Anthrax lethal toxin-induced inflammasome formation and caspase-1 activation are late events dependent on ion fluxes and the proteasome

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Abstract

SUMMARY—Anthrax lethal toxin (LT) is cytotoxic to macrophages from certain inbred mouse strains. The gene controlling macrophage susceptibility to LT is Nalp1b. Nalp1b forms part of the inflammasome, a multi-protein complex involved in caspase-1 activation and release of interleukin (IL)-1β and IL-18. We confirm the role of caspase-1 in LT-mediated death by showing that caspase inhibitors differentially protected cells against LT, with the degree of protection corresponding to each compound's ability to inhibit caspase-1. Caspase-1 activation and cytokine processing and release were late events inhibited by elevated levels of KCl and sucrose, by potassium channel blockers, and by proteasome inhibitors, suggesting that inflammasome formation requires a protein degradation event and occurs downstream of LT-mediated potassium efflux. In addition, IL-18 and IL-1β release was dependent on cell death, indicating that caspase-1-mediated cytotoxicity is independent of these cytokines. Finally, inducing Nalp3-inflammasome formation in LT-resistant macrophages did not sensitize cells to LT, suggesting that general caspase-1 activation cannot account for sensitivity to LT and that a Nalp1b-mediated event is specifically required for death. Our data indicate that inflammasome formation is a contributing, but not initiating, event in LT-mediated cytotoxicity and that earlier LT-mediated events leading to ion fluxes are required for death.

INTRODUCTION

Bacillus anthracis pathogenicity is principally mediated through the production of three polypeptides that combine to form two bipartite toxins, edema toxin (ET) and lethal toxin (LT). Protective antigen (PA), the receptor-binding component common to both toxins, combines with the enzymatic components edema factor (EF) or lethal factor (LF) to form ET or LT, respectively (Leppla, 2006). EF is a calmodulin-dependent adenylate cyclase (Leppla, 1982), and LF is a zinc metalloprotease that cleaves members of the mitogen-activated protein kinase family (MEKs) (Duesbery et al., 1998; Pellizzari et al., 1999; Vitale et al., 2000). All tested eukaryotic cells possess PA receptors and effectively internalize EF and LF (Leppla, 2006). Murine macrophages from certain inbred strains undergo a unique, rapid death induced by LT (< 90 min), while other strains harbor LT-resistant macrophages (Friedlander et al., 1993; Singh et al., 1989). Although the pathway of toxin entry into cells is well characterized, the mechanisms of LT-induced macrophage killing remain unresolved. Cleavage of the MEK
proteins is not sufficient to cause macrophage death, as these proteins are also cleaved by LT in resistant macrophages (Kim et al., 2002; Pellizzari et al., 1999).

The genetic polymorphism responsible for differences in macrophage sensitivity to LT was previously mapped to a single mouse chromosome 11 locus (Ltxs1) (Roberts et al., 1998; Watters and Dietrich, 2001), subsequently identified as Nalp1b (Boyden and Dietrich, 2006). Nalp family proteins are scaffold proteins that associate with the adaptor protein ASC and caspase-1 to form a multiprotein signaling complex known as the inflammasome (Mariathasan et al., 2004; Martinon et al., 2002; Tschopp et al., 2003). The inflammasome responds to a variety of stimuli including bacterial RNA (Kanneganti et al., 2006), cytoplasmic flagellin (Franchi et al., 2006), gout-associated uric acid crystals (Martinon et al., 2006), bacterial pathogens (Mariathasan et al., 2006), and ion fluxes (Perregaux and Gabel, 1994; Walev et al., 1995). A number of Nalp proteins have been reported to form inflammasomes, and the Nalp3 inflammasome is the most well characterized activator of caspase-1, forming in response to most of the stimuli listed above (Martinon and Tschopp, 2006). Following Nalp activation and inflammasome formation, the 45-kDa precursor of caspase-1 (p45) is cleaved through a proximity-induced, autocatalytic mechanism to produce 20-kDa (p20) and 10-kDa (p10) fragments, which combine to form the tetrameric active enzyme (Thornberry et al., 1992; Yamin et al., 1996). Caspase-1 then cleaves precursor forms of proinflammatory IL-1β and IL-18, and the mature cytokines are released extracellularly (Black et al., 1988; Fantuzzi and Dinarello, 1999).

Following LT treatment, caspase-1 is activated only in sensitive macrophages (Boyden and Dietrich, 2006; Cordoba-Rodriguez et al., 2004). Caspase-1 deficient macrophages harboring the Nalp1b sensitivity allele were also shown to be resistant to LT (Boyden and Dietrich, 2006). These results show that LT susceptibility requires both the sensitive Nalp1b allele and caspase-1, but it is currently unknown how either of these proteins participates in LT-induced killing.

In this study, we establish that LT-induced inflammasome formation is a relatively late event, beginning at 50-60 min, when compared to the early (20-40 min) cleavage of the MEK proteins, indicating that toxin delivery to these cytoplasmic substrates precedes caspase-1 activation. Furthermore, our data demonstrate that macrophage death is not dependent on IL-1β or IL-18 processing or release. We show that inflammasome formation in macrophages is dependent on the proteasome, on LT-induced ion fluxes (Hanna et al., 1992), and on the release of intracellular potassium. Therefore, caspase-1 activation appears to be a downstream consequence of earlier LT-mediated cellular events that result in the breakdown of intracellular proteins and changes in plasma membrane permeability. Finally, we show that general caspase-1 activation is necessary but not sufficient to induce macrophage sensitivity in resistant cells, suggesting that other Nalp1b-specific events are required for cell death.

RESULTS

Caspase inhibitors show varying degrees of protection against LT

Genetic evidence suggests that caspase-1 is required for LT-induced macrophage death (Boyden and Dietrich, 2006). We tested a panel of irreversible, cell-permeable peptide-based caspase inhibitors Livingston, 1997; Thornberry and Molineaux, 1995) for protection against LT-mediated macrophage cytotoxicity. LT-sensitive RAW264.7 cells and BALB/cJ BMDM were treated with various concentrations of the pan-caspase inhibitor Z-VAD-FMK and the caspase-1-specific inhibitors Ac-YVAD-CMK and Z-WEHD-FMK for 1 h prior to LT addition. The inhibitors showed different degrees of protection against LT in a dose-dependent manner (Fig. 1A). The protective ability of the inhibitors was also evident when the compounds were added to cells 40 min after LT addition (Fig. 1A, lower panel). At the range of
To explain the differential ability of the inhibitors to protect against LT, we compared the inhibition of LT-induced caspase-1 autocatalytic activation and IL-1β maturation after treatment with each inhibitor. BALB/cJ BMDM and RAW264.7 cells were treated with LPS to induce the synthesis of pro-IL-1β (as baseline levels of this cytokine in these cells are not detectable, even by Western blot), followed by 60-min pretreatment with the inhibitors. In LPS-primed cells treated with LT, IL-1β maturation was strongly inhibited by Z-VAD-FMK, while Ac-YVAD-CMK and Z-WEHD-FMK showed intermediate levels of inhibition (Fig. 1B). Similar effects were seen in both cell types. These inhibitors covalently react at the active site of the p10/p20 tetrameric form of active caspase-1 (Rano et al., 1997; Scheer et al., 2006; Walker et al., 1994; Wilson et al., 1994) and have no effect on caspase-1 autocatalytic activation as detected by p10 subunit formation (Fig. 1B). Instead, our results demonstrate that the inhibitors block caspase-1 catalytic activity, as shown by the different levels of mature IL-1β produced in the presence of each inhibitor (Fig. 1B). The level of protection afforded by the caspase inhibitors against LT-induced death was directly related to their ability to inhibit caspase-1, with the highest protection against LT corresponding to the almost complete inhibition of caspase-1 activity seen with Z-VAD-FMK. However, since the two caspase-1 specific inhibitors do not strongly protect against LT-mediated cell death, while the pan-caspase inhibitor is most effective, it is possible that the inhibition of other cellular proteases by Z-VAD-FMK also contributes to protection.

Timing of LT-induced inflammasome formation in sensitive macrophages

We next examined the timing of caspase-1 activation and IL-1β and IL-18 maturation and release to better understand their potential role in LT-induced cell death. BALB/cJ BMDM, 129S1 peritoneal macrophages, and RAW264.7 cells were treated with LT for various lengths of time, and the appearance of activated caspase-1 (p10 subunit), mature IL-1β, and mature IL-18 was monitored in cell lysates by Western blotting using antibodies that recognize both the proform and active forms of the proteins. Culture supernatants of cells treated with toxin were analyzed for IL-1β and IL-18 release by both ELISA and Western blot. Caspase-1 activation and subsequent IL-18 maturation were first observed in BMDM cell lysates approximately 50-60 min after LT addition (Fig. 2A). We were unable to detect caspase-1 p10 or mature IL-18 in RAW264.7 cells treated with LT by Western blotting due to low levels of the proteins (data not shown). Therefore, the presence of intracellular mature IL-1β was used as a readout for inflammasome formation. RAW264.7 cells contained no measurable pro-IL-1β (data not shown), but LPS-priming allowed detection of this cytokine. Similar to BMDM, intracellular mature IL-1β was first seen after 50-60 min of LT treatment in LPS-primed RAW264.7 cells (Fig. S1A). This late timing is in contrast to MEK cleavage, which can be observed as early as 20 min after LT addition (shown after 30 min in Fig. 2A and S1A).

We next examined the release of IL-18 and IL-1β from cells treated with LT. In cell supernatants of BMDM treated with LT, IL-18 was detected by ELISA in a toxin treatment timeand concentration-dependent manner (Fig. 2B). Western blot analysis of the same supernatant samples showed that both mature and pro-IL-18 were present in the supernatant of cells treated with LT (Fig. 2B). This cytokine release, however, was only seen at later time points and was directly correlated to the loss of cell viability as assessed by MTT staining. No IL-1β was detected in the supernatant of LT-treated cells (data not shown). LPS priming and the resultant upregulation of pro-IL-1β prior to LT treatment allowed for the detection of the cytokine's release from cells in a pattern identical to that seen with IL-18, with higher levels.
of IL-1β associated with increasing cell death (Fig. 2C). Control cells treated with LPS alone for similar times showed no IL-1β release or mortality (data not shown). Cytokine release from LT-treated RAW264.7 cells followed the same pattern as seen in BMDM (Fig. S1).

Importantly, the release of IL-18 from unprimed RAW264.7 cells (Fig. 1SB) indicates that LPS treatment did not alter LT-mediated caspase-1 activation. Finally, thioglycolate-activated BALB/cJ peritoneal macrophages indicated the presence of mature IL-1β in cell lysates after a 60 min LT treatment and in the supernatant (by ELISA) after 75 min in a manner directly correlated to cell viability (data not shown).

Our data show that LT-induced caspase-1 activation is a late event (50-75 min), relative to MEK cleavage (20-40 min) and occurs immediately prior to cell death (85-90 min). Likewise, IL-1β and IL-18 release is a very late event occurring only in dying cells and likely occurs passively via cell lysis. The conclusion that late cytokine release is not required for cell death was further supported by the observation that IL-1β neutralizing antibodies do not protect BALB/cJ macrophages against LT (data not shown).

LT-induced inflammasome formation is dependent on ion fluxes

Because no pathway has been proposed that connects the LF-mediated cleavage of MEKs to inflammasome formation or caspase-1 activation, we attempted to place these events in the context of other early LT-induced events previously observed in macrophages. LT has been shown to increase membrane permeability and induce ion fluxes in sensitive macrophages beginning at 45 min (Hanna et al., 1992). Ion fluxes and potassium efflux in particular are the most extensively documented signals reported to induce inflammasome formation. Thus, agents such as extracellular ATP (Ferrari et al., 1997; Kahlenberg and Dubyak, 2004; Laliberte et al., 1999; Perregaux and Gabel, 1994) or the K+/H+ ionophore nigericin (Cheneval et al., 1998; Kahlenberg and Dubyak, 2004; Perregaux et al., 1992; Perregaux and Gabel, 1994) have been classically employed to stimulate caspase-1 activation and IL-1β maturation and release via the Nalp3 inflammasome (Pettrilli et al., 2007). Additionally, ion fluxes induced by classic pore-forming toxins such as Staphylococcus α-toxin (Walev et al., 1995), Aeromonas aerolysin (Gurcel et al., 2006), Listeria listeriolysin O (Mariathasan et al., 2006; Ozoren et al., 2006), and maitotoxin (Mariathasan et al., 2006; Verhoef et al., 2004), as well as antimicrobial peptides (Perregaux et al., 2002) have also been implicated in inflammasome activation.

Given the kinetic evidence presented above, we asked whether LT-induced ion fluxes occurred upstream of inflammasome formation in macrophages. BALB/cJ BMDM and LPS-primed RAW264.7 cells were treated with LT in the presence of 130 mM KCl or 300 mM sucrose in the extracellular medium. Elevated levels of KCl prevent potassium efflux while elevated sucrose prevents the influx of water into cells and inhibits generalized ion fluxes. LPS-primed BMDM were treated with nigericin as a positive control for Nalp3 inflammasome formation. Inhibition of ion fluxes by elevated levels of KCl has been shown to prevent nigericin-induced inflammasome formation (Pettrilli et al., 2007), and we confirm this result (Fig. 3A). Treatment of macrophages with LT in the presence of elevated KCl and sucrose prevented LT-mediated lysis (Fig. 3B) and, furthermore, inhibited LT-induced caspase-1 activation and IL-18 maturation in BMDM (Fig. 3A) as well as IL-1β maturation in LPS-primed cells RAW264.7 cells (Fig. S2A). Treatment of the cells with LT in the presence of the potassium channel blockers quinidine and TEA similarly inhibited LT-mediated caspase-1 activation and associated cytokine processing (Fig. 3C and S2B), but neither compound protected cells against LT-induced death at the highest tested concentration (data not shown). At the tested concentrations, sucrose, KCl, TEA, and quinidine had no effect alone on cell viability (data not shown) or LT-induced MEK1 cleavage, indicating no effects on toxin uptake or delivery to the cytosol (Fig. 3A and C and Fig. S2). These results demonstrate the explicit requirement
of ion fluxes, specifically potassium efflux, for LT-induced inflammasome formation and suggest that an early LT-mediated event leading to ion fluxes initiates late caspase-1 activation.

**LT-induced inflammasome formation is dependent on the proteasome**

Proteasome inhibitors protect against LT-induced death (Tang and Leppla, 1999), showing that the degradation of intracellular protein(s) by the proteasome is required for cell death. Caspase-1 activation induced by LPS and ATP treatment was reported to be dependent on the proteasome (Sadoul et al., 1996), and a protein degradation event has been reported to be involved in caspase-1 activation induced by the bacterial pathogens *Shigella flexneri* (Hilbi et al., 2000) and *Chlamydia trachomatis* (Lu et al., 2000). We tested whether LT-induced inflammasome formation similarly requires the proteasome and found that LT-induced caspase-1 activation in BMDMs was prevented in the presence of the proteasome inhibitor lactacystin (Fig. 4A). In contrast to LT-induced caspase-1 activation, the NALP3-inflammasome induced by LPS and nigericin treatment (Mariathasan et al., 2006; Petrilli et al., 2007) was unaffected by lactacystin (Fig. 4B). Therefore distinctly different events are required for the formation of these inflammasomes and proteasome inhibition can distinguish the LT-mediated inflammasome from the classic Nalp3-inflammasome.

**Caspase-1 activation alone does not sensitize LT-resistant macrophages**

LT-resistant macrophages harboring the *Nalp1b* resistance allele (such as those from DBA/2J and C57BL/6J mice) do not activate caspase-1 or release IL-1β in response to LT, but do possess other functional Nalp proteins capable of forming caspase-1-activating inflammasomes in response to various stimuli (Mariathasan et al., 2006). Therefore, we tested whether general caspase-1 activation alone through the Nalp3-inflammasome would sensitize BMDM derived from these inbred strains to LT. As evidenced by the presence of caspase-1 p10 and mature IL-1β, LPS-nigericin treatment successfully induced inflammasome formation in these cells (Fig. 5A), but did not sensitize them to killing by LT (Fig. 5B). While not disputing the requirement of caspase-1 activity for cell death, our results show that caspase-1 activation alone cannot account for LT-sensitivity.

**DISCUSSION**

Recent studies have shown that Nalp1b, a member of the Nalp family of proteins involved in inflammasome assembly and caspase-1 activation, controls macrophage sensitivity to LT. Macrophages from caspase-1 knockout mice were not susceptible to LT, even when carrying the sensitivity-conferring *Nalp1b* gene, showing that caspase-1 is required for LT-mediated cell death (Boyden and Dietrich, 2006). Previous studies investigating the potential role of caspases in macrophage death were confined to the use of caspase inhibitors, with such studies reporting either no protection from LT (Kassam et al., 2005; Tang and Leppla, 1999) or a reduction in LT toxicity (Kirby, 2004; Popov et al., 2002). Our studies with caspase inhibitors indicated that only the pan-caspase inhibitor Z-VAD-FMK was able to protect against LT, despite equivalent LT-mediated autocatalytic activation of caspase-1 in the presence of each tested inhibitor. The differences in inhibitor effects is likely due to differences in the efficiency of their inhibition of activated caspase-1, as reflected by the generation of different levels of mature IL-1β. The inhibitors’ ability to prevent IL-1β maturation in response to a classic inflammasome-inducing treatment (LPS/nigericin) was similar to that observed with LT treatment (data not shown). Since we also show that LT-mediated caspase-1 activation is a very late event which requires proteasome activity and ion fluxes induced by toxin, it is possible that the more effective inhibitors are actually inhibiting a number of cellular proteases, some of which may be involved in earlier steps of the toxic process.
Compared to the cleavage of MEK proteins (beginning at 20 min), caspase-1 activation is a late LT-induced event (50-60 min), dependent on potassium efflux and proteasome activity. Following LT-induced caspase-1 activation, IL-1β and IL-18 are processed intracellularly. However, we found the release of these cytokines to be a passive late event, resulting from cell death and lysis. Moreover, the concurrent presence of equal levels of both the pro- and mature forms of these cytokines in the supernatant confirms that they are being released from dying cells and not from intact cells. Thus, the release of these cytokines is not required for macrophage death.

Caspase-1 activation alone is insufficient for LT-mediated macrophage death because nigericin-induced Nalp3 inflammasome formation did not sensitize resistant macrophages to LT. Although potassium efflux is the common trigger for formation of the LT- and nigericin-induced inflammasomes (Petrilli et al., 2007), the LT-induced inflammasome is dependent on proteasome activity while the nigericin-induced Nalp3-inflammasome is not. Moreover, while both inflammasomes similarly activate caspase-1, the downstream effects of this activation may depend on the stimulus and the particular Nalp protein present in the inflammasome, with only the LT-induced inflammasome leading to cell death. Therefore, while the inability of LPS/nigericin to sensitize cells to LT confirms that caspase-1 activation alone is not sufficient for sensitization to LT, it is clear that LT-mediated death requires caspase-1 activation in conjunction with Nalp1b-mediated events.

The role of Nalp1b in LT-mediated macrophage killing is unclear. The actual protein components of the LT-induced inflammasome have yet to be identified, and while it has been reported that LT triggers formation of a Nalp1b inflammasome, no reports have explicitly shown that the Nalp1b inflammasome to be the sole LT-induced inflammasome. The lack of caspase-1 activation in macrophages harboring resistant Nalp1b alleles (Boyden and Dietrich, 2006) is used as evidence that LT specifically activates a Nalp1b-specific inflammasome in LT-sensitive cells. The absence of caspase-1 activation in resistant macrophages, however, could possibly be attributed to the parallel absence of ion fluxes as the necessary signaling event for inflammasome formation. Therefore, although Nalp1b may indeed be a required component of the LT inflammasome, additional Nalp proteins may also be activated in response to LT-induced ion fluxes. Furthermore, Nalp1b could play a role in LT-mediated cytotoxicity events upstream of LT-induced ion fluxes since expressing the sensitive Nalp1b allele in resistant macrophages is sufficient to sensitize cells to LT-mediated killing (Boyden and Dietrich, 2006). The crucial LT-induced early events which lead to the ion fluxes and subsequent inflammasome formation remain unknown and may include the degradation of protein(s) by the proteasome, the cleavage of yet unidentified LF substrates or downstream effects of MEK cleavage which differ between resistant and sensitive macrophages. In this model, inflammasome formation and caspase-1 activation function secondarily in LT-mediated killing as essential required sequelae of the early events that induce potassium release (Fig. 6).

Following caspase-1 activation by Nalp1b and/or other Nalp family proteins, the mechanism of the caspase-1-dependent cell death induced by LT is unknown. Unlike other proapoptotic caspases, caspase-1 is primarily associated with inflammation and rarely linked to apoptosis. Nevertheless, caspase-1 has been previously implicated in some cell death studies. Overexpression of caspase-1 in fibroblasts has been shown to induce apoptosis (Miura et al., 1993). Other bacterial pathogens, including Salmonella (Brennan and Cookson, 2000; Hersh et al., 1999; Monack et al., 2001; van der Velden et al., 2003), S. flexneri (Chen et al., 1996; Edgeworth et al., 2002; Hilbi et al., 1998; Hilbi et al., 1997), Burkholderia pseudomallei (Sun et al., 2005), Francisella tularensis (Mariathasan et al., 2005), and Actinobacillus actinomycetemcomitans (Nonaka et al., 2001), have been reported to induce a novel form of cell death via a caspase-1-dependent mechanism. Matching our observations, a number of these studies (Hersh et al., 1999; Sun et al., 2005; van der Velden et al., 2003) reported partial
protection from the pathogens using caspase inhibitors and more complete protection in caspase-1-deficient macrophages. Also agreeing with our conclusion that IL-1β is not involved in LT-mediated cytotoxicity, the caspase-1-dependent cell deaths associated with Salmonella (Monack et al., 2001), F. tularensis (Mariathasan et al., 2005), and S. flexneri (Chen et al., 1996) have been shown to be IL-1β independent by using IL-1β deficient macrophages, neutralizing antibodies to IL-1β, or IL-1β receptor antagonists, respectively.

Pyroptosis is a recently introduced term to describe the caspase-1-mediated macrophage death associated with these infections (Fink and Cookson, 2005). The mechanism of cell death during pyroptosis is not well defined, but the pathway is characterized by the formation of plasma membrane pores, and it has been reported that in Salmonella infection, this pore formation is dependent on caspase-1 (Fink and Cookson, 2006). It is possible that the important events mediated by caspase-1 in these other bacterial infections have similarities to those seen with LT treatment.

In summary, the late timing of LT-mediated inflammasome formation along with the requirement of ion fluxes for its assembly suggests that caspase-1 does not initiate macrophage death. However, caspase-1 is essential to cell death by participating in a step that follows the early LT-mediated events that instigate potassium efflux. LT-induced death appears to be dependent on a unique proteasome-dependent caspase-1 activation induced specifically by Nalp1b. The particular step at which Nalp1b is required remains unknown. Future work will focus on identifying the early LT events that initiate ion fluxes, proteins that are degraded by the proteasome in response to LT treatment, cellular proteases as well as caspase-1 substrates that may be required for macrophage death and the role of Nalp1b in cell death.

**EXPERIMENTAL PROCEDURES**

**Materials**

PA and LF were purified in our laboratory as described previously (Park and Leppla, 2000; Ramirez et al., 2002; Varughese et al., 1998). In all assays, working stocks of toxin were prepared in Dulbecco’s modified Eagle medium (DMEM). Concentrations of LT refer to the amount of each component (i.e., 1 μg/ml LT is 1 μg LF plus 1 μg PA/ml). Caspase-1 p10 (sc-514), EF-2 (sc-25634), and actin (sc-1616) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit (611-131-003) and anti-goat (605-731-125) infrared-dye-conjugated secondary antibodies (IRDye 800CW IgG) were from Rockland Immunochemicals (Gilbertsville, PA). Anti-murine IL-18 polyclonal antibody (JM-5180-100) was from MBL International (Woburn, MA). Anti-mouse IL-1β antibody (AF-401-NA) and the caspase inhibitors Z-WEHD-FMK (Z-W-E(OMe)-H-D(OMe)-FMK) and Z-VAD-FMK (Z-V-A-D(OMe)-FMK) were from R&D Systems (Minneapolis, MN). Ac-YVAD-CMK, nigericin, lactacystin, and anti-MEK1 NT antibody were from Calbiochem (San Diego, CA). Ac-DEV-D-CHO and DEVD-FMK were purchased from Bachem (King of Prussia, PA). Lipopolysaccharide (LPS) from E. coli serotype 011:B4, tetraethylammonium chloride (TEA), and quinidine were from Sigma (St. Louis, MO).

**Cell culture**

RAW264.7 cells and L929 mouse fibroblast cells were grown in DMEM supplemented with 10% fetal bovine serum, 10 mM HEPES, and 50 μg/ml gentamicin (all from Invitrogen, Carlsbad, CA) at 37°C in 5% CO₂. Bone marrow-derived macrophages (BMDM) and peritoneal macrophages were derived from BALB/cJ, 129S1/SvImJ, DBA/2J, and C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). Bone marrow cells were plated in DMEM (supplemented as described above) containing 30% L929 cell culture supernatant, grown for 5-8 days, and used in assays at 90% confluence following removal of unattached cells.
Peritoneal macrophages were elicited by intraperitoneal injection of 2 ml 4% Brewer modified thioglycolate (Becton Dickinson Microbiology Systems, Cockeysville, MD) 72 h prior to harvesting by peritoneal lavage. Peritoneal macrophages were plated and allowed to attach overnight prior to use.

**Cytotoxicity assays**

RAW264.7 cells and BALB/cJ BMDM were grown in 96-well plates to 90% confluence. For protection assays, cells were treated with LT (1 μg/ml) in the presence of 130 mM KCl or 300 mM sucrose or pretreated with caspase inhibitors (1 h) or potassium channel blockers (25 min) at various concentrations prior to the addition of LT at a set concentration of 1 μg/ml. Alternatively, the caspase inhibitors were added to cells concurrently with LT or 40 min after LT addition. Following a 2.5-3 h toxin treatment, MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO) was added at a final concentration of 0.5 mg/ml for 35 min to determine cell viability. In some experiments, pictures of the stained cells were taken using a light microscope. Alternatively, the production of formazan by viable cells was measured by removing medium and dissolving cells in 50 μl/well 0.5% (wt/vol) SDS, 25 mM HCl in 90% (vol/vol) isopropanol. The A₅₇₀ was read using a microplate reader, and cell viabilities were calculated relative to controls. In other experiments, DBA/2J and C57BL/6J BMDM were primed with 1 μg/ml LPS for 2 h followed by the addition of nigericin at a range of concentrations for 25 min. LT was then added at a final concentration of 1 μg/ml, and cell viability was determined as above following a 2.5-h toxin treatment.

**MEK, Caspase-1, IL-1β, and IL-18 cleavage assays**

RAW264.7 cells, BALB/cJ BMDM, and 129S1 peritoneal macrophages grown in DMEM were treated with 1 μg/ml LT at 37°C for various lengths of time in the presence or absence of quinidine (250 or 125 μM), TEA (100 or 80 mM), 15 μM lactacystin, 130 mM KCl, 300 mM sucrose, or caspase inhibitors (100 μM). Where indicated, RAW264.7 cells and BMDM were primed with 1 μg/ml LPS for 2 h prior to medium removal and LT addition. In these experiments, LT-treated and untreated controls were lysed at the same time to ensure all cells received identical LPS treatments. In other experiments, BMDM were primed with 1 βg/ml LPS for 2 h, and following medium removal, cells were further incubated with nigericin at the indicated concentrations for 25 min in the presence or absence of 130 mM KCl, 300 mM sucrose, 15 μM lactacystin, or caspase inhibitors (all at 100 μM). Cell lysates were prepared after three washes with ice-cold PBS using RIPA lysis buffer (1% Nonidet, 0.5% sodium deoxycholate, 0.1% SDS in PBS) containing EDTA-free Complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Protein concentrations were quantified using a bicinchoninic acid assay (BCA) (Pierce Biotechnology, Rockford, IL) and equal amounts of proteins were loaded for SDS-PAGE. For Western blot analyses, blots were probed with anti-MEK1 NT (1:7500), anti-caspase-1 p10 (1:500), anti-IL-18 (1:1000), or anti-IL-1β (1:800) antibodies. Anti-EF2 (1:1000) and anti-actin (1:1000) antibodies were used to show equal loading. Primary antibodies were detected using IR-dye conjugated secondary IgG antibodies (anti-rabbit: 1:5000; anti-goat: 1:5000) and the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

**Assay for IL-1β and IL-18 Release**

RAW264.7 cells 129S1 peritoneal macrophages and BALB/cJ BMDM were plated in 96-well plates (100 βl/well) and treated with LT at the indicated concentrations for various times. In some experiments, BMDM and RAW264.7 cells were first primed with 1 μg/ml LPS for 2 h prior to beginning LT treatment. For every toxin treatment time, cells were treated with LPS alone for a similar time. Culture supernatants were collected and tested (50 μl) for IL-1β (R&D Systems, Minneapolis, MN) and IL-18 (MBL International, Woburn, MA) levels by ELISA.
following the manufacturer’s protocols, and results were analyzed using GraphPad Prism 4.0 software (San Diego, CA). To determine the form of IL-18 and IL-1β present in the supernatant, SDS sample buffer was added to the collected supernatant, samples (20 μl) were run on gels, and the cytokines were detected by Western blotting as described above. Viability of the cells was assessed by MTT as described above immediately after supernatant removal.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**ACKNOWLEDGEMENTS**

This research was supported by the Intramural Research Program of the NIH, National Institute of Allergy and Infectious Diseases. We thank Dr. Antonio Rothfuchs for a gift of L929 cells and Jason Wiggins and Devorah Crown for help with peritoneal macrophage and bone marrow isolation.

**References**


Cell Microbiol. Author manuscript; available in PMC 2009 February 1.


Figure 1. Protection from LT-induced macrophage death with caspase inhibitors

(A) RAW264.7 cells and BALB/cJ BMDM were treated with caspase inhibitors for 1 h prior to LT addition (1 µg/ml), and cell viability was assessed by staining with MTT at 3 h. In a similar experiment, ZVAD-FMK was added to RAW264.7 cells 1 h prior to LT addition (1 µg/ml), at the same time as LT addition, or 40 min after LT addition. Data shown are from a single experiment representative of the protection curves from five separate assays.

(B) LPS-primed RAW264.7 cells and LPSprimed BALB/cJ BMDM were pretreated with the indicated caspase inhibitors (all at 100 µM, 1 h) prior to LT treatment (1 µg/ml) for 70 min (RAW) or 85 min (BMDM). Cell lysates were prepared and analyzed for the presence of the p10 fragment of active caspase-1 or mature IL-1β by Western blotting.
Figure 2. Timing of inflammasome formation in LT-sensitive BALB/cJ BMDM
(A) BMDM were treated with 1 μg/ml LT for the indicated times (min). Western blot analyses were used to follow inflammasome formation, as evidenced by the appearance of the p10 fragment of active caspase-1 and mature IL-18. MEK cleavage in the same lysates was following using an N-terminal antibody whose epitope maps to the LF cleavage site of MEK1. Blots were reprobed with an antibody to EF-2 to verify equal loading. (B) BMDM were treated with LT at various concentrations for the indicated times. Culture supernatants were collected and analyzed for the presence of IL-18 by ELISA and Western blot. After supernatant removal, cells were stained with MTT to determine cell viability. (C) LPS-primed BMDM were treated with LT as in (C), and culture supernatants were analyzed for the presence of IL-1β by ELISA. Cell viability was determined by MTT staining. Filled triangles in (B) and (C) mark the LT treatments that induced the highest levels of IL-18 or IL-1β release and the cell viabilities that correspond to these treatments. In all gels, NT refers to no-treatment control cells.
Figure 3. Inflammasome formation is prevented in the presence of KCl, sucrose, quinidine and TEA
(A) BALB/cJ BMDM were treated with LT (1 μg/ml) for the indicated times (min) in DMEM alone or DMEM containing 130 mM KCl or 300 mM sucrose. In the indicated control lanes, cells were primed with LPS (1 μg/ml; 2 h) and treated with 20 μM nigericin for 25 min. Caspase-1 activation and IL-18 maturation were monitored in cell lysates by Western blotting. (B) RAW264.7 cells were treated with 1 μg/ml LT in the presence or absence of 130 mM KCl or 300 mM sucrose for 2.5 h. Cells were then stained with MTT, and pictures of stained cells were taken using a light microscope. (C) BALB/cJ BMDM were pretreated with quinidine or TEA at the indicated concentrations for 25 min prior to LT treatment (1 μg/ml) for 85 min. Active caspase-1 and mature IL-18 were then detected in cell lysates by Western blotting. In all gels, NT refers to no-treatment control cells.
Figure 4. LT-induced caspase-1 activation requires active proteasomes

(A) BALB/cJ BMDM were pretreated with lactacystin (15 μM) for 30 min prior LT treatment (1 μg/ml) for the indicated times (min). Active caspase-1 was detected in cell lysates by Western blot. (B) BALB/cJ BMDM were primed with LPS (1 μg/ml) for 1.5 h prior to lactacystin (15 μM) addition 30 min prior to addition of 20 μM nigericin for 25 min. Mature IL-1β was detected in cell lysates by Western blot.
Figure 5. Caspase-1 activation alone does not sensitize resistant macrophages to LT
(A) C57BL/6J BMDM were primed with LPS for 2 h, followed by medium removal, and addition of nigericin (5 μM) for 25 min. Caspase-1 and IL-1β were monitored in cell lysates by Western blotting. (B) DBA/2J and C57BL/6J BMDM were treated with LPS and 5 μM nigericin as in (A). LT was then added, and cell viability was determined 2.5 h later by MTT staining.
Figure 6. A model of LT-induced macrophage death

Following entry into cells, LF is released from late endosomes and cleaves the MEK proteins in the cytosol (20-40 min) in both LT-sensitive and resistant macrophages. In a series of unknown events, possibly involving the cleavage of additional LF substrates, downstream effects of MEK inactivation or direct involvement of the sensitive allele of Nalp1b, LF induces increases in plasma membrane permeability, resulting in ion fluxes in LT-sensitive, but not resistant cells. These ion fluxes are sensed by functional Nalps in the macrophage, possibly including Nalp1b, and lead to caspase-1 recruitment, inflammasome formation, and caspase-1 activation (50-60 min) in sensitive cells only. Active proteasomes are required in an unknown step that precedes caspase-1 activation. Caspase-1 activity is then required in unknown late events that lead to cell lysis. In a pathway not required for cell death, caspase-1 cleaves IL-1β and IL-18, and the mature forms of the cytokines are subsequently released.