Sensitizing anthrax lethal toxin-resistant macrophages to lethal toxin-induced killing by tumor necrosis factor-α

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SUMMARY

Macrophages from different inbred mouse strains exhibit striking differences in their sensitivity to anthrax lethal toxin (LeTx)-induced cytolysis. While LeTx-induced cytolysis of macrophages plays an important role in the outcome of anthrax infection, the sensitivity of macrophages in vitro does not correlate with in vivo susceptibility to infection of \textit{B. anthracis}. This divergence suggests that additional factors other than LeTx are involved in the cytolysis of LeTx-resistant macrophages in vivo. We found that LeTx-resistant macrophages became sensitive to LeTx-induced cytolysis when these cells were activated by bacterial components. Tumor necrosis factor-\alpha (TNF) induced by bacterial components is a key factor that cooperates with LeTx in inducing LeTx-resistant macrophage death. TNF+LeTx-induced death of LeTx-resistant macrophages is dependent on mTor (mammalian target of rapamycin) but independent from caspases. Our data indicates that host responses to anthrax infection contribute to cytolysis of LeTx-resistant macrophages.
INTRODUCTION

Anthrax infections are initiated by endospores of *Bacillus anthracis* (1). These spores germinate after they are phagocytosed by macrophages and begin to express virulence toxins that lead to a systemic immune response, shock, and death (1). It has been shown that the three toxins, edema factor (EF), lethal factor (LF), and protective antigen (PA) produced by anthrax bacilli, are principally responsible for provoking the host responses (1,2). PA functions as a molecular transporter by facilitating the entry of LF and EF into cells through endocytosis and translocation of EF and LF into cytosol (3,4). LF, a zinc metalloprotease (4), together with PA is termed as lethal toxin (LeTx). LeTx is the major contributor of virulence in infected animals (1,5). EF, an adenylate cyclase (3), functions as an enhancer which enhances the cytotoxicity of LeTx on macrophages (6).

Much is known about the mechanisms of action and cellular entry of LF and EF. PA binds to a cell surface receptor (7), where it is proteolytically processed on the cell surface (8,9). The processed PA heptamerizes and binds 3 molecules of either EF or LF, resulting in either a PA-EF or a PA-LF complex which is internalized through receptor-mediated endocytosis (10). PA then forms a hole in the acidified endocytic vesicle through which EF or LF is delivered to the cytosol (11). While it is known that EF exerts its effects through a calmodulin-dependent adenylate cyclase activity, the mechanisms of LF intoxication are less clear. LF can be delivered to the cytosol of cells, but cytolysis as a consequence of the intracellular LF is observed only in macrophages from certain mouse strains (5). Conflicting results have been reported regarding whether LeTx induces cytokine production in macrophages (12-14). The toxic activity of LF
depends on its protease activity (4) suggesting that proteolysis of one or more cellular protein(s) unleashes a cascade of events that results in the death of the intoxicated macrophages. Indeed, cleavage of several mitogen-activated protein kinase kinases (MKKs) has been observed (15-17). However, the physiological importance of the MKKs proteolysis relative to macrophage cytolysis cannot be established because similar cleavage was observed in macrophages that were resistant to LeTx (16,17).

Inbred mouse strains exhibit striking differences in the sensitivity of their cultured macrophages to the effects of LeTx (5). For example, C3H mouse macrophages lysed by LeTx concentrations of 1 ng/ml were 100,000 times more sensitive than those from A/J mice (5). Macrophages from CBA/J and Balb/C were sensitive, while those from C57BL/6J and DBA/2J were resistant. Direct binding studies revealed that the affinity and number of PA receptors per cell were the same in sensitive and resistant cells (5). Proteolytic activation of PA was also the same in both sensitive and resistant macrophages (5). Resistant macrophages were not cross-resistant to other toxins and viruses which, like lethal toxin, require vesicular acidification for activity. This implies that resistance is not due to defects in vesicular acidification (5). As mentioned earlier, the proteolysis of MKKs were comparable in sensitive and resistant cells (16,17). Thus, resistance is due to a defect at a stage occurring after proteolysis. Watters et al. identified a mutation on a gene named Kif1C that is responsible for the different sensitivities of macrophages to LeTx (17). Kif1C encodes a kinesin-like motor protein of the UNC104 subfamily (18). It is clear that proper function of Kif1C is required for LeTx resistance, but how
this protein is involved in LeTx elicited changes in macrophages is unknown (17). Kif1C protein has been excluded as a target of LF-mediated proteolysis (17).

It was believed that LeTx induced cytolysis of macrophages played an important role in the overall outcome of anthrax infection. Injection of LeTx into CBA/J mice whose macrophages are sensitive to LeTx resulted in a more rapid death than A/J mice whose macrophages were resistant to LeTx-induced cytolysis (5). However, the sensitivity of macrophages to LeTx in vitro does not correlate with in vivo susceptibility to infection of either encapsulated or nonencapsulated strains of \textit{B. anthracis} (5,16,17). Since macrophages can be activated by encounters with infected bacteria (19,20), we addressed whether macrophage activation had an effect on cell viability in LeTx treated LeTx-resistant macrophages. We have found that the treatment of macrophages with bacterial components can make LeTx-resistant macrophages become sensitive to LeTx-induced cytolysis. Tumor necrosis factor-\(\alpha\) (TNF) induced by bacterial components is at least one of the factors that cooperate with LeTx in inducing macrophage death. Our data suggests that the autocrine/paracrine effect of TNF plays a key role in LeTx-resistant macrophage death in vivo.
EXPERIMENTAL PROCEDURES

Materials. Recombinant PA and LF were prepared as described (15). Murine TNF-α was provided by Dr. Vladimir Kravchenko (The Scrips Research Institute). Anti-TNF antibodies were raised in rabbits by using recombinant TNF. Lipopolysaccharides, peptidoglycan and poly-D-glutamic acid were from Sigma (St. Louis, MO). IL-1β, IL-6, IFN-γ and human TNF-α were from R&D Systems (Minneapolis, MN). Mouse TNF-α Elisa assay kits were from R&D Systems. All chemicals were from Sigma or as indicated in text.

Preparations of peritoneal macrophages. Peritoneal macrophages were obtained from mice by saline lavage as previously described (21). Mice received i.p. injections of 3% thioglycollate 4 days before the preparation of peritoneal macrophages. After being anesthetized with I.M injections of a mixture of ketamine (80 mg/kg/body weight) and xylazine (16 mg/kg/body weight), peritoneal macrophages were harvested from the mice through lavage of the peritoneal cavity with 5 ml of saline. The cells were then washed with RPMI-1640 media, resuspended in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (supplemented DMED), and 1x10^5 or 2x10^6 cells were plated on 96-well microtiter or 6-well plates, respectively. After incubation at 37 °C for 2 h, the non-adherent cells were removed by washing three times with fresh DMEM and cultured overnight in supplemented DMED.
Cell viability assays. The extent of cell death was measured using crystal violet uptake assays and propidium iodide exclusions (22). Briefly, cell culture medium was removed from plates by immersing them vertically in a beaker containing 2 liters of 0.9% saline. After immersion, all liquid was tapped out onto paper towels. To stain cells, 80 µl of 0.5% crystal violet solution containing 25% methanol in saline was added to each well and incubated for 5 min at room temperature. Excess stains were removed by immersing plates in saline as is listed above repeating twice. After removing all liquid as before, 100 µl of 50% acetic acid was added to each well to dissolve all the stained cellular materials. Plates were then placed on a shaker for about 30 min and the O.D. at 590 nm was measured. PI staining was performed using trypsinized and resuspended cells in PBS with 1 µg/ml PI. The level of PI incorporation was quantified by flow cytometry on a FACScan flow cytometer.

HOE-33258 and annexin V staining. Nuclear condensation and fragmentation were determined by staining the cells with HOE-33258 (St. Louis, MO) as described previously (22). Phosphatidylserine exposure to the outer leaflet were analyzed by staining cells with FITC-conjugated annexin V (Roche Molecular Biochemicals, Indianapolis, IN) as suggested by the manufacturer’s manual. Briefly, cells were resuspended in Annexin labeling solution containing 10 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM CaCl2 and FLUOS-conjugated annexin V for 25 min. After washing twice with PBS, cell pellets were resuspended in PI (1 µg/ml) containing PBS and analyzed by flow cytometry.
**DNA ladder assay.** Cells were collected, lysed in lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% SDS, 25 mM EDTA and 0.1 mg/ml protease K), and incubated at 50°C overnight. DNA was phenol/chloroform extracted, precipitated by adding NaCl to 0.3M and 2 volumes of ethanol. The DNA pellets were resuspended in TE buffer with 0.1 mg/ml RNase I for 1h at room temperature then treated with 0.1 mg/ml protease K overnight at 37°C. DNA was further phenol/chloroform extracted and precipitated as above and resuspended in 20 µl TE buffer. Equal amounts of DNA were separated on 2% agarose gel and stained with ethidium bromide for visualization.

**Total cell lysate preparation and Western blot analysis.** Extraction of total cell lysate and Western blot analysis for MEK1 were performed as previously described (22). Briefly, cells were lysed in ice-cold cell lysis buffer containing 20 mM MOPS, 15 mM EGTA, 2 mM EDTA, 1 mM Na3VO4, 1 mM dithiothreitol, 75 mM β-glycerophosphate, 0.1 mM PMSF, 1 μg/ml aprotinin, 10 μg/ml pepstatin A, 1 μg/ml leupeptin and 1% Triton X-100, then sonicated on ice. Cell extracts were obtained by centrifuging the homogenate at 13,000 rpm for 10min. These extracts were electrophoretically resolved in Ready-made 10% SDS-PAGE gels (Bio-Rad, Hercules, CA), followed by transfers onto nitrocellulose membranes. Membranes were subsequently blocked with 5% skim milk for 30min, immunoblotted with antibodies, and developed by using an enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) detection system. MEK1 N-terminal antibodies were purchased from Upstate Biotechnology Incorporated.
RESULTS

LeTx-resistant macrophages are selectively resistant to LeTx-induced cell death. We used macrophages from C3H/HeN, 129svj and C57BL/6J mice to repeat published results that macrophages from different mouse strains had a difference in the sensitivity to LeTx-induced cell killing in vitro. As shown in Figure 1A, the macrophages of C3H/HeN and 129svj are sensitive to LeTx-induced killing while the macrophages from C57BL/6J are resistant. The maximum cell death of sensitive cells was reached 3-4h after the cells were treated with LeTx [PA(500 ng/ml)+LF(500 ng/ml)], whereas most of the resistant macrophages were still viable after 16h of the same treatment (Fig. 1B). Cytolytic dose of LF for the macrophages of C3H/HeN (Fig. 1C) and 129 svj (data not shown) was about 10 ng/ml when a saturated PA (500 ng/ml) was present. The resistance of C57BL/6J macrophages to LeTx induced cytolysis was not dependent on the dose of LF used in the experiments since up to 50-fold increase of LF concentration still cannot produce more cytolysis (Fig. 1C). The cell viability was measured using crystal violet uptake of live cells and propidium iodide exclusion (data not shown) with comparable results.

It is known that LF cleaves certain intracellular proteins and that proteolysis may be essential for cell death. Recent reports have shown that the cleavage of MEK2 and MKK3 also occurs in LeTx resistant macrophages (16,17). This suggests that the resistance to LeTx induced cytolysis in resistant macrophages is not due to a defect of proteolysis mediated by LF. We tested the cleavage of MEK1 in the macrophages from C57BL/6J and C3H/HeN. MEK1 was cleaved in the macrophages of both strains when treated with LeTx (Fig. 1D).
It was known that LeTx-resistant macrophages were not resistant to other toxins (5). We further tested whether LeTx-resistant macrophages are generally resistant to death stimuli. Macrophages from C3H/HeN, 129svj and C57BL/6J were treated with lipopolysaccharides (LPS) plus interferon-γ (IFN-γ), LPS plus benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD), sodium nitroprusside (SNP), and etoposide. All of these reagents have been shown to cause macrophage death (22,23). As shown in Figure 1E, the macrophages from the three strains were equally killed by these death stimuli. Thus, there is no general resistance to death in C57BL/6J macrophages.

**Cellular activation can sensitize the resistant macrophages to LeTx-induced cell death.** Available data strongly supports the role of macrophage cytolysis in eliciting shock and death in infected animals (1). Since the mice, whose macrophages were resistant to LeTx induced cytolysis in vitro, still died after infection, we decided to test whether macrophage activation by bacterial components, which should happen during bacterial infection, had any effect on the viability of LeTx-treated resistant macrophages. Macrophages isolated from C57BL/6J mice (LeTx-resistant macrophages) were used in the experiments. The cells were treated with LeTx (PA+LF) in the presence or absence of the major component of *B. anthracis* capsule poly-D-glutamic acid (PGA) (24) (100 µg/ml), cell wall component of gram-positive bacteria peptidoglycan (PG) (10 µg/ml), or cell wall components of gram-negative bacteria LPS (10 ng/ml). We also treated the cells with retrovirus (MLV, ~10⁶ pfu/ml), or zymosan A (100 µg/ml) for comparison. Cell viability was measured 16 h later and is shown in Figure 2A. The presence
of PGA promoted LeTx-induced C57BL/6J macrophage death. LPS and PG also significantly enhanced LeTx-induced cell death. Retrovirus and zymosan A slightly enhanced cell death. The results of an experiment with more controls is shown in Figure 2B. LPS, PG or PGA alone did not cause cell death. LPS, PG or PGA combined with PA or LF alone did not cause cell death. The cell death of resistant macrophages started around 5h of treatment and reached a maximum around 16h (Fig. 2C). 

*B. anthracis* is a gram-positive bacteria whose capsule should interact with macrophages, and its cell wall component PG can activate macrophages if the capsule is removed. Thus, the death of LeTx-treated resistant macrophages could be promoted by cell activation with bacterial capsule or cell wall components in vivo.

**Phenotype of LeTx-induced death of activated macrophages.** LeTx-induced cytolysis of macrophage is more like necrosis than apoptosis (13,25), although treatment of macrophages with sublytic amounts of LeTx can trigger some intracellular events of apoptosis (26,27). To determine whether C3H/HeN- and C57BL/6J-macrophages treated with LPS plus a cytolytic dose of LeTx had phenotype of apoptosis, three apoptotic features were examined. Nuclear labeling with HOE-33258 is a commonly used method to detect chromatin condensation, a feature of apoptosis. Macrophages from C3H/HeN and C57BL/6J were treated with nothing (control) or LeTx+LPS and stained with HOE-33258. Chromatin condensation was not seen throughout the course of cell death of both C3H/HeN- and C57BL/6J-macrophages (Fig. 3A and data not shown). As a positive control, chromatin condensation was observed when C57BL/6J- or C3H/HeN-macrophages were treated with LPS+INF-γ (Fig. 3A and data not shown). DNA
fragmentation is a typical feature of apoptosis and was examined in the macrophages treated with LPS plus LeTx. No DNA ladder was detected in C57BL/6J- and C3H/HeN-macrophages treated with LeTx plus LPS (Fig. 3B). Treatment of C57BL/6J-macrophages with LPS+IFN\(\gamma\) was used as positive control for DNA fragmentation (Fig. 3B, right lane). Translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer is a common early event in apoptosis. Annexin V staining of PS was observed in macrophages treated with LPS+IFN\(\gamma\) but not in the macrophages treated with LPS plus LeTx (Fig. 3C). Thus, LeTx+LPS-induced death of LeTx-resistant macrophages did not have apoptotic phenotype and LPS stimulation did not change the mode of cell death induced by cytolytic doses of LeTx.

**TNF plays a role in sensitizing LeTx-resistant macrophages.** Activated macrophages secrete a number of cytokines including TNF, IL-1\(\beta\), IL-6 and INF-\(\gamma\). We treated C57BL/6J macrophages with these cytokines of murine origin in the presence or absence of LeTx and measured cell viability. As shown in Figure 4A, these cytokines alone did not affect the viability of macrophages. Interestingly, dramatic cell death was induced in LeTx-treated cells in the presence of TNF, but not other cytokines tested. Thus, TNF can sensitize LeTx-resistant macrophages to LeTx-induced cell death.

We measured TNF production in C57BL/6J macrophages after stimulation with LPS, PG, and PGA. LPS stimulation led to the highest production of TNF (Fig. 4B). PG stimulated TNF production was about half of the LPS-induced TNF and PGA was less potent in inducing TNF production. The treatment of cells with LeTx reduced but did not abolish TNF production. The
level of TNF production (Fig. 4B) was correlated with the death of LeTx-treated resistant macrophages promoted by these bacterial components (Fig. 2A) suggesting that TNF is a mediator of bacterial components-promoted death of LeTx-treated cells.

To determine whether the sensitization of LeTx-resistant macrophages to LeTx-induced death by bacterial components was mediated by TNF, we used a neutralizing antibody to block TNF. As shown in Figure 4C, ~50% inhibition of the TNF+LeTx-induced cell death was observed when an anti-TNF antiserum was included in the cell culture medium. Pre-bled serum had no effect indicating that the antibody can block the function of TNF. This antibody also inhibited LPS+LeTx, PG+LeTx or PGA+LeTx-induced cell death, supporting the idea that TNF is at least one of the factors that are responsible for sensitizing the resistant macrophages to LeTx-induced cytolysis. Thus, the autocrine/paracrine effect of TNF plays a role in LeTx-resistant macrophage death.

The bioactivity of tumor necrosis factor (TNF) is mediated by two TNF receptors, TNF-RI and TNF-RII. Although human TNF (hTNF) and murine TNF (mTNF) are very homologous, hTNF binds only to mTNF-RI (28). We treated C57BL/6J macrophages with hTNF plus LeTx and found that, in contrast to mTNF, hTNF cannot sensitize LeTx-resistant macrophages to LeTx-induced cell death (Fig. 4D), suggesting that TNFRII mediated signaling is required for sensitizing LeTx-resistant macrophages.

C57/10ScCr is a mouse strain that has a deletion of the toll-like receptor 4 (TLR4) gene and will not respond to LPS (29). A comparable strain with wildtype TLR4 gene is C57/ScSn. The macrophages isolated from these two strains of mice are resistant to LeTx (Fig. 4E).
Treatment of LPS in the presence of LeTx leads to cell death in C57/ScSn macrophages but not C57/10ScCr macrophages confirming that TLR4 signaling is required for LPS promoted death of LeTx treated cells. In contrast, TNF+LeTx caused cell death in both macrophages, which is consistent with the idea that bacterial components promote the death of LeTx treated cells through the induction of TNF.

TNF can induce the death of a number of different cells. Although TNF alone had no effect on the viability of macrophages, it is possible that intracellular signaling activated by TNF can augment the death of C3H/HeN-macrophages induced by LeTx. To examine this possibility, we tested whether TNF can promote death of macrophages treated with sublytic dose of LF. Macrophages from C3H/HeN and C57BL/6J were treated with 500 ng/ml PA and different doses of LF in the presence or absence of TNF. The level of cytolysis of cells were analyzed. As shown in Figure 5A, TNF stimulation did not enhance the cell death of C3H/HeN-macrophages treated with sublytic or cytolytic doses of LF. In contrast, TNF increased the cell death of C57BL/6J-macrophages treated with cytolytic dose (Fig. 5B). The sensitization of LeTx-induced cell death by TNF appears to be a unique feature of LeTx-resistant macrophages.

**TNF+LeTx-induced cell death in LeTx-resistant macrophages requires proteolytic activity of LF and mTor signaling.** LF(E687C), an inactive LF mutant, has been shown to be unable to mediate the proteolysis of proteins in vitro and in cells. Also, LF(E687C) is incapable of causing cytolysis of LeTx-sensitive macrophages (15). To determine whether the protease activity of LF is required for TNF+LeTx-induced death of resistant macrophages, we compared the effect of
wildtype LF and LF(E687C) on the cell viability of resistant macrophages in the presence of TNF (Fig. 6A). No cell death was observed in LF(E687C) treated cells indicating that the protease activity of LF is required for TNF+LeTx-induced death of resistant macrophages.

Caspases have been implicated to play an important role in TNF-induced cell death of many different cells (30). To test whether caspase is involved in TNF+LeTx-induced death of LeTx-resistant macrophages, we used a pan-caspase inhibitor zVAD. As shown in Figure 6B, zVAD itself did not have an effect on the viability of resistant macrophages nor did zVAD together with LeTx have any effect on cell viability. Therefore, caspases are not required for TNF+LeTx-induced death of LeTx-resistant macrophages.

TNF activates a number of signaling pathways that are involved in cell survival or death of a number of different cells (32-35). In order to understand how TNF sensitizes LeTx-resistant macrophages, we initiated our search on whether the known signaling pathways that are activated by TNF are involved in sensitizing LeTx-resistant macrophages. We used available inhibitors to inhibit the signaling pathways activated by TNF. The inhibitors (U0126, JNK inhibitor 2, and SB203580) that inhibit the three MAP kinase pathways, extracellular regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, did not have any inhibitory effect on TNF+LeTx induced cell death, but rather enhanced cell death (Fig. 6C). The other inhibitors that enhanced cell death are protein kinase C inhibitor bisindolylmaleimide II, tyrosine kinase inhibitor genistein and herbimycin, PI3 kinase inhibitor wortmannin. Since oxidative burst was implicated to be responsible for LeTx-induced cell death (13), we also tested butylated hydroxyanisole (BHA), a scavenger of free radicals, and found no effect on the death of resistant
macrophages (data not shown). The only inhibitor we tested that inhibited cell death was rapamycin (Fig. 6C). Maximum inhibition of TNF+LeTx-induced cell death by rapamycin was reach at 10 nM. The known target of rapamycin is mTor (mammalian target of rapamycin, also named FRAP or RAFT1) and no non-specific effect of rapamycin has been reported at 10 nM. Thus mTor is most likely involved in the cell death.

It is known that the proteolysis by LF impairs the two mitogen activated protein (MAP) kinase pathways, the ERK and p38 pathways. It was proposed that disruption of certain intracellular pathways by LF impairs cell survival mechanisms and thus promotes cell death (15-17, 27). To test whether disruption of the ERK and/or p38 pathways by LeTx is involved in TNF+LeTx induced death of LeTx-resistant macrophages, we used chemical inhibitors to mimic the inhibitory effect of LF on the ERK and p38 pathways. Inhibition of the ERK pathway by U0126 and the p38 pathway by SB203580 either independent or together did not influence cell viability of TNF-treated LeTx-resistant macrophages (Fig. 6D and data not shown), suggesting that cleavage of MEK1/2 and MKK3/6 by LF is either not involved in TNF+LeTx-induced cell death or is insufficient to reduce the ability of cell survival. We further tested inhibitors for other pathways. We used c-Jun N-terminal kinase (JNK) inhibitor 2 to inhibit the JNK pathway, another MAP kinase pathway, and did not find any change in the viability of TNF-treated LeTx-resistant macrophages (Fig. 6D). Similarly, inhibition of NF-κB, PKC, or PI3K pathways had no effect on the cell viability of TNF-treated macrophages (Fig. 6D). We tested whether inhibition of all three MAP kinase pathways had effect on the viability of macrophages treated with TNF. As shown in Figure 6D, ~20% cell death was observed when ERK, p38, and JNK were inhibited.
at the same time. This data suggests that simultaneous inhibition of multiple MAP kinase pathways may have a role in TNF+LeTx induced macrophage death. However, the possible non-specific effect of these inhibitors in influencing cell viability cannot be excluded. Inhibition of NF-κB, or PKC and the three MAP kinase pathways at the same time did not further enhance the death of TNF-treated C57BL/6J macrophages (Fig. 6D). Inhibition of PI3K pathway together with the three MAP kinase pathways further enhanced cell killing (Fig. 6D). Collectively, these data suggested that impairing anyone of the known pathways is not sufficient for sensitizing LeTx-resistant macrophage. However, it is possible that disruption of multiple signaling pathways by LF can impair cellular survival mechanisms, though a conclusion cannot be made due to limited information on the cellular targets of LF.
DISCUSSION

We have examined the effect of cellular activation of macrophages on the cell viability of LeTx-treated LeTx-resistant macrophages. We found that treatment of macrophages with different bacterial components made LeTx-resistant murine macrophages susceptible to LeTx-induced cell death. TNF produced by activated macrophages is a key mediator that sensitizes LeTx-resistant macrophages to LeTx-induced death. We have determined that the protease activity of LF and mTor activity in cells are required for TNF+LeTx-induced resistant macrophage death and that this type of cell death is caspase-independent. Sensitizing LeTx-resistant macrophages to LeTx-induced cytolysis suggests that host responses to anthrax infection participate in the macrophage death in vivo.

LeTx induced cytolysis of macrophages plays an important role in the outcome of anthrax infection. Lethality in the mice of different strains resulting from direct injections of LeTx mimicked the in vitro sensitivity of macrophages to LeTx-induced cell death (5). Contribution of host responses to the lethality of anthrax infection can also be deduced from the study of Welkos et al. They showed that C3H/HeN and C3H/HeJ mouse strains were killed by B. anthracis Vollum 1B strain (wildtype) infection (5) with similar LD_{50} (5-6 spores). However, the LD_{50} of the nonencapsulated toxin-producing strain Sterne was significantly different. The LD_{50} of C3H/HeN mice is 8x10^6 spores while C3H/HeJ mice were completely resistant to the Sterne strain at the highest dose used (2x10^7). These two strains have similar genetic backgrounds except that C3H/HeJ mice have a point mutation in the Toll-like receptor (TLR) 4 which impairs signaling initiated by some bacterial cell wall components such as LPS. It is possible that
capsulated *B. anthracis* can escape TLR4 recognition and therefore the TLR4 mutation does not alter the host responses to anthrax infection. “Naked” *B. anthracis*, however, can be detected by TLR4 and the TLR4 deficiency impaired macrophage responses thereby increasing levels of LD$_{50}$. Although wildtype *B. anthracis* does escape phagocytosis by macrophages and some other host defense reactions, certain levels of host response should occur. The capsule should interact with macrophages and we have shown here that the major components of the capsule such as PGA can stimulate macrophages to produce TNF. Although the capsule may prevent PG from interacting with the macrophages, this interaction would occur if the capsule was released or damaged. Since macrophages are producers of TNF, the local concentration of TNF around macrophages can be high. We suggest that macrophage-produced TNF may be responsible for cytolysis of LeTx-resistant macrophages in vivo. This contention is consistent with two published in vivo studies using neutralizing antibodies of TNF. When BALB/C mice were administered with the TNF antibody, there was no protective effect from an injection of LeTx (14). In contrast, administration of the TNF antibody delayed the death of C57BL/6J mice infected with *B. anthracis* (36). When the macrophages from BALB/C and C57BL/6J mice were analyzed for their sensitivity to LeTx in vitro, BALB/C macrophages were found to be sensitive to LeTx but C57BL/6J were not. It is possible that because LeTx can directly trigger cytolysis of macrophages in BALB/C mice, inhibition of TNF does not help the survival of the mice. While TNF is involved in the cytolysis of macrophages in anthrax-infected C57BL/6J mice, blocking TNF delays death.
Life or death of a cell is determined by a balance between death and survival pathways. TNF is a pleiotropic cytokine produced mainly by macrophages. TNF is also recognized by macrophages and regulates the pattern of gene expression (35). TNF-induced cellular responses are mediated by either one of the two TNF receptors, TNF-RI (p55) and TNF-RII (p75) (37). Macrophages express both receptors (33). Since TNF-R1 knockout mice had the same sensitivity to anthrax infection as wildtype mice (36), the sensitization of LeTx-resistant macrophage to LeTx-induced death by TNF is most likely through TNF-R2. This notion was consistent with the observation that human TNF, which is only able to bind with TNF-RI on the murine cells cannot promote death of LeTx-resistant macrophages (Fig. 4D). TNF can induce caspase-dependent and -independent cell death (38-40). It is clear that TNF+LeTx-induced cell death is independent from caspases since zVAD did not inhibit cell death (Fig. 6B). Because the mechanism of caspase-independent cell death by TNF is largely unknown, it is unclear whether the same method was used in TNF+LeTx-induced macrophage death.

Even though TNF induces cell death in many cells, some cell types such as macrophages proliferate in response to TNF (33). TNF activates various kinases of the MAP kinase family and induces various transcription factors such as NF-κB in macrophages (32-35). Activation of MAP kinase has been shown to involved in both apoptosis and cell survival of macrophages depending on the cell death stimuli (41,42). Recently, Park et al showed that LPS stimulation of macrophages treated with sublytic dose of LF and saturated PA63 (cleaved active form of PA) resulted in apoptosis. The dismantling of the p38 MAP kinase pathway by sublytic dose of LF was implicated to be partly responsible for the apoptosis of activated macrophages (27).
showed here that inhibition of ERK, p38, JNK, PKC, tyrosine kinases, and PI3K pathways enhances TNF+LeTx-induced death of LeTx-resistant macrophages (Fig. 6C), which supports the idea that damages of certain intracellular signaling pathways by LF may impair cellular survival mechanisms (27). Inhibition of any single pathway did not have effect on the viability of TNF-treated macrophages (Fig. 6D), suggesting disruption of a single signaling pathway cannot mimic LF effect of survival mechanisms. This speculation was supported by the observation that a combination of inhibitors for MAP kinases and PI3K significantly induced cell death in TNF-treated macrophages (Fig. 6D). Different from reported effect of sublytic dose of LF (27), apoptosis features cannot be found in macrophage treated with cytolytic doses of LeTx in the presence of LPS stimulation (Fig. 3). It appears that the phenotype of cell death is determined by the level of damage that LF made rather than cellular activation because sublytic dose of LeTx alone already triggers apoptotic features (26).

Rapamycin was the only inhibitor we tested that can prevent TNF+LeTx-induced macrophage death (Fig. 6C). Rapamycin target mTor is a controller for regulatory metabolic responses (43). The PI3K/Akt pathway can regulate mTor in some systems (43). It is unlikely that PI3K/Akt pathway is responsible for mTor regulation in our system because wortmannin or LY294002 did not have the same effect as rapamycin, but rather had an opposite effect. Since rapamycin did not inhibit LeTx-induced cytolysis of LeTx-sensitive macrophages (data not shown), the mTor is most likely involved in TNF signaling in TNF+LeTx-induced death of LeTx-resistant macrophages. How mTor was regulated and how mTor function in TNF+LeTx-induced LeTx-resistant macrophage death is a subject for further investigation.
LeTx causes the proteolysis of certain cellular proteins by triggering the cytolysis of LeTx-sensitive macrophages. Because of a difference of three amino acids in the kinesin-like motor protein Kif1C, this proteolysis cannot cause cell death in LeTx-resistant macrophages. Kif1C was suggested to be involved in retrograde vesicle transport and TNF may influence this transport to sensitize LeTx-resistant macrophages. It is also possible that TNF sensitizes LeTx-resistant macrophages through a mechanism independent from Kif1C. LeTx leads to proteolysis of certain proteins that may damage some cellular functions. TNF-induced cellular responses under this condition could be imbalanced and result in cell death. It was reported that human macrophages are sensitive to LeTx-induced cytolysis in vitro (13). However, we have tested human macrophages from two donors and found that both of them were resistant to LeTx-induced cell death and could be sensitized through TNF treatment as was observed in murine LeTx-resistant macrophages (data not shown). This result fits with a predication that human macrophages should be resistant to the effect of LeTx because the human Kif1C gene has the same amino acid sequence as C57BL/6J and other resistant strains in the region that determine LeTx-sensitivity (17). It appears that different human populations exhibit differences in the sensitivity of their macrophages due to the effect of LeTx. Thus, our study on LeTx-resistant macrophage death is relevant to anthrax infection in humans and provides valuable insight into the anthrax pathogenesis.
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FIGURE LEGEND

Figure 1. LeTx(PA+LF)-sensitive and –resistant macrophages. (A) Peritoneal macrophages were isolated from C3H/HeN, 129svj and C57BL/6J mice and treated with PA (500 ng/ml) plus LF (500 ng/ml). Cell viability was measured 3h after treatment using crystal violet uptake assay. (B) Macrophages of C57BL/6J and C3H/HeN were treated with PA (500 ng/ml) plus LF (500 ng/ml) for different periods of time and the cell viability was measured. (C) Macrophages of C57BL/6J and C3H/HeN were treated with PA (500 ng/ml) plus different concentrations of LF as indicated for 16h. Cell viability was measured. (D) Macrophages of C57BL/6J and C3H/HeN were treated with PA (500ng/ml) plus LF (500 ng/ml) for different periods of time and MEK1 and Erk1/2 proteins were analyzed by immunoblotting using anti-MEK1 N-terminal and Erk antibodies. (E) Macrophages of C3H/HeN, 128svj, and C57BL/6J were treated with LPS (10 ng/ml) + zVAD (10 µM), LPS+IFN-γ (100 u/ml), SNP (500 µM), or etoposide (200 µM). Cell viability was determined 24h after the treatments. The results represent means ± SE (n=3-6).

Figure 2. Bacterial components-promoted the death of LeTx-treated LeTx-resistant macrophages. (A) C57BL/6J (LeTx-resistant) macrophages were treated with PA+LF (500 ng/ml) in the presence of nothing (control), MLV virus (~10^6 pfu/ml), zymosan A (100 µg/ml), peptidoglycan (PG, 10 µg/ml), lipopolysaccharides (LPS, 10 ng/ml), or poly-D-glutamic acid (PGA, 100 µg/ml). Cell viability was assayed 16h after treatment. (B) C57BL/6J macrophages were treated with nothing (control), PA (500 ng/ml), LF (500 ng/ml), or PA+LF in the presence of LPS, PG or PGA. Cell viability was measured 16h after treatment. (C) C57BL/6J
macrophages were treated with PA(500ng/ml)+LF(500 ng/ml)+LPS(10 ng/ml) for different periods of times and cell viability was measured.

**Figure 3.** Phenotype of LeTx-induced death of activated macrophages. (A) C3H/HeN and C57BL/6J macrophages were treated with nothing (control), PA(500 ng/ml)+LF(500 ng/ml)+LPS (10 ng/ml). C3H/HeN or C57BL/6J cells were fixed and stained with HOE-33258 at 2h or 12h after the treatment, respectively. As a positive control, C57BL/6J-macrophages were treated with INF-γ(10 u/ml)+LPS(100 ng/ml) for 24 hr and stained with HOE-33258. (B) Macrophages of C3H/HeN and C57BL/6J were treated with PA+LF, or PA+LF+LPS as in (A) but for 5h or 24h, respectively. The positive control was C57BL/6J-macrophages treated with LPS+IFN-γ for 36h. Genomic DNA was isolated from C3H/HeN- or C57BL/6J-macrophages and analyzed by 2% agarose gel electrophoresis followed by ethidium bromide straining. (C) Macrophages of C3H/HeN and C57BL/6J were treated as in (A). The cells were stained with PI and annexin V. The percentage of PI negative and annexin V positive cells was determined by FACS analysis (n=2-3).

**Figure 4.** Sensitizing LeTx-resistant macrophages to LeTx-induced death by TNF. (A) C57BL/6J macrophages were treated with nothing (control), murine TNF (200 pM), IL-1β (10 u/ml), IL-6 (250 u/ml), or IFN-γ (100 u/ml) in the presence or absence of PA(500 ng/ml)+LF(500 ng/ml). Cell viability was determined 16h after treatment. (B) C57BL/6J macrophages were treated with nothing (control), PG, PGA, or LPS in the presence or absence of PA+LF for 16h.
TNF concentrations in cultural medium were measured by ELISA. (C) C57BL/6J macrophages were treated with PA+LF+TNF, PA+LF+LPS, PA+LF+PG, or PA+LF+PGA in the presence or absence of anti-TNF antiserum (1:500 dilutions) for 16h. The extent of cell death was analyzed. The percentage of inhibition of cell death was calculated by (cell death in the absence of antibody – cell death in the presence of antibody)/cell death in the absence of antibody. (D) Macrophages from C57BL/6J mice were treated with human TNF (hTNF, 500 pM) or murine TNF (mTNF) in the presence of PA+LF. Cell viability was determined 16h after treatment. (D) Macrophages from C57/ScSn or C57/10ScCr mice were treated with PA+LF, PA+LF+LPS, or PA+LF+mTNF. Cell viability was determined 16h after treatment.

**Figure 5.** Dose curve of LF in mediating cell death of TNF-activated C3H/HeN- and C57BL/6J-macrophages. C3H/HeN-macrophages (A) or C57BL/6J-macrophages (B) were treated with PA(500 ng/ml) and different dose of LF in the presence or absence of TNF(500 pM) for 16h. Cell viability was measured.

**Figure 6.** TNF+LeTx-induced death of resistant macrophages requires protease activity of LF and is independent from caspases. (A) C57BL/6J-macrophages were treated with nothing (control), PA, LF, LF(E687C), PA+LF, or PA+LF(E687C) in the presence of TNF (500 pM). Cell viability was measured 16h after treatment. (B) C57BL/6J-macrophages were treated with nothing (control), TNF, zVAD, PA+LF, and their combinations. Cell viability was measured 16h after treatment. (C) C57BL/6J macrophages were treated with TNF+PA+LF in the presence
of U0162 (10 μM), SB203580 (20 μM), JNK inhibitor 2 (8 μM), bisindolylmaleimide II (3 μM), genistein (50 μM), herbimycin (100 ng/ml), wortmannin (1 μM), LY294002 (10 μM), rapamycin (20 nM). Cell viability was measured 16h after treatment. (D) C57BL/6J macrophages were treated with or without TNF in the presence of U0162 (10 μM), SB203580 (20 μM), JNK inhibitor 2 (8 μM), NF-κB SN50 cell- permeable inhibitor peptide (18 μM), bisindolylmaleimide II (3 μM), LY294002 (10 μM) and their combinations. Cell viability was measured 16h after treatment.
A

% of survival

Control  Virus  Zymosan  PG  LPS  PGA

+PA/LF

B

% of survival

Control  PA  LF  PA/LF  Control  PA  LF  PA/LF  Control  PA  LF  PA/LF

+LPS  +PG  +PGA

C

% of survival

0  5  10  15  20  25

Time (h)

0  50  100
A

C3H/HeN

Control

PA+LF+LPS

C57BL/6J

Control

PA+LF+LPS

LPS+IFN-γ

B

C

% of annexin V Positive Cells

C3H/HeN

C57BL/6J

Control

PA+LF

PA+LF+LPS

LPS+IFN-γ

Control

PA+LF

PA+LF+LPS

LPS+IFN-γ

0

10

20

30