial marker gene, and CMG2, human capillary morphogenesis protein 2 – the two proteins share about 60% amino acid identity, require metal ion for PA binding and are upregulated on endothelium of both normal and tumor vasculature [8, 9]. Northern blot analysis indicates that both receptors are expressed in a wide array of human tissues, thus making it difficult to predict which is more relevant for pathogenesis. The identification of the ATR has been very helpful for design of toxin inhibitors or antitoxins, leading to new avenues in anthrax treatment [10].

Mode of action of anthrax LT

LF is a zinc-dependent metalloprotease which impairs the MAPK signaling pathway [5]. It cleaves MAPK-activating enzymes (MAPKKs 1–4, 6, 7) near their amino terminal [10–12]. The MAPKK cleavage prevents phosphorylation



Figre 1. The impairment of the MAPK pathway by anthrax lethal toxin causes multisystem damage to the host. LT cleaves the MAPKK enzymes of the MAPK signaling pathway inhibiting phosphorylation of downstream MAPK p38, ERK and JNK. The ubiquitous and essential nature of the MAPK pathway results in multisystem dysfunction in the host.

of downstream components (p38, ERK, JNK), leading to inhibition of the MAPkinase pathway (fig. 1). This activity of the LT results in widespread, multisystem damage, including cytotoxicity, although to date a direct causal relationship between inactivation of the MAPK pathway and cytotoxicity is not established. Figure 1 gives an overview of the effect of LT-impaired MAPK function on different systems of the body.

LT and the immune system

An understanding of the interaction of *B. anthracis* with the cells of the immune system is imperative to gain insights into the potential immune evasion mechanisms employed by the bacillus, and to define new therapeutic opportunities. LT appears critical for pathogenesis, since bacterial strains lacking the toxin gene are not found to be lethal in mice [14]. Yet until recently, there was very little information about how LT interacts with the cells of the immune system. The effect MAPK inactivation by LT has on different cells of immune system is summarized in table 1. It is also crucial to understand how the ET acts in tandem with the LT to further aid the destruction of the immune system. ET has been shown to elevate cyclic AMP (cAMP) levels in susceptible cells which are known to cause changes in membrane permeability, leading to edema. The increase in cellular AMP levels in neutrophils by ET inhibits their phagocytic activity [15, 16]. Furthermore, ET-induced accumulation of cAMP also blocks tumor necrosis factor α (TNF-α) production by monocytes, thus impairing the microbicidal activity of human monocytes [17].

Macrophages and dendritic cells (DCs) are antigen-presenting cells which serve important functions in the immune system. DCs, in particular, are endowed with the unique capacity of being able to initiate, tune and suppress the adaptive immune response [18–20]. They are scattered in an immature state throughout the body and at portals of entry of microbes. DCs can sense infections by the expression of pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs) [18–20]. Encounter with a pathogen leads to the migration of DCs to secondary lymphoid organs. During migration they undergo maturation, resulting in upregulation of costimulatory molecules and major histocompatibility complex (MHC) class II. The antigen is then presented to the T cells activating the adaptive arm of the immune system. In addition, DCs can modulate the nature of the immune response through differential cytokine secretion in response to activation of different TLRs [21, 22]. DCs are thus key players in tuning the immune response, and it is critical to understand the effect the anthrax bacillus has on these cells. The impairment of the MAPkinase pathway, which is downstream of most TLRs, by LT thus disarms the innate component of the immune system. The following sections will consider the effects of LT on macrophages and DCs.

Table 1. Effect of MAPK activation by LT on different cells of the immune system.

Cell type	Effect of LT		Mechanism	Ref.
	In vitro	In vivo		
1) Mouse macrophages	lysed within 4 h (depending	macrophage depletion leads to		3, 12
a) Peritoneal and circulating	on strain of mice); inhibition of proinflammatory cytokine secretion	resistance to anthrax lethality; circulating monocyte numbers decreased	MAPK inactivation	27–30
b) Fixed tissue	inhibition of proinflammatory cytokine secretion.	fixed tissue macrophage numbers not affected		30
2) Human peripheral blood monocytes	early apoptotic changes in cell membrane	not known		41
3) Mouse DCs	cell viability not affected; inhibition of cytokine secretion, downregulation of costimulatory molecules; impaired T cell priming	impaired T cell priming and antibody responses	MAPK Inactivation	35
4) Human monocyte derived DCs	cell viability not affected; inhibition of proinflammatory cytokine secretion; downregulation of costimulatory molecules and impaired T cell priming;			35
5) Human endothelial cells	become apoptotic and die within 2–3 days		MAPK inactivation	36
6) Liver cells, hepatoma cell line	represses the transcriptional activity of glucocorticoid receptor	represses the transcriptional activity of glucocorticoid receptor	MAPK impairment	37

Macrophages

The effect of LT on macrophages is an area of intense research, but much of the evidence obtained so far is somewhat conflicting in nature. Macrophages are important for anthrax pathogenesis at all stages of infection, from the time the spores enter the body to the death of the host. The spores entering through the inhalation route most likely initially interact with pulmonary macrophages, and DCs, which engulf them and ferry them to the mediastinal and peribronchial lymph nodes [2, 3], where they germinate to produce vegetative bacteria. Successful infection might require that the bacilli should escape phagocytosis from macrophages to multiply and disseminate in the host. However, two different studies proposed an opposing point of view. Dixon et al. show that *B. anthracis* escapes the phagolysome to multiply within the macrophage cytoplasm [23]. Toxins do not contribute to this, and bacilli are released by lysis of the cell [23]. Guidi-Rontani et al., however, claim that there is no multiplication of the bacilli within macrophages; however, there is an absolute requirement of the toxin for germination of spores within the macrophages [24]. Toxin-deficient bacilli strains do not survive in the macrophages. The use of different strains of bacillus and mice might account for the differences in observations. It is interesting that toxin proteins PA, LF and EF are all expressed at the spore stage, suggesting a potential role of the toxins in early bacilli survival which needs to be further clarified [25].

The effect LT has on different subsets of macrophages like those from spleen, blood or peritoneal cavity, and their consequences on anthrax pathogenesis, is also a subject of debate.

An important role for macrophages in the pathogenesis of *B. anthracis* was suggested by two major initial findings: (i) certain inbred mice, such as DBA/2J and C57BL/6, were found to be resistant to LT-induced lethality, which correlated with their macrophages also being resistant to LT-mediated lysis [26], and (ii) the observation by Hanna et al., that the depletion of macrophage with silica particles resulted in protection of mice from LT-induced death [27]. Initial pathological findings indicated that death in anthrax is due to cytokine-induced shock. This correlated well with the fact that sublytic doses of LT-activated macrophages secreted interleukin (IL)-1β and Tumor necrosis factor (TNF-α), and lysis of macrophages at high LT doses released of cytokines in the blood, causing death. Furthermore, treatment of mice with anti-IL-1 and anti-TNF sera was shown to provide protection against LT-induced death [27]. However, more recent studies indicate that LT suppressed cytokine production from macrophages [12, 13, 28–30]. TNF and inducible nitric oxide (iNOS) receptor knockout mice were also found to be susceptible to LT-mediated killing [31]. The discovery by Duesbury et al. that LT cleaves the MAPKKs to inactivate the MAPK pathway further supported the notion that LT-inhibited cytokine production from macrophages [11, 12]. Moayari et al. have done extensive analysis of cytokines and histopathology of various tissues, after LT injection into mice, and this clearly demonstrates that death is not due to proinflammatory cytokine shock. There were signs of major liver necrosis, pleural edema and hepatic dysfunction [30]. Hypoxia led to liver failure, causing death, and susceptibility of macrophages to lysis by toxin did not have any role in this LT-mediated killing of the host. Though the effect of LT on macrophages is not responsible for host death, it still is necessary to understand the effect it has on macrophage function and its consequences on the immune response to anthrax infection.

Recent studies have thrown further light on MAPK inactivation and macrophage lysis. Activation with TNF-α or lipopolysaccharide (LPS) appears to render even the macrophages from LT-resistant mouse strains, sensitive to LT-mediated cell death [32–34]. Park et al. showed that LT causes apoptosis of LPS-treated macrophages [33]. LT inactivates ERK, p38 and JNK components of the MAPK pathway, but inhibition of only p38 induced apoptosis in LPS activated macrophages. Macrophages lacking I kappa B kinase (IKK) and Nuclear factor kB (NF-kB) activity were also shown to be sensitive to LPS-induced apoptosis [33]. The activation of macrophages through TLRs results in induction of the antiapoptotic pathway through synergistic action of MAPK and NF-kB. The disruption of either one of the pathways by LT thus renders the macrophage apoptotic. Recently, protein kinase PKR, which is downstream of TLR-4 activation, has been found to be responsible for the apoptosis of macrophages. Heat-killed *B. anthracis* was found to go through TLR-4- and LT-mediated impairment of MAPK. A macrophage survival pathway thus kills the macrophages through PKR, enabling the bacillus to evade the innate immune response [34].

Despite intense research, the role of macrophages in anthrax pathogenesis is still unclear. One problem may lie with the fact that most studies are done in vitro using macrophage cell lines, and in vivo studies with real infection are few and far between. Clearly, further work that address the precise roles played by distinct subsets of macrophages, in different tissues and microenvironments, at different stages of the disease is warranted.

Dendritic cells

Recent work from our lab has determined the effect of LT on mouse and human DCs [35]. Our work suggests that LT impairs the MAPK pathway in DCs in vitro suppressing TLR-mediated induction of proinflammatory cytokines and costimulatory molecules. As a consequence, DCs exposed to LT were found to be poorly stimulatory

to T cells, in vitro. Antigen-specific priming of T-helper cells was determined in vivo using an ovalbumin (OVA)-specific T-cell receptor (TCR) transgenic system. DCs exposed to LT and pulsed with OVA when injected into mice were unable to prime OVA-specific transgenic T cells. Although the T cells showed initial signs of activation through upregulation of activation markers, they failed to differentiate into memory T cells, probably due to the absence of the second signal due to downregulation of costimulatory molecules on DCs. The effect of LT on antigen-specific B cell priming was even more profound. Injection of OVA adsorbed on alum after LT exposure resulted in a significant decrease in the OVA-specific antibody titer at day 14 post injection. The acquired immunity was thus also found to be compromised in the mice exposed to LT. It is well known that the MAPkinase pathway is activated on engagement of the TLRs by the microbes. The activation of different TLRs by different pathogens governs the nature of the adaptive immune response. The impairment of the MAPK signaling cascade by LT thus appears to impair both the innate and adaptive immune response by paralyzing DCs. To what extent such a mechanism might contribute to the pathogenesis of *B. anthracis* deserves further evaluation in an animal model of the disease.

LT and the circulatory system: effect of LT on endothelial cells

The damaging effect of LT is not restricted to the immune system but is also evident in the vascular system, in which it causes apoptosis of the endothelial cells lining the interior of the blood vessels [36]. Primary endothelial cell monolayers derived from both large and small blood vessels were found to be positive for annexin V, a marker for apoptosis, after 24-h exposure to LT [36]. Addition of Z-VAD-FMK, a broad-spectrum caspase inhibitor, prevented apoptosis and promoted cell survival. Even though LT-induced cleavage of MKKs and suppression of phosphorylation of all three downstream MAPkinases (p38, ERK and JNK) was observed, the apoptotic changes in the endothelial cells were attributed mainly to the inhibition of ERK kinase, since the effect could be mimicked using an ERK inhibitor [36]. JNK and p38 inhibition, on the other hand, were shown to promote survival of endothelial cells. Interestingly, ERK inhibitor when added together with p38 and JNK inhibitors still exerted considerable cytotoxicity towards these cells [36].

In agreement with these observations, clinical observations of infected human subjects have shown signs of vascular damage, e.g. tissue hemorrhages, gastrointestinal bleeding and so on. Autopsy findings also show destruction of both large and small blood vessels [3]. However, no report of vascular injury is reported with the injection of LT alone in vivo in experimental models [30]. This could simply be due to a lack of other virulent cofactors such as the capsule. Further studies are required to confirm whether indeed LT damages endothelial cells in vivo and its consequences on anthrax pathogenesis.

LT and the endocrine system: effects on the glucocorticoid receptor

Glucocorticoid receptor is a member of the nuclear receptor family of hormone-activated transcription factors that plays a central role in defending the body against many forms of stress, including inflammation. Webster et al. showed that LT represses the transcriptional activity of the glucocorticoid receptor by inhibiting p38 MAPkinase phosphorylation [37]. Activity of several other nuclear hormone receptors such as progesterone and estrogen, was also shown to be blocked [37]. The potential effect of this suppression on anthrax pathogenicity is still unclear, but one can speculate that since glucocorticoids are one of the most pervasive hormones in the human organism, the effects of this suppression would be manifold, affecting nearly all systems of the body. These steroid molecules, which reach all tissues, including the brain, readily penetrate the cell membrane and interact with ubiquitous cytoplasmic/nuclear glucocorticoid receptors, through which they exert markedly diverse actions. One of the major functions of glucocorticoids is to mobilize and shape the immune response during any kind of stress, be it infectious, physical or psychological [38]. Through their inhibitory actions on the NF-kB signaling pathway, glucorticoids suppress the inflammation caused by the stress, returning it back to baseline levels [38, 39]. Indeed, in the absence of glucorticoids the mice show clear signs of inflammatory disorders [40]. Inhibition of the glucorticoid receptor is thus of major advantage to the anthrax bacillus.

Conclusion

It is likely that disruption of the MAPK pathway by LT allows the bacteria to highjack the body's major defense systems. However, we are still unaware of the effects of LT on other signaling pathways. Indeed, certain LT related effects such as hypoxia and vascular damage, may not be explained solely by MAPK inactivation. To fully understand the nature of LT action in conjunction with other bacterial components, especially the capsule and ET, further in vivo studies with live strains of *B. anthracis* are required.

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