

Report

Anthrax Lethal Toxin Kills Macrophages in a Strain-Specific Manner by Apoptosis or Caspase-1-Mediated Necrosis

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KEY WORDS

anthrax, lethal toxin, caspase-1, necrosis, apoptosis, macrophage, interleukin 18, inflammasome

ABBREVIATIONS

LT	lethal toxin
LF	lethal factor
PA	protective antigen
MAPKK	mitogen activated kinase kinase
BMM	bone marrow derived macrophages
DC	dendritic cell
PBMC	peripheral blood mononuclear cells
LPS	lipopolysaccharides
MDM	monocyte derived macrophages

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ABSTRACT

Murine macrophages have been classified as either susceptible or nonsusceptible to killing by anthrax lethal toxin (LT) depending upon genetic background. While considered resistant to LT killing, we found that bone marrow-derived macrophages (BMMs) from DBA/2, AKR, and C57BL/6 mice were slowly killed by apoptosis following LT exposure. LT killing was not restricted to in vitro assays, as splenic macrophages were also depleted in LT-injected C57BL/6 mice. Human macrophages, also considered LT resistant, similarly underwent slow apoptosis in response to LT challenge. In contrast, LT triggered rapid necrosis and broad protein release in BMMs derived from BALB/c and C3H/HeJ, but not C57BL/6 mice. Released proteins included processed interleukin-18, confirming reports of inflammasome and caspase-1 activation in LT-mediated necrosis in macrophages. Complete inhibition of caspase-1 activity was required to block LT-mediated necrosis. Strikingly, minimal residual caspase-1 activity was sufficient to trigger significant necrosis in LT-treated macrophages, indicating the toxicity of caspase-1 in this process. IL-18 release does not trigger cytolysis, as IL-18 is released late and only from LT-treated macrophages undergoing membrane perturbation. We propose that caspase-1-mediated macrophage necrosis is the source of the cytokine storm and rapid disease progression reported in LT-treated BALB/c mice.

INTRODUCTION

Bacillus anthracis, the causative agent of anthrax disease, produces two exotoxins, lethal toxin (LT) and edema toxin (ET), which are the primary cause of morbidity and mortality associated with anthrax infections.^{1,2} The bipartite LT is composed of lethal factor (LF) and protective antigen (PA). Injection of LT into mice produces morbidity similar to that observed in mice following *B. anthracis* challenge.^{3,4}

LF is a zinc-dependent metalloprotease, and its proteolytic activity is required for LT-mediated killing of specific target cells and pathogenicity in LT-treated mice.⁵⁻⁸ LF specifically cleaves mitogen-activated protein kinase kinases (MAPKKs), thereby disrupting three MAPK signaling pathways.⁹⁻¹³ Due to the ubiquitous expression of anthrax toxin receptors (ATRs),¹⁴⁻¹⁶ LT uptake and subsequent MAPKK cleavage occur in all mammalian cells tested.¹⁷ Despite the broad entry and activity of LT, it selectively kills only a few cell types.^{2,18-24} As MAPKK cleavage also occurs in cells that are not killed by LT, MAPKK cleavage is not sufficient for LT killing, and possibly not even required for this process.²⁴

We have previously shown that human and murine dendritic cells (DCs) are killed by LT in vitro.¹⁸ LT killing of murine dendritic cells is dependent upon their genetic background. DCs derived from the BALB/c strain undergo rapid necrosis following LT treatment, while DCs from the C57BL/6 strain and human PBMCs die by slow apoptosis upon LT exposure.¹⁸ A corresponding depletion of splenic DCs in LT-treated mice indicated in vivo killing of these cells.¹⁸

LT-mediated killing of antigen-presenting cells, including macrophages and dendritic cells, presumably leads to impairment of immunity. LT-mediated MAPKK cleavage, which renders specific cells nonresponsive to stimulation by mitogens, might further diminish the immune response.^{11,25-27} Immune impairment likely promotes bacterial proliferation in hosts infected with *B. anthracis*.²⁸⁻³¹

The susceptibility of murine macrophages to LT-mediated killing is strain-dependent. For example, C3H/HeJ and BALB/c-derived macrophages, the prototypical cells in anthrax research, are highly susceptible to killing by LT.²⁴ LT treatment of these

macrophages triggers rapid induction of necrosis, which normally occurs within 2 to 4 hours of treatment.²⁴ In contrast, macrophages from C57BL/6 mice have largely served as the prototype for LT-resistant macrophages.^{24,32-34}

A recent report suggested that a highly polymorphic gene, Nalp1b, controls LT susceptibility in murine macrophages.³² Nalp1b is a critical immune protein that functions in assembling the inflammasome, a multimeric complex, which contains and activates caspase-1.^{32,35} Macrophages deficient in caspase-1 show resistance to LT-mediated necrosis.³² However, the theory that Nalp1b and caspase-1 control LT-induced necrosis in murine macrophages is challenged by studies using caspase-1 inhibitors, which failed to block LT killing.^{18,36,37}

Here we show that macrophages derived from C57BL/6, DBA/2 and AKR strains, previously described as "resistant" to LT-mediated cell killing, are actually susceptible to LT-induced cell death by apoptosis. This finding appears to be applicable in vivo, as LT triggered depletion of splenic macrophages in C57BL/6 mice. We found that LT induced rapid necrosis in macrophages derived from BALB/c and C3H/HeJ strains, and a depletion of macrophage-like Kupffer cells in LT-injected BALB/c mice. LT triggered processing and a passive release of interleukin 18 (IL-18) in BALB/c and C3H/HeJ macrophages. IL-18 processing and LT killing was blocked by complete caspase-1 inhibition, indicating the caspase-1 dependence of these processes.

MATERIAL AND METHODS

Animals, cell culture and reagents. C57BL/6, C3H/HeJ, BALB/c, AKR and DBA/2 mice were obtained from Jackson Laboratories (Bar Harbor, MN). Murine bone marrow-derived macrophages (BMMs) were maintained in DMEM supplemented with 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 0.1 mM MEM non-essential amino acids, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% FBS and 10% L929 preconditioned media. The J774A.1 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA), and maintained in DMEM supplemented with 2 mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FBS. Human macrophages were cultured in RPMI Medium 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 2 mM L-Glutamine, 55 µM 2-mercaptoethanol, 0.1 mM MEM nonessential amino acids, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% human AB serum (Gemini Bio-Products, Woodland, CA) and 10 ng/ml Human M-CSF (PeproTech, Inc, Rocky Hill, NJ). Caspase inhibitors Ac-YVAD-cmk, zVAD-fmk, Boc-D-fmk, and Boc-D-cmk (Calbiochem, San Diego, CA) were reconstituted in DMSO and used at a concentration of 40 µM. Recombinant anthrax LF and PA were obtained from List Biological Laboratories (Campbell, CA). PA and LF were reconstituted in water, and were used at 500 ng/ml PA and 250 ng/ml LF. LPS stimulation was performed using 1 µg/ml of pure LPS from *E. coli*, serotype EH100 (Alexis Biochemicals, Lausen, Switzerland).

Generation of murine BMMs. BMMs were prepared as described previously.³⁸ Bone marrow cells were flushed from femora and tibiae of C57BL/6, C3H/HeJ, BALB/c, AKR and DBA/2 mice. Cells were differentiated into macrophages (BMMs) by incubation for six days in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 2.0 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 0.1 mM MEM non-essential amino acids, 10% FBS) and 20% conditioned medium

from a confluent culture of L929 fibroblasts as a source of CSF-1 (LCCM). After removal of non-adherent cells, macrophages were recovered by washing plates with cold PBS containing 5 mM EDTA. BMMs were assayed by flow cytometry using standard methods to verify the macrophage phenotype. Murine BMMs were uniformly positive for F4/80-FITC (clone BM8, Cell Sciences, Canton, MA) and CD11b-APC (clone M1/70, ATCC) staining.

Generation of human macrophages. Human leukocyte concentrates obtained from volunteer blood donors were separated over a Ficoll-Paque™ PLUS gradient (Amersham Biosciences AB, Uppsala, Sweden) to yield peripheral blood mononuclear cells (PBMC). Adherent monocytes were obtained by culturing PBMC (2 x 10⁷/plate) in serum-free DMEM for 1 hour at 37°C. Medium containing nonadherent cells was removed by aspiration, and plates were washed twice with 10 ml DPBS to remove any residual nonadherent cells. Medium was then replaced with 10 ml fresh RPMI 1640 supplemented with 2 mM L-Glutamine, 55 µM 2-mercaptoethanol, 0.1 mM MEM non-essential amino acids, 10 mM Hepes, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% human serum AB (Gemini Bio-Products) and 20 ng/ml human M-CSF (PeproTech). Following a 5-day incubation period, nonadherent cells were removed, and human macrophages were recovered by washing plates with cold PBS containing 5 mM EDTA. Human macrophages were assayed by flow cytometry, and were uniformly positive for CD11b/Mac-1-APC (clone ICRF44, BD Pharmingen, San Diego, CA) staining.

Cell death and viability assays. Cell viability was analyzed by MTT assay as described previously.^{18,45} Cells were either untreated or exposed to LT (500 ng/ml PA + 250 ng/ml LF) for the lengths of time indicated. Following LT treatment, MTT solution (5 mg/ml MTT in PBS) was added directly to wells and incubated at 37°C for 4 hours. The reaction was stopped, and the dye solubilized, using MTT solubilization solution (25 mM HCl and 0.5% SDS in isopropanol). Absorbance was measured at 570 nm.

Cell death was analyzed by trypan blue exclusion, performed in triplicate for each group. Media was removed from cells following LT treatment, and cells were suspended in 0.4% trypan blue solution (Sigma-Aldrich, St. Louis, MO) in DPBS. The suspension was placed on a hemacytometer, and trypan blue exclusion was determined by the fraction of cells remaining unstained. TUNEL assays were performed as previously described.³⁹

For analysis of membrane integrity, cells were detached in a solution of 2.6 µM TO-PRO-3 (Invitrogen, Carlsbad, CA) in PBS. TO-PRO-3 was quantified by flow cytometry.

Western blotting. Western blotting was performed on both cell culture supernatants and cell lysates. For collection of supernatants, 2.5 x 10⁵ cells were plated in 24-well plates in 250 µl of media, followed by treatment with LT and caspase inhibitors. At indicated time points, plates were centrifuged at 200 g for 10 minutes at 4°C. Supernatants were collected, and passed through 0.22 µm PES syringe filters. 200 µl of filtered supernatant was added to 200 µl of SDS sample buffer, and incubated at 100°C for 3 minutes. 15 µl of supernatant preparation were then run on 15 well BioRad SDS-Tris HCl polyacrylamide gels (Bio-Rad, Hercules, CA), and transferred to PVDF membranes (Amersham, Piscataway, NJ). For collection of cell lysates, 2.5 x 10⁵ cells were plated in 24-well plates in 500 µl of media, followed by treatment with LT and caspase inhibitors. At indicated time points, media were removed, and the cells were lysed in RIPA buffer supplemented with a cocktail of protease inhibitors (Roche, Basel, Switzerland). Lysate was incubated with SDS sample buffer at 100°C for 3 minutes. 20 µg of protein in 15 µl of lysate was size

fractionated on 15-well SDS-Tris HCl polyacrylamide gels (BioRad, Hercules, CA), and transferred to PVDF membranes (Amersham).

Membranes were probed with an anti-MEK-3 polyclonal antibody, (Santa Cruz Biotechnology, Santa Cruz, CA; #Sc-960) anti-actin monoclonal antibody, (Sigma; #Ac-40) and anti-murine IL-18 polyclonal antibody (BioVision, Mountain View, CA.) Polyclonal HRP-conjugated anti-rabbit antibodies were used as secondary antibodies (Santa Cruz Biotechnology; #Sc-2313), and blots were developed using ECL Plus solution (Amersham).

Analysis of splenic macrophages. Eight to ten-week old C57BL/6 female mice were anesthetized by isoflurane inhalation, and were subsequently injected intraperitoneally with 1 mL of PBS or 100 μ g of LT (100 μ g PA + 100 μ g LF) in 1 ml of sterile PBS using a 1 ml syringe fitted with a 27-gauge needle. Mice were sacrificed 24 hours post injection. Spleens from PBS or LT-treated mice were mechanically dissociated following collagenase D treatment to generate single-cell suspensions in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% FBS. The resulting splenocyte suspensions were then washed and resuspended in RPMI-1640 medium supplemented with 10 mM HEPES, 2.0 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 0.1 mM MEM nonessential amino acids, 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin. Aliquots of these splenocyte suspensions were immediately used for determination of total splenocyte cell counts and analysis by flow cytometry.

Analysis of kupffer cells. Pairs of eight-week old male BALB/c and C57BL/6 mice were given intravenous tail vein injections of 35 μ g LT (35 μ g LF + 35 μ g PA) or in the case of two C57BL/6 mice, 100 μ g LT (100 μ g LF + 100 μ g PA) or PBS, and sacrificed at 6, 16, 24, 30, 48 and 72 hours. Tissues were harvested and placed in 10% formalin for 24 hours, followed by two washes with PBS, and storage in 70% ethanol overnight at 4°C. Tissues were processed by ethanol dehydration and embedded in paraffin by Histoserv Inc (Gaithersburg, MD) according to standard protocols. Kupffer cell levels were determined by immunohistochemistry.

Immunohistochemistry. Immunohistochemistry was performed as described previously.⁴⁰ Four to five μ m sections of paraffin-embedded liver were placed on Super-Frost Plus slides (Fisher, Pittsburgh, PA) for immunohistochemistry. Paraffin sections were deparaffinized in xylene, hydrated through a graded series of ethanol washes and placed in PBS. Trypsin digestion (Sigma) was performed for 30 minutes at 37°C. Sections were rinsed with water, and endogenous peroxidase activity was quenched by incubation with peroxidase blocking reagent (DAKO, Glostrup, Denmark). Sections were incubated overnight with monoclonal anti-F4/80 (BMA, #T-2008). After being washed, the slides were incubated with a biotin-conjugated anti-Rat IgG antibody (Vector Laboratories, Burlingame, CA). The slides were then incubated with horseradish peroxidase-conjugated streptavidin (DAKO). Bound antibodies were visualized using diaminobenzidine as the substrate. The sections were then counterstained with hematoxylin and dehydrated. The slides were mounted with a xylene-based mounting medium, Micromount, (Surgipath, Richmond, IL) and analyzed with an Olympus IX 70 inverted microscope.

Flow cytometry. Events were acquired on a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA) or a Becton Dickinson LSR II, and were analyzed using WinMDI (Windows Multiple Document Interface for Flow Cytometry).

ELISA. TNF release in response to LPS was quantified using an ELISA assay, as described previously.⁴¹ The ELISA was performed according to manufacturer's instructions (eBioscience, San Diego, CA).

RESULTS

Susceptibility of murine macrophages to LT killing. Macrophages from different murine strains have been traditionally characterized as either susceptible or nonsusceptible to LT killing: bone marrow-derived macrophages from BALB/c mice have been described as highly susceptible, while those from C57BL/6 mice were considered resistant to LT killing.^{32,33} In recent years a more nuanced picture has emerged. Macrophage susceptibility to LT killing appears to be dependent upon maturation state, and certain external stimuli, like lipopolysaccharide (LPS), can confer susceptibility to C57BL/6 macrophages.^{24,42,43} Moreover, we recently showed significant LT killing of another myeloid lineage cell type, dendritic cells (DCs), derived from BALB/c and C57BL/6 mice, as well as human PBMCs.¹⁸

Due to the similar responses of macrophages and DCs from BALB/c mice to LT treatment, we hypothesized that, like C57BL/6 DCs, C57BL/6-derived macrophages may be susceptible to LT-mediated cell killing. Previous studies analyzing the susceptibility of these macrophages have not looked for cell killing beyond 16 hours post-LT treatment.^{24,33} To test the LT susceptibility of C57BL/6, AKR and DBA/2-derived macrophages, which are considered resistant to LT, we generated BMMs from these strains. As positive controls, we generated macrophages from BALB/c and C3H/HeJ mice, which have been described as LT-sensitive.^{24,32,33}

Isolated primary BMMs were either untreated or treated with a dose of LT cytotoxic to BALB/c and C57BL/6 DCs (250 ng/ml LF and 500 ng/ml PA). Consistent with findings obtained with C57BL/6-derived DCs,¹⁸ BMMs from C57BL/6, AKR and DBA/2 strains were susceptible to LT-mediated cell killing. These macrophages died with similar kinetics: 80% of C57BL/6 and DBA, and 60% of AKR BMMs were killed within three days of LT treatment, as determined by MTT assay (Fig. 1B and C). LT killing kinetics of C57BL/6, AKR, and DBA/2 BMMs mirrored those described for C57BL/6 DCs. Consistent with previous reports,²⁴ BALB/c and C3H/HeJ-derived BMMs were killed within two hours of LT treatment (Fig. 1A).

We hypothesized that, as reported for murine DCs,¹⁸ slowly killed murine macrophages die by apoptosis, rather than the necrosis that is characteristic of rapid LT killing. As predicted, LT-treated C57BL/6, AKR and DBA/2 macrophages showed significant TUNEL staining, indicating cell death by apoptosis (Fig. 2 and data not shown). As expected, LT-treated BALB/c (Fig. 2) and C3H/HeJ (data not shown) macrophages remained TUNEL-negative. These data suggest that murine macrophages are susceptible to LT killing regardless of their genetic background, and should be divided according to cell death pathways activated by LT, and not by LT susceptibility.

LPS responsiveness of LT-treated C57BL/6 BMMs. It has been reported that LT treatment renders murine dendritic cells non-responsive to LPS by inactivating MAPK signaling pathways.^{11,25,26} Additionally, BALB/c-derived transformed macrophage lines are similarly nonresponsive to LPS stimulation following LT treatment.⁵¹ We predicted that LT exposure has the same effect on slowly killed murine macrophages, and impairs the LPS response prior to the onset of cytopathic effects. To test this, we exposed C57BL/6 BMMs to LT for four hours, and evaluated TNF release in response to LPS stimulation. Supernatants from these BMMs were collected 12 hours post-LPS stimulation, and assayed for TNF production using an ELISA capture assay. As expected, C57BL/6 macrophages pre-treated with LT did not respond to LPS, as indicated by control level TNF production (Fig. 3). In the absence of LT, macrophages

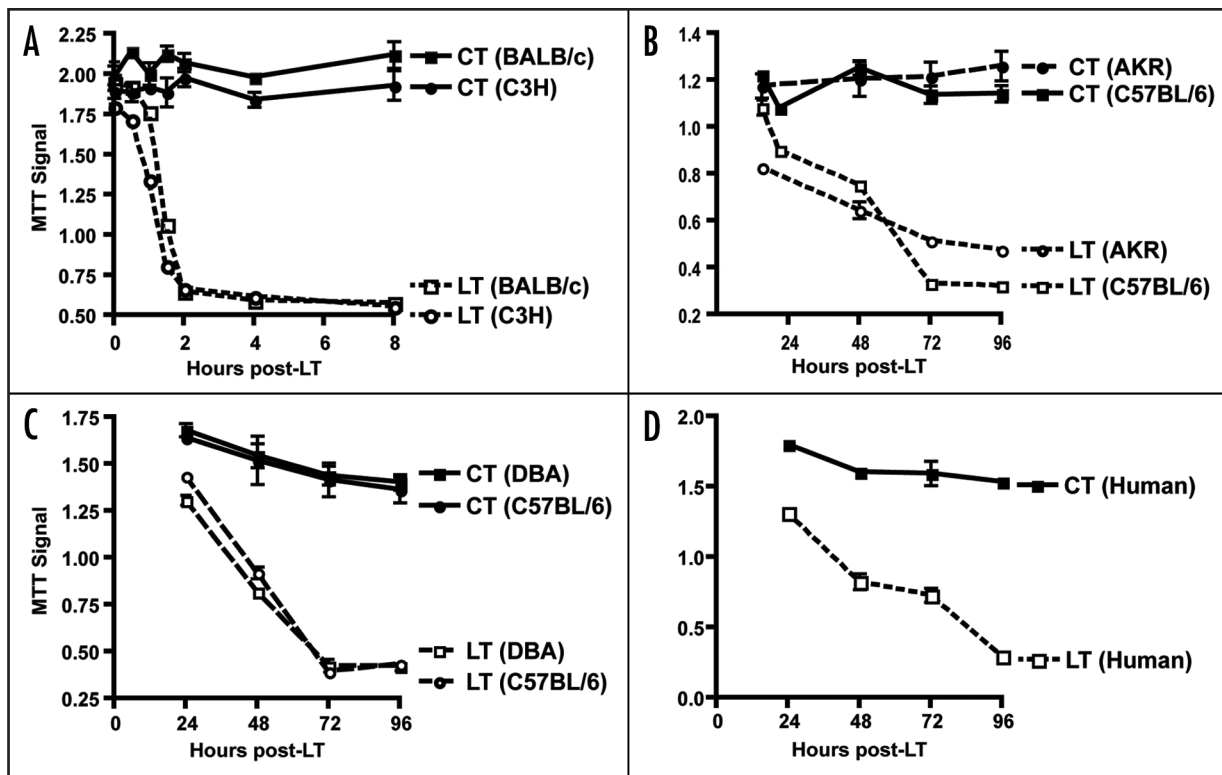


Figure 1. Killing of murine and human macrophages by LT. BMMs derived from BALB/c and C3H/HeJ (A) C57BL/6 and AKR (B) and DBA/2 (C) mice remained either untreated or were treated with LT, (500 ng/ml PA + 250 ng/ml LF) and cell viability was determined by MTT assay. (D) Human macrophages either remained untreated, or were treated with LT, and cell viability was determined by MTT assay. CT indicates untreated control cells.

released high levels of TNF following LPS exposure (Fig. 3). Taken together, LT treatment rendered C57BL/6 macrophages nonresponsive to LPS stimulation, indicating impairment of these cells prior to the onset of cytopathic effects.

Human macrophages are susceptible to LT-mediated cell killing. Unstimulated human macrophages are considered resistant to LT-mediated cell killing. We investigated whether human macrophages are, like human DCs,¹⁸ susceptible to slow LT-mediated cell killing. To test this, we generated monocyte-derived macrophages (MDMs) from human peripheral blood monocytes. Human MDMs were treated with LT, and cell killing was assayed by MTT and TUNEL assays. LT-treated MDMs were TUNEL-positive (data not shown), and died with similar kinetics as C57BL/6 BMMs (Fig. 1D). Taken together, our results showed that human and murine macrophages were susceptible to LT killing.

In vivo depletion of macrophages. A depletion of splenic macrophages has been described in BALB/c mice injected with LT.¹⁸ Because C57BL/6 macrophages are actually susceptible to LT killing in *in vitro* assays, we tested whether these cells are also depleted in C57BL/6 mice injected with LT. Towards this, we injected 10-week-old C57BL/6 mice intraperitoneally with LT (100 μ g LF + 100 μ g PA). Mice were sacrificed 24 hours post-injection, and bulk splenocytes were analyzed by flow cytometry. Indeed, a 75% depletion of splenic macrophages was noted in LT-injected C57BL/6 mice (Figs. 4A and B). This indicated that LT-mediated killing of C57BL/6 macrophages was not limited to *in vitro* systems, but also occurred in LT-injected C57BL/6 mice.

Similarly, we found depletion of macrophage-like Kupffer cells in LT-treated BALB/c mice. Kupffer cells are bone marrow-derived specialized macrophages in the liver.⁴⁴ Sections of BALB/c liver

collected at 16, 24, and 48 hours post-LT injection were devoid of Kupffer cells, while the six-hour sections appeared to have normal Kupffer populations (Fig. 4C). In contrast to BALB/c mice, we detected no depletion of Kupffer cells in C57BL/6 mice at any time point post-LT injection (data not shown). A lack of Kupffer cell depletion was observed in these mice even after intravenous challenge with increasing amounts of LT (35 and 100 μ g) (data not shown). Therefore, cytopathic effects did not extend to Kupffer cells following LT injection of C57BL/6 mice.

LT-mediated necrosis in BALB/c macrophages induces a passive release of cytokines. A spike in the levels of peripheral blood inflammatory cytokines has been observed in BALB/c and C3H/HeJ strains following LT injection, but not in C57BL/6, AKR, and DBA/2 strains.^{3,33} Strikingly, the release of cytokines is restricted to mice harboring macrophages susceptible to rapid LT-mediated necrotic cell death (BALB/c and C3H/HeJ strains): no cytokine release was observed in mice containing macrophages that undergo apoptosis induction following LT exposure (C57BL/6 and AKR strains).³³ These results suggest that the means of macrophage killing, specifically the induction of necrosis, is responsible for the cytokine spike.

To test the role of the two LT-mediated cell death pathways on cytokine release, we analyzed protein release from LT-treated BALB/c, C3H/HeJ, C57BL/6, and AKR BMMs. As predicted, we found that LT-treated BALB/c and C3H/HeJ BMMs release a broad range of proteins following LT exposure. Starting at 1.5 hours, and reaching maximal levels 2 hours post-LT exposure, we detected an efflux of the structural protein actin into the supernatant of LT-treated BALB/c and C3H/HeJ BMMs (Fig. 5A). This release coincided perfectly with the kinetics of membrane perturbation in these cells, as determined by propidium iodide exclusion (data not shown). We detected

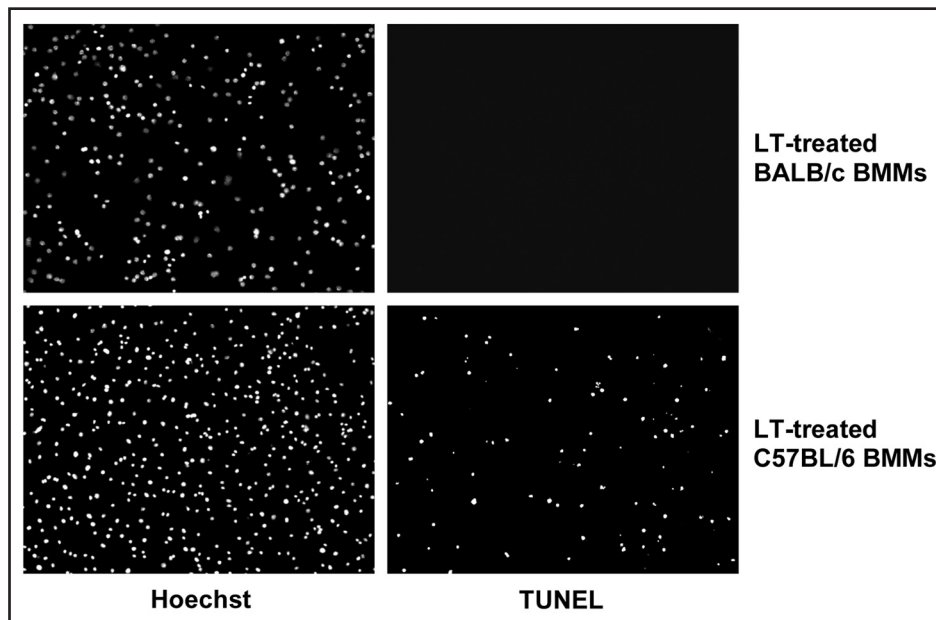


Figure 2. LT triggers apoptosis in C57BL/6-derived macrophages. BALB/c and C57BL/6 BMMs were treated with LT for 6 and 48 hours, respectively, and apoptosis was determined by TUNEL staining. Hoechst staining was used to visualize the total number of macrophages per field.

no protein release in either C57BL/6 or AKR BMMs even after 72 hours of LT exposure, despite over 90% cell killing by that time point (Fig. 5A and B).

Concurrent with actin release, we found release of the constitutively expressed cytokine IL-18 from LT-treated BALB/c and C3H/HeJ macrophages (Fig. 5B). As expected, C57BL/6 and AKR macrophages did not release IL-18 at any time point following LT exposure. Intriguingly, LT-treated BALB/c and C3H/HeJ BMMs released both the active and unprocessed forms of IL-18, indicating IL-18 processing in these cells. This is consistent with previous reports of IL-18 processing in LT-treated macrophages.^{3,36} Active

caspase-1, which has been linked to LT killing,^{32,35} is required for IL-18 processing. Notably, IL-18 processing peaked 1-hour post-LT exposure (Fig. 5C), while IL-18 release occurred 2 hours post-LT treatment, concurrent with actin release (Fig. 5A and B). This indicated that the majority of the active IL-18 is passively released during cell lysis, and not immediately following processing.

IL-18 processing occurs with LT-induced necrosis, not apoptosis. We then tested whether caspase-1 activation and subsequent IL-18 processing is restricted to macrophages undergoing necrosis following LT exposure. To address this, we performed western blotting for IL-18 in lysates of LT-treated BALB/c and C57BL/6 BMMs. Indeed, IL-18 processing (indicating caspase-1 activation) occurred at 1-hour post-LT treatment in BALB/c BMMs, but did not happen in LT-treated C57BL/6 macrophages (Fig. 5C). Taken together, caspase-1 activation and IL-18 processing, as well as the subsequent protein release, was restricted to macrophages that undergo necrosis, but not apoptosis, following LT exposure. This may explain the strain-specific cytokine spike observed in LT-injected BALB/c mice.^{3,33}

Caspase-1 activation is a critical and necessary event in LT-induced necrosis. Processing of IL-18 indicated caspase-1 activation in LT-treated BALB/c macrophages. While macrophages derived from caspase-1-deficient mice show LT resistance, broad caspase inhibitors fail to block rapid cell death in LT-treated hematopoietic cells.^{18,32,36,37} To reconcile these findings, we applied a panel of caspase-1 inhibitors to LT-treated J774A.1 macrophages, and assayed cell viability by trypan blue exclusion. The BALB/c-derived macrophage cell line J774A.1 undergo, like their corresponding primary cells, necrosis in response to LT challenge.^{36,45,46} The inhibitors tested included the broad caspase inhibitors zVAD-fmk and Boc-D-fmk, as well as the caspase-1 specific inhibitors Boc-D-cmk and Ac-YVAD-cmk. Intriguingly, of all caspase inhibitors tested, only Boc-D-cmk blocked LT-induced necrosis, while zVAD-fmk, Ac-YVAD-cmk, and Boc-D-fmk offered only intermediate, or no protection (Fig. 6A).

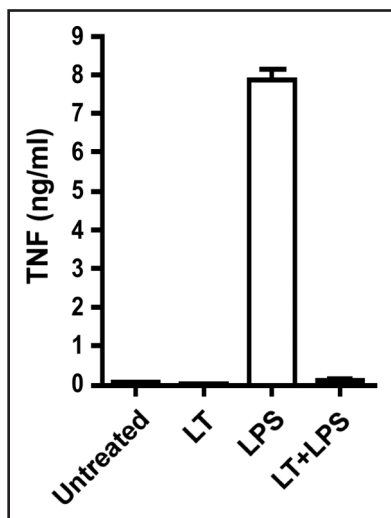


Figure 3. LT treatment impairs the LPS response in macrophages. C57BL/6 BMMs remained untreated, or were pre-treated with media only or with LT for four hours, followed by addition of 1 µg/ml LPS. Supernatants were collected 12 hours post-LPS treatment, and TNF release was quantified by ELISA capture assay.

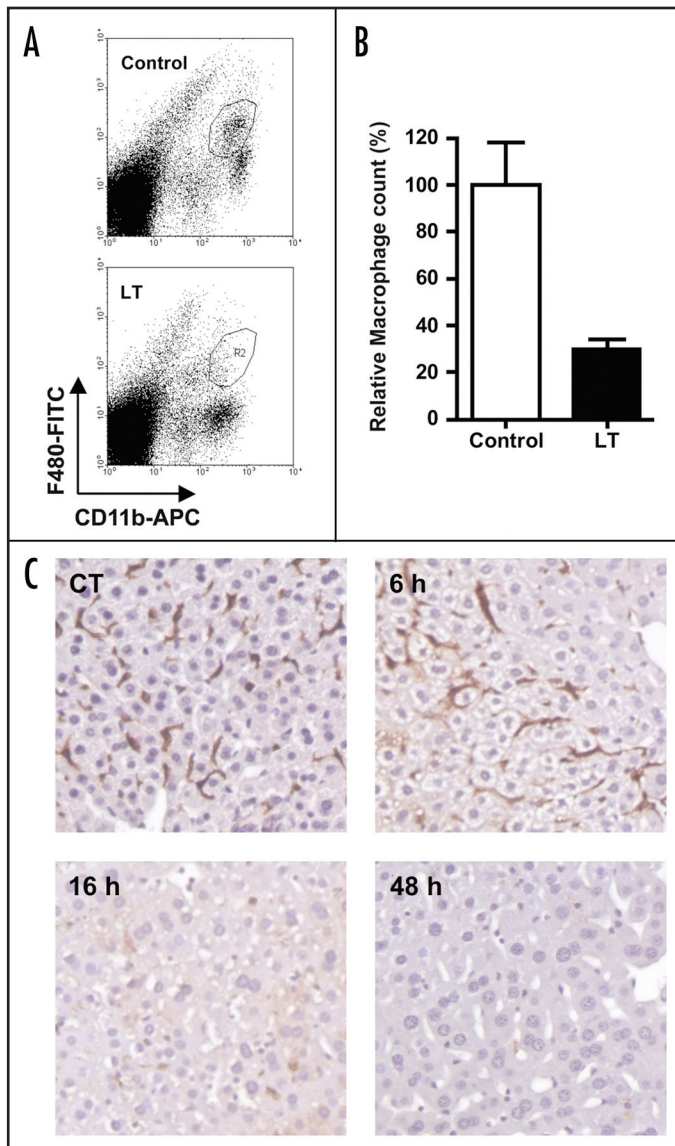


Figure 4. In vivo macrophage depletion following LT exposure of BALB/c and C57BL/6 mice. (A and B) C57BL/6 mice were injected with LT, and levels of macrophages in the spleens 24 hours post-injection were determined by flow cytometry. A representative experiment is shown (A), as well as data averaged for three independent experiments (B). (C) Kupffer cell depletion post-LT injection in BALB/c mice, as determined by immunohistochemistry using anti-F4/80 staining. Representative experiments were performed in triplicates.

We established the potency of Boc-D-cmk at inhibiting LT-mediated necrosis by applying the inhibitor to LT-treated J774A.1 macrophages at a range of concentrations, from 1–10 μ M. Necrosis was assessed two hours post-LT treatment by flow cytometry measuring staining with the membrane impermeable DNA-intercalating dye TO-PRO-3. As little as 1 μ M Boc-D-cmk was sufficient to prevent most LT induced necrosis, and concentrations greater than 2.5 μ M provided complete protection against LT-killing (Fig. 6B).

To rule out the possibility that Boc-D-cmk interfered with uptake or enzymatic activity of LF, we treated J774A.1 macrophages with the caspase inhibitors, followed by LT exposure. LT uptake and LF activity was tested by analysis of MKK-3 cleavage using immunoblotting. Complete MKK-3 cleavage was observed in LT-treated

macrophages in the presence of all four caspase inhibitors, indicating that these do not interfere with LF uptake and activity (Fig. 6C).

We then tested whether the differences in the ability of caspase inhibitors to block LT killing stemmed from varying degrees of inhibitor efficacy. Towards this, the inhibitors were applied to LT-treated J774A.1 macrophages. Western blotting using antibodies against IL-18 was performed to analyze caspase-1 activity in the presence of different caspase inhibitors. Indeed, only Boc-D-cmk completely blocked IL-18 processing, while the others showed only partial inhibition of caspase-1 (Fig. 6D). These results indicated that LT killing was dependent upon caspase-1 activation, and that complete caspase-1 inhibition was required to block LT-mediated necrosis.

DISCUSSION

Here we show that macrophages isolated from C57BL/6, DBA/2 and AKR mice, which are associated with resistance to LT killing, were efficiently killed by LT treatment. Human macrophages, also considered resistant to cell killing by LT, died similarly following LT-treatment. The kinetics and cell death pathways associated with LT treatment of C57BL/6, DBA/2, AKR and human macrophages were markedly different from those obtained with macrophages considered highly susceptible to LT killing. Rather than dying by rapid necrosis like BALB/c and C3H/HeJ macrophages, C57BL/6, DBA/2, AKR, and human macrophages died by slow apoptosis. Slow LT killing kinetics are not restricted to human macrophages and specific murine macrophage strains, but have also been observed in endothelial cells and C57BL/6-derived dendritic cells.^{18,21} In the case of C57BL/6 macrophages, these findings were not restricted to in vitro assays, as splenic macrophages were also depleted in LT-injected C57BL/6 mice. These findings suggest that macrophages, regardless of their genetic background, are susceptible to LT killing. We propose that macrophages previously termed resistant to LT toxicity should instead be considered resistant to rapid necrosis.

Susceptibility of macrophages to rapid necrosis is likely a critical determinant of disease progression. Unprocessed and processed inflammatory cytokines were released from LT-treated necrotic macrophages, but were conspicuously absent in supernatants from macrophages undergoing apoptosis. Our results suggest that the cytokine release observed in BALB/c mice following LT exposure^{3,33} is caused by a necrotic burst of hematopoietic cells. Released cytokines from LT-treated BALB/c, but not from C57BL/6 macrophages, likely cause a permeability shift in endothelial beds, and might contribute to the vascular collapse observed in LT-injected BALB/c mice.³ Cytokine release may also account for the enhanced disease progression in LT-treated BALB/c mice when compared to C57BL/6 mice. This is further supported by a previous study, which showed that C57BL/6 mice become more susceptible to LT-induced mortality when they express susceptible macrophages.³³

Our findings are consistent with previous studies which showed that caspase-1 is activated in LT-treated macrophages undergoing necrosis.^{32,36} While caspase-1 deficient macrophages show reduced sensitivity to LT-mediated necrosis,^{32,35} several studies suggest that caspase activation, while associated with LT-induced necrosis, is not a required event in this process.^{36,37,45} In this study, we were able to address this issue, and found that complete inhibition of caspase-1 was needed to block rapid LT-killing. We found that the caspase-1 inhibitor Boc-D-cmk blocked IL-18 processing, as well as subsequent necrosis, in LT-treated macrophages. Therefore, caspase-1 activation

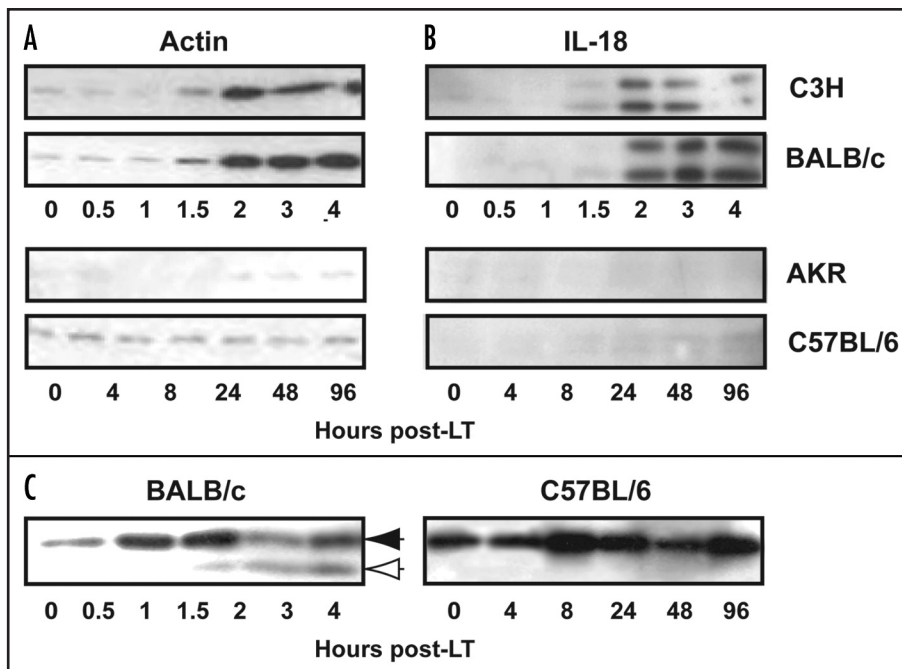


Figure 5. Release of cytoplasmic proteins and activation of caspase-1 is associated with necrosis induction in LT-treated macrophages. (A and B) Supernatants from C3H/HeJ, BALB/c, AKR, and C57BL/6 BMMs were collected at the indicated time points post-LT treatment. Supernatant levels of actin (A) and IL-18 (B) were determined by immunoblotting. (C) Lysates were collected from LT-treated BALB/c and C57BL/6 BMMs, and IL-18 processing was determined by immunoblotting. Pro-IL-18 and activated IL-18 are indicated by black and white arrows, respectively.

and LT-mediated necrosis appear to be directly linked, making the processing of inflammatory cytokines a fundamental hallmark of LT-induced rapid macrophage cell killing. Our findings suggest that caspase-1 activation is required for LT induced necrosis, which is consistent with reduced LT sensitivity of caspase-1-deficient murine macrophages.³² We also found that complete inhibition of caspase-1 activity was required to prevent LT-mediated necrosis of macrophages. It is surprising that residual caspase-1 activity is sufficient to kill these macrophages, considering that only a minor fraction of the enzyme is activated in LT-treated macrophages. This indicates that caspase-1 activation is a highly toxic event in these cells.

Genetic mapping and inhibition studies identified the inflammasome protein Nalp1b as the genetic determinant for LT sensitivity of murine macrophages. Expression of the *Nalp1b* allele from susceptible murine strains in 'LT-resistant' mice confers macrophage sensitivity to LT-mediated necrosis.³² Strikingly, the human ortholog NALP1 controls caspase-1 activation,⁴⁷⁻⁴⁹ suggesting that murine Nalp1b has a similar function. It remains to be shown how LT activates the inflammasome and caspase-1. It is also unclear how caspase-1 activation triggers cell death. It is conceivable that interleukin processing is involved in cytopathic effects. However, efficient LT killing occurs in the presence of protein biosynthesis inhibitors,⁴⁵ and cytokines are released only after cytolysis. This is consistent with the high susceptibility of macrophages lacking IL-1 β or IL-18 to caspase-1-mediated necrosis.⁵⁰ These findings indicate that cytokine release is associated with, but alone does not induce lysis. This would suggest that caspase-1 targets proteins other than interleukins to promote cytolysis in LT-treated macrophages. Future experiments might identify caspase-1 targets responsible for induction of necrosis.

While caspase-1 was not activated at any point during LT-induced macrophage apoptosis, other caspases might be involved in LT killing

of C57BL/6 macrophages. We have recently shown that caspase-3 is activated in C57BL/6-derived DCs following LT exposure.¹⁸ It is reasonable to assume that caspase-3 activation triggers apoptosis in C57BL/6 macrophages and DCs, while caspase-1 activation drives necrosis of BALB/c macrophages following LT exposure.

It is conceivable that both cell-death pathways, necrosis and apoptosis, are activated in LT-treated BALB/c macrophages. Rapid necrosis took place within one hour following inflammasome activation in these cells. We assume that should inflammasome assembly not occur in BALB/c macrophages following LT treatment, these cells would undergo the slow apoptosis observed in C57BL/6 macrophages. Presumably, the dominance of the necrotic pathway in cells exhibiting inflammasome activation was due to the rapid killing kinetics. These findings may shed light on the mechanism behind a previously reported phenomenon. Stimulation of C57BL/6 macrophages prior to LT treatment has been shown to confer susceptibility to rapid LT killing.^{24,43} As stimulation of macrophages, particularly by lipopolysaccharides, can induce caspase-1 activation, we hypothesize that inflammasome activation prior to LT treatment might be responsible for the increase in LT susceptibility following LPS stimulation.

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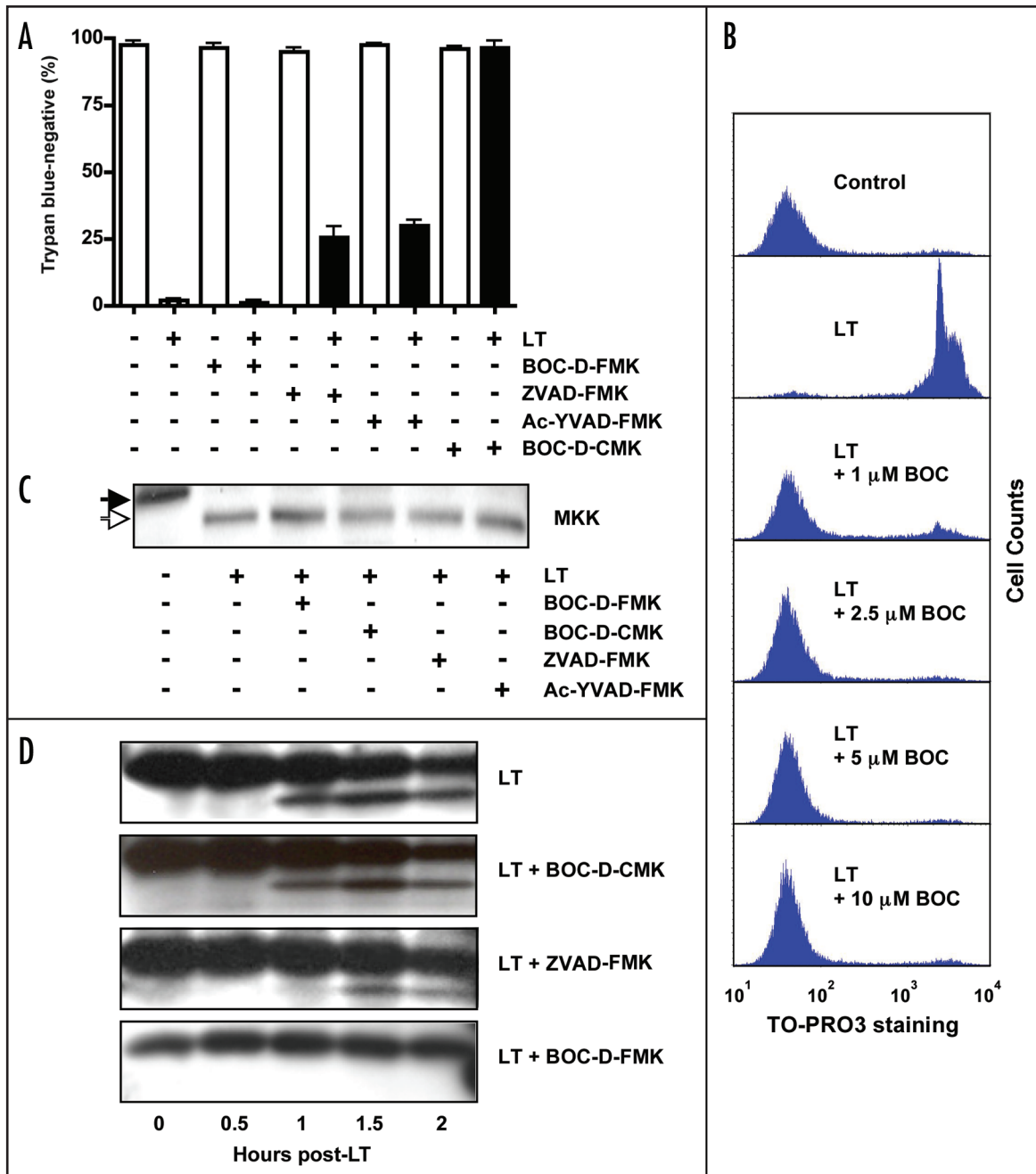


Figure 6. Caspase-1 inhibition blocks LT-mediated necrosis. (A) LT-induced necrosis in J774A.1 macrophages in the presence of the pan-caspase inhibitors Boc-D-fmk and zVAD-fmk, and the caspase-1 inhibitors Ac-YVAD-cmk and Boc-D-fmk, was determined by trypan blue staining three hours post-LT treatment. (B) Survival of LT-treated J774A1 macrophages was determined in the presence of increasing amounts of Boc-D-cmk using flow cytometry with TO-PRO3 staining. (C) LT-mediated MEK3 cleavage in the presence of the caspase-inhibitors was determined by immunoblotting. Cells were lysed after 1-hour post LT-treatment. Uncleaved and cleaved MEK3 are indicated by black and white arrows, respectively. (D) LT-induced IL-18 processing by caspase-1 in the presence of caspase inhibitors. Immunoblotting of J774A.1 cells treated with LT was performed in the presence of Boc-D-fmk, zVAD-fmk, or Boc-D-cmk.

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