Leukotoxin (Leukothera®) Targets Active Leukocyte Function Antigen-1 (LFA-1) Protein and Triggers a Lysosomal Mediated Cell Death Pathway*

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Background: Leukotoxin is a bacterial protein that kills WBCs expressing the β2 integrin, leukocyte function antigen-1 (LFA-1).

Results: Leukotoxin binds active LFA-1 and induces lysosomal mediated cell death.

Conclusion: Leukotoxin kills activated WBCs by two mechanisms involving caspases and lysosomes.

Significance: Leukotoxin is the first bacterial toxin shown to be internalized and localized to the lysosomal compartment.

Leukotoxin (LtxA) is a protein toxin that is secreted from the oral bacterium, Aggregatibacter actinomycetemcomitans. LtxA targets specifically the β2 integrin, leukocyte function antigen-1 (LFA-1) on white blood cells (WBCs) and causes cell death. LtxA preferentially targets activated WBCs and is being developed as a therapeutic agent for the treatment of WBC diseases such as hemato logic malignancies and autoimmune/inflammatory diseases. However, the mechanism by which interaction between LtxA and LFA-1 results in cell death is not well understood. Furthermore, how LtxA preferentially recognizes activated WBCs is not known. We show here that LtxA interacts specifically with LFA-1 in the active (exposed) conformation. In THP-1 monocytes, LtxA caused rapid activation of caspases, but LtxA could overcome the inhibition of caspases and still intoxicate. In contrast, inhibiting the vesicular trafficking pathway or cathepsin D release from the lysosome resulted in significant inhibition of LtxA-mediated cytotoxicity, indicating a more potent, lysosomal mediated cell death pathway. LtxA caused rapid disruption of the lysosomal membrane and release of lysosomal contents into the cytosol. Binding of LtxA to LFA-1 resulted in the internalization of both LtxA and LFA-1, with LtxA localizing specifically to the lysosomal compartment. To our knowledge, LtxA represents the first bacterial toxin shown to localize to the lysosome where it induces rapid cell death.

Leukotoxin (Leukothera™; LtxA) is a naturally secreted repeats in toxin protein produced by the oral bacterium Aggregatibacter actinomycetemcomitans (for recent review, see Ref. 1). LtxA targets specifically the β2 integrin, leukocyte function antigen-1 (LFA-1) on white blood cells (WBCs) and causes cell death. The primary functional role of LFA-1, which is a heterodimer of CD11a and CD18, is to bind intercellular adhesion molecules (ICAM-1, -2, and -3) located on vascular endothelial cells (2). LFA-1 can only bind ICAM-1 after activation of the integrin by cell signaling molecules or divalent cations (3, 4). Activation of LFA-1 results in a change from a closed conformation to an open, exposed conformation on the surface of cells. Interaction between activated LFA-1 and ICAM-1 leads to adhesion and then migration of the WBCs out of the blood vessel and into the surrounding tissue.

Because of its high specificity for LFA-1, LtxA is being developed as a targeted biologic agent for the treatment of WBC diseases including hematologic malignancies (5), autoimmune/inflammatory diseases (6), and HIV infection (7). We showed that resting, nondiseased WBCs are minimally affected by LtxA, whereas malignant and activated cells become susceptible to killing by LtxA (5–7). In humanized animal models for leukemia and psoriasis, LtxA has demonstrated significant therapeutic efficacy with no observed toxicities (5, 6). LtxA is strongly synergistic with standard leukemia chemotherapeutics in vitro, demonstrating its potential utility in combination treatment to reduce chemotoxicity, resistance, and relapse (8). In nonhuman primates, LtxA was specific for a subset of WBCs and did not change red blood cell (RBC) counts, platelet counts, hemoglobin levels, or markers of toxicity (BUN, creatinine, alkaline phosphatase) (5). In addition, we recently showed that LtxA caused significant depletion of HIV-infected T cells ex vivo, and we proposed that LtxA may be able to purge HIV-infected cells from patients (7).

Several cellular changes are induced by LtxA such as loss of mitochondrial membrane potential and activation of caspases (9). LtxA-mediated death has been studied in several cell types, including 293 T cells infected with HIV, where LtxA strongly synergistic with standard leukemia chemotherapeutics.
and it is not clear whether LtxA intoxicates all cell types the same way. In Jurkat T cells, intoxication appears to begin with the clustering of LFA-1 in lipid rafts and modification of the cytoskeleton, leading to apoptosis (10). In JY B cells, LtxA was shown to cause the loss of mitochondrial membrane potential, production of reactive oxygen species, release of cytochrome c from the mitochondrial intermembrane space, and activation of caspases 3, 7, and 9 (11). Kelk et al. reported that LtxA lyases healthy monocytes by activation of inflammatory caspase 1 and causes release of IL-1β (12, 13). In addition, it is reported that at low doses, LtxA induces apoptosis whereas high doses of LtxA cause rapid necrosis of cells (14–16).

Typically, agents that bind integrins do not induce cell death. For example, interaction between LFA-1 and its natural ligands, such as ICAM-1, initiates a signaling cascade that leads to cellular adhesion and migration. Therapeutic anti-LFA-1 monoclonal antibodies, such as efalizumab (17), prevent binding and migration of WBCs, but are not cytotoxic. Another monoclonal antibody therapeutic, natalizumab, targets the α4 integrin and prevents migration of WBCs, but does not cause cell death (18).

In this study, we sought to determine how LtxA targets specifically malignant and activated WBCs and decipher how binding of LtxA to LFA-1, in contrast to other LFA-1-binding agents, can lead to cell death. We report here that LtxA recognizes specifically the active form of LFA-1 and is delivered to the lysosome, resulting in its leakage and loss of acidity. In the end, the interaction between LtxA and its host cell induces multiple cell death pathways. The importance of elucidating the biological mechanism underlying LtxA-mediated cell death is to understand better the clinical utility of the agent. This work also contributes to the understanding of the role that β2 integrins play in immune cell function and cell death.

**EXPERIMENTAL PROCEDURES**

**Primary Human Cells and Cell Lines**—Human peripheral blood “leukocyte units” from anonymous donors were provided by the New York Blood Center. WBCs were isolated as theuffy coat layer and were diluted with an equal volume of PBS in a separate tube. The dilutedbuffy coat layer was then layered slowly over the same volume of Ficoll (Lymphocyte Separation Medium; Cellgro) in a separate tube followed by centrifugation at 1,200 rpm for 30 min. Peripheral blood mononuclear cells and granulocyte layers were then collected together and washed once with PBS. The cells were finally resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Human cell lines (THP-1, Jurkat, RL) were purchased from ATCC and maintained in RPMI 1640 medium supplemented with 10% FBS at 37 °C, 5% CO2.

**Purification of Leukotoxin—**LtxA was purified from culture supernatants of strain A. actinomycetemcomitans NJ4500 as described previously (19).

**Inhibitors, Antibodies, and Reagents**—The inhibitors used were Z-VAD-FMK (R&D Systems), necrostatin-1, 3-methylad- enine (3-MA), 2-(1H-indol-3-yl)-3-pentylamino-maleimide (IM-54) (Calbiochem), and pepstatin A (Sigma). Cell death was measured using annexin V-FITC, propidium iodide, and 7-aminoactinomycin (7-AAD) (Biolegend). Caspase activation was determined using the polycaspase FAM-VAD-FMK FLICA reagent (Invitrogen). Lysosomal pH indicator LysoTracker Green DND-26 (Invitrogen) and lysosomal marker anti-human LAMP-1-PE (Biolegend) were used for flow cytometric analysis involving the lysosome. Monoclonal antibody to LtxA was isolated from mouse hybridoma cells after injecting BALB/c mice with purified LtxA (ProMab Biotechnologies). Anti-AIF-1 (Abgent), anti-LAMP-1 (Biolegend), anti-cathepsin D (BD Biosciences) primary antibodies were used for immunoblot analysis or flow cytometry. The secondary antibody used for detection of cathepsin D was goat anti-mouse PE (Biolegend). Detection of active LFA-1 was carried out using anti-LFA-1 antibody mAb24 (Abcam), which was tagged with FITC using the Anatag 5-FITC Microscale Protein labeling kit (Anaspec), and anti-CD11a-APC or CD11a-FITC clone HI111 (Bioleg-end). For staining and flow cytometry, anti-LFA-1 antibodies were diluted 1:50 in cell suspension. Camptothecin B (Calbi-ochem) was prepared in dimethyl sulfoxide.

**Activation and Fixation of WBCs—**12-O-Tetradecanoyl-phorbol-13-acetate (TPA; Alexis Biochemicals) was diluted in dimethyl sulfoxide and added to primary WBCs or THP-1 cells at a final concentration of 10 ng/10⁶ cells and incubated at 37 °C. For fixation, the activated cells were washed once with PBS and treated with 1% paraformaldehyde for 20 min at room temperature and washed with PBS before incubation with LtxA.

**Cell Death Assays—**For evaluating cell death, 5 × 10⁵ cells were plated, and inhibitors (75 μM Z- VAD-FMK, 100 μM necrostatin-1, 5 μM IM-54, 10 mM 3-MA, 25 μM pepstatin A) were added to cells for 1 h prior to treatment with LtxA. Cell death was measured by staining with annexin V-FITC and 7-AAD and analyzed using a FACSCalibur flow cytometer (BD Biosciences). Ten thousand events were recorded per sample. In some experiments, 5 μM camptothecin B was used as a positive control for apoptosis.

**Activation of Caspases and Loss of Lysosomal Integrity—**5 × 10⁵ cells were plated, treated with LtxA, and washed with PBS. Cells were stained with either LysoTracker Green DND-26 (75 nM) or poly caspase FAM-VAD-FMK FLICA reagent (Invitrogen) for 1 h according to the manufacturer’s instructions. Increase in lysosomal pH or caspase activation was determined by flow cytometry as described above, and mean fluorescence intensity (MFI) was determined using FlowJo software (Tree Star). Determination of surface and intracellular LAMP-1 was done by treating 5 × 10⁵ cells with LtxA for 90 min and then washed with PBS. Surface staining was performed by incubating the cells with anti-human LAMP-1-PE antibody for 30 min before analyzing with flow cytometry. Intracellular staining was performed by fixing cells in 0.01% formaldehyde and permeabilizing the cells with Tween 20 (0.5% in PBS) for 15 min. Cells were then washed and stained with anti-human LAMP-1-PE or anti-human cathepsin D for 30 min before analyzing with flow cytometry. Anti-cathepsin D antibody was detected with secondary anti-mouse PE antibody.

**Mitochondrial and Lysosomal Purification—**THP-1 cells (1 × 10⁶) were treated with 1 μg/ml LtxA for 1 or 2 h, and mitochondria were separated from the cytosol using the Cell Fractionation kit (MitoSciences) according to the manufacturer’s instructions. For lysosomal isolation, 2 × 10⁷ cells were
treated with 5 μg/ml, and lysosomal fractions were obtained using the Lysosome Enrichment Kit for Tissue and Cultured Cells (Thermo Scientific) according to the manufacturer’s instructions. Total protein was determined by the BCA protein assay (Thermo Scientific).

Western Blot Analysis—Organelles were isolated as described above, and fractions were resuspended in SDS loading dye and equal amounts of protein/sample were resolved on an SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane using an iBlot Dry Blotting transfer system (Invitrogen) and treated with anti-LtxA-HRP (1:50,000), anti-AIF-1 (1:5,000), or anti-LAMP-1 (1:5,000) primary antibody overnight. HRP-conjugated secondary antibodies were used for detection of anti-AIF-1 and LAMP-1 primary antibodies. For all Western blots, 30 μg of protein was loaded into each well.

Confocal Microscopy—THP-1 cells were stained with LysoTracker Green and propidium iodide and treated with 1 μg/ml LtxA. Cells were imaged by time lapse confocal microscopy (Nikon A1Rs confocal microscope) for 60 min, collecting one image every 1 min. The microscope is equipped with an in vivo scientific environmental chamber for live-cell imaging.

Internalization of LFA-1—THP-1 cells were pretreated with anti-human CD11a-FITC antibody for 25 min and subsequently treated with 1 μg/ml LtxA for 15, 30, and 60 min at 37 °C. Cells were washed with PBS and fixed with 2% formaldehyde. Some samples were treated with 10 mM 3-MA before the antibody staining. Imaging flow cytometry was carried out using the Amnis ImageStream Analyzer (Amnis Corp.), and data were analyzed using the Amnis IDEAS software. Cells with an internalization value >0 were determined to have internalized LFA-1. Internalization of LFA-1 was confirmed by following the protocol above, but instead of fixation, cells were treated with trypan blue (0.4%; EMD Chemicals) for 15 min to quench all extracellular fluorescence. Fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences). Ten thousand events were recorded per sample, and MFIs were determined using FlowJo software.

Statistical Analysis—For statistical analyses, data were subjected to a paired Student’s t test, with p < 0.05 considered to be statistically significant.

RESULTS

LtxA Recognizes Active LFA-1—We have shown previously that activated cells are more susceptible to LtxA-mediated cell death than nonactivated cells (5–7). To determine how LtxA preferentially recognizes activated cells, we examined the interaction between LtxA and LFA-1 on WBCs using mAb24, an antibody that recognizes specifically the active, open conformation of LFA-1 (20). Incubation with TPA increased the percentage of CD11a (Fig. 1A). Activated WBCs were then incubated with LtxA, and we observed a decrease in the percentage of CD11a+ mAb24+ cells (Fig. 1B) whereas the percentage of CD11a+ mAb24− cells (nonactivated) cells increased (Fig. 1C). These results suggest that LtxA preferentially recognizes the active form of LFA-1.

To demonstrate that LtxA binds specifically to the active conformation of LFA-1, we used the LtxA-sensitive leukemia cell line, THP-1. THP-1 cells were incubated with or without TPA and then fixed with paraformaldehyde to prevent LtxA-mediated killing. We tested whether pretreatment of fixed cells with LtxA could block binding of mAb24. We found that LtxA prevented binding of mAb24 to activated THP-1 cells (Fig. 1D).
contrast, LtxA did not block binding by HI111 (22), an antibody that preferentially binds to the closed conformation of nonactivated CD11a (Fig. 1D). These results indicate that the LtxA binding site on LFA-1 overlaps the mAb24 binding site, which is exposed only in the active conformation.

**LtxA-induced Cell Death**—Interaction between LtxA and LFA-1 leads to rapid cell death. To examine the kinetics and type of cell death induced by LtxA, we stained THP-1 cells with annexin V and 7-AAD after treatment with different doses of LtxA. Annexin V staining is a marker of apoptosis whereas 7-AAD stains cells in late apoptosis or necrosis. We found that after only 30 min, the majority of the cells were annexin V-positive and 7-AAD-negative, even at high doses of LtxA (Fig. 2, left). After 3 h, the cells became primarily annexin-positive and 7-AAD-positive (Fig. 2, right).

Based on the annexin V cell staining data and other reports, we considered the possibility that cells were undergoing rapid apoptosis. We assayed the activation of caspasess using the fluorescent reagent, FAM-VAD-FMK FLICA and found a significant shift in the population after treatment with LtxA (Fig. 3A). Treatment with LtxA for 90 min resulted in a dose-dependent increase in positive cells, indicating that caspasess were activated. To determine whether LtxA-mediated intoxication requires caspase activity, we pretreated cells with the pancaspase inhibitor, Z-VAD-FMK. The inhibitor did not significantly block killing by LtxA at the 1.0 μg/ml and 0.2 μg/ml doses of LtxA, but showed partial and statistically significant inhibition at the 0.1 μg/ml doses (Fig. 3B). However, Z-VAD-FMK was able to significantly inhibit cell death induced by the known apoptotic-inducing agent camptothecin B (Fig. 3B). This result suggests that high doses of LtxA can overcome inhibition of the caspase-dependent pathway and that we thus considered this possibility. Translocation of the proapoptotic mitochondrial membrane protein AIF-1 from the mitochondria to the cytosol and nucleus is indicative of caspase-independent apoptosis and was evaluated by Western blot analysis. No release of AIF-1 from the mitochondria into the cytosol (Fig. 3C) or nucleus (data not shown) was observed after incubation with LtxA even though cell death was observed under these conditions. These results suggest that LtxA is not inducing a mitochondrial-mediated caspase-independent apoptotic pathway.

To explore other mechanisms of cell death induced by LtxA, we screened inhibitors of several cell death pathways (23, 24). Inhibition of these pathways using necrostatin 1 (necroptosis) or IM-54 (necrosis) failed to prevent LtxA-mediated cell death regardless of the concentration (data not shown). However, when we pretreated cells with the phosphatidylinositol kinase (PI3K) inhibitor 3-MA, we achieved significant inhibition of LtxA-mediated cell death at all concentrations of LtxA at both 3 h (Fig. 4A) and 24 h (Fig. 4B).  

**Involvement of Lysosomes in LtxA-mediated Intoxication**—The inhibitor 3-MA has been used to inhibit lysosomal mediated cell death pathways such as autophagy. Because of our results with 3-MA, the role of the lysosome was explored further using the stain LysoTracker Green, which detects acidic lysosomes. A decrease in fluorescence indicates an increase in pH. Flow cytometry revealed a rapid and complete loss of lysosomal acidity (Fig. 5A), and confocal microscopy showed that the increase in lysosomal pH occurred before propidium iodide staining (Fig. 5B), suggesting that the lysosome has an early role in LtxA-mediated death. In addition, the micrographs revealed membrane blebbing and altered cellular morphology early on, even prior to loss of lysosomal acidity. In contrast, cells that were treated with buffer did not change in appearance or staining properties over time (data not shown). This result could imply a mechanism involving degranulation of the cells, as suggested by Johansson et al. (25). Presentation of the lysosomal
protein LAMP-1 (CD107a) on the cell surface is characteristic of cellular degranulation (26), but we did not observe cell surface expression of LAMP-1 after LtxA treatment (Fig. 5C). Instead, when the cells were permeabilized and stained for LAMP-1, there was a significant and dose-dependent increase in intracellular staining in the LtxA-treated cells compared with the untreated cells (Fig. 5D). This result was confirmed by detecting LAMP-1 in cytosolic fractions by Western blot analysis after LtxA treatment (Fig. 5E). Furthermore, we were able to detect the release of cathepsin D, a lysosomal aspartyl protease, from the lysosome to the cytosol in a time-dependent manner by flow cytometry (Fig. 6A). To determine whether the release of cathepsin D is necessary for LtxA-mediated killing, a cathepsin D inhibitor (pepstatin A) was used to prevent cell death (Fig. 6, B–D). Significant inhibition of killing was achieved after 2 or 24 h with LtxA. Taken together, these results suggest that LtxA treatment causes disruption of lysosomes and release of their contents into the cytosol in a process that is distinct from degranulation.

Internalization of LtxA and LFA-1—After binding LFA-1, LtxA may be taken up by the cells and shuttled to the lysosome where it initiates damage. To examine whether LtxA is internalized and localizes to the lysosome, we isolated lysosomal fractions and probed these fractions for LtxA, LAMP-1, and AIF-1. Because we showed that AIF-1 does not translocate out of the mitochondria (Fig. 3C), AIF-1 served as our mitochondrial marker. LtxA localized to the lysosomal fraction and the presence of LAMP-1 and lack of AIF-1 staining confirmed that our lysosome preparation was not contaminated with other organelles (Fig. 7A). In addition, the Western blot shows that the amount of lysosomal LAMP-1 decreased after LtxA treatment, which further supports our flow cytometry and Western blot data showing a LtxA-mediated increase in cytosolic LAMP-1 (Fig. 7A). We next considered a receptor-mediated endocytosis mechanism for uptake of LtxA and transport to the lysosome. To explore the possibility that LtxA was inducing the uptake of LFA-1, cells were stained with anti-CD11a-FITC mAb and then treated with LtxA and analyzed using imaging flow cytometry (27). LtxA caused a rapid increase in the percentage of cells with internalized LFA-1 compared with the buffer-treated sample (Fig. 7B and C). In addition, 3-MA prevented uptake of LFA-1 (Fig. 7B). We further confirmed LFA-1 internalization using an agent, trypan blue, which quenches extracellular fluorescence. Cells were stained with anti-CD11a-FITC mAb, incubated with LtxA or buffer, quenched with trypan blue, and then analyzed by flow cytometry (Fig. 7D). After 45 min with buffer, the fluorescence was quenched (MFI = 20.8), indicating that CD11a was on the surface of the cell (Fig. 7D). In contrast, LtxA treatment caused an increase in MFI in a time-dependent manner (MFI = 55.3 after 45 min),
indicating that LFA-1 was being internalized and protected from the quenching agent. Using this quenching assay we also confirmed that 3-MA prevented internalization of LFA-1 (data not shown). Taken together, these data support the hypothesis that the LtxA-LFA-1 complex is internalized and LtxA is delivered to the lysosome via a mechanism dependent on PI3K.

LtxA-mediated Cell Death in T and B Lymphocytes—Studies on LtxA have also included T lymphocytes and B lymphocytes, and various cell death pathways have been shown to be activated. We asked whether lysosomes are also involved in LtxA-mediated cell death of lymphocytes. In contrast to THP-1 monocytes, 3-MA did not inhibit killing of Jurkat T cells (Fig. 8A, left) and RL B cells (Fig. 8A, right). In addition, we observed very little shift in the LysoTracker signal (Fig. 8B), even though at least 50% cell death could be detected according to annexin V/7-AAD staining (data not shown). For Jurkat cells, the MFI changed from 99.4 with buffer to 63.7 with the highest dose of LtxA and for RL cells, the MFI changed from 100 with buffer to 89.5 with the highest dose of LtxA. This result is in contrast to the large shift that was observed for THP-1 cells (Fig. 5A; MFI = 244 for buffer and 34.6 for LtxA). These results indicate that a lysosomal mediated mechanism is not the primary cell death pathway induced by LtxA in lymphocytes.

DISCUSSION

In this report, we show that LFA-1-targeting LtxA binds preferentially to the active, open conformation of LFA-1 and kills cells that express this active form. After binding, LtxA causes rapid death of cells and induces multiple cell death pathways including a novel lysosomal mediated pathway. Although anthrax lethal toxin has been shown to cause permeabilization of the lysosomal membrane through its inhibitory activity on MAP kinase kinases (28, 29), to our knowledge, LtxA is the first bacterial toxin shown to localize specifically to the lysosomal compartment where it causes release of contents into the cytosol.

Recently, Paccani et al. (30) showed that adenylate cyclase toxin from Bordetella pertussis also binds LFA-1 in its active conformation on T cells and disrupts the immune synapse. Targeting specifically the active form of LFA-1 is an elegant strat-
energy for a pathogen to evade the host immune system. Indeed, we found that healthy nonactivated WBCs are minimally affected by LtxA. Elimination of the most immunologically relevant cells precludes complete immune suppression, which could kill the host and eliminate a long term reservoir for the pathogen. For a pathogen, death of the host often means termination of its life cycle.

Treatment of THP-1 cells with LtxA displayed a significant increase in lysosomal pH and concomitant release of LAMP-1 and cathepsin D into the cytosol, indicating breakdown of the lysosomal membrane. Johansson et al. (25) reported that cells underwent degranulation after LtxA treatment; however, our data do not support this claim because LAMP-1 was never detected on the surface of cells. It is important to note that this group examined the effect of LtxA on total primary peripheral blood mononuclear cells rather than a single cell type. Thus, degranulation could be occurring in certain cell types or may have been a secondary effect of cell death.

Using numerous methods, we showed that LtxA is itself internalized by cells and mediates the internalization of LFA-1. Inside the cell, LtxA localized specifically to the lysosome. We propose that from within the lysosome, LtxA causes breakdown of the lysosomal membrane and release of damaging lysosomal contents, such as oxygen radicals and lysosomal enzymes. LAMP-1 is normally found in the lysosomal membrane, and so its loss from the lysosome and detection in the cytosol after LtxA treatment supports the hypothesis that LtxA disrupts the lysosomal membrane. The disappearance of LtxA from the lysosome after 2 h of treatment also supports the theory that lysosomal contents are being released into the cytosol. To demonstrate conclusively that the primary cell death pathway involves lysosomes, we showed that inhibiting release of cathepsin D from the lysosome blocked LtxA-mediated cytotoxicity. This effect was not overcome even after a 24-h incubation with LtxA. Thus, interaction between LtxA and the lysosome is a critical step in the process of LtxA-mediated killing (31).

Interestingly, we previously reported that LtxA interacts with Cu,Zn superoxide dismutase from *A. actinomycetemcomitans* (32). In this publication, we proposed that the Cu,Zn superoxide dismutase may prevent damage to LtxA and bacterial cells from oxygen radicals released from dying WBCs. However, given the new results presented here, Cu,Zn superoxide dismutase may also protect LtxA within the lysosomes where the protein is in close contact with oxygen radicals.

Others have reported that LtxA causes apoptosis at low doses and necrosis at high doses (14–16). However, we found that THP-1 cells did not undergo necrosis even at high doses, indicated by the majority of cells being annexin V-positive and 7-AAD-negative. After longer incubation times with LtxA, most of the cells became 7-AAD-positive, including at the lowest dose tested. Thus, we conclude that the 7-AAD-positive cells at later times represent late apoptosis or secondary necrosis and question whether necrosis is ever a primary mechanism of LtxA-mediated cell death. Inhibition of caspases with a general caspase inhibitor had only partial effect on LtxA-mediated killing of THP-1 at the lowest dose of LtxA. At the higher doses...
of LtxA, the inhibitor did not significantly inhibit LtxA-mediated killing, suggesting that caspases are not required for cell death at the doses tested. We also could not obtain evidence that a mitochondrial mediated caspase-independent pathway was solely responsible for cell death even though LtxA treatment results in disruption of the mitochondrial membrane potential. However, inhibiting internalization of LFA-1 and transport of LtxA to the lysosome with 3-MA and inhibition of cathepsin D release had the pronounced inhibitory effects on LtxA-mediated killing. Thus, our results suggest that the primary cell death pathway initiated by LtxA involves the lysosome.

The compound 3-MA has also been used as an inhibitor for the process of autophagy (33). Despite its role in the inhibition of autophagosome formation by acting on PI3K, we do not believe that monocytes die by an autophagic pathway. The rapid kinetics of cell death, the dynamic changes in morphology of the cells, in addition to the population of annexin V−/H11001−/7-AAD− cells point to a nonautophagic mechanism. Instead, we propose that by blocking PI3K, 3-MA is affecting the trafficking of LtxA to the lysosomes because LFA-1 is unable to be internalized as we have shown (33, 34). We thus excluded the possibility of autophagy by identifying the role of 3-MA in preventing the earliest step of LtxA-mediated cell death. We propose that most of the cellular changes that are triggered by LtxA in monocytes, such as activation of caspases and staining by 7-AAD, are likely secondary events that arise because of the initial uptake and transport of LtxA to the lysosome. Nonetheless, these numerous other pathways likely contribute to and ensure death of the cell and explain why high doses of LtxA can overcome individual inhibitors. It will be of considerable interest to determine whether other important related tox-

![FIGURE 7. Intracellular localization of LtxA and LFA-1.](image)

A. THP-1 cells were treated with 5 μg/ml LtxA for 1 or 2 h, and then lysosomes and mitochondria were isolated by biochemical fractionation as described under “Experimental Procedures.” The presence of LtxA, LAMP-1, and AIF-1 were determined by Western blot analysis. Equal total protein was loaded into each lane. B. cells were incubated with anti-CD11a-FITC mAb, treated with 1 μg/ml LtxA for the indicated times, and analyzed by imaging flow cytometry. A separate sample was pretreated with 10 mM 3-MA for 1 h, prior to incubation with antibody and LtxA. Data are representative of two independent experiments. C. data obtained from imaging flow cytometry were analyzed. Histogram identifies cells that had surface-localized LFA-1 (left) and internalized LFA-1 (right). D. cells were stained with anti-CD11a-FITC mAb, treated with 1 μg/ml LtxA for the indicated times, and quenched with trypan blue. Cells were analyzed by flow cytometry.

![FIGURE 8. Role of lysosomal mediated cell death in malignant B and T cells.](image)

A. Jurkat T cells (left) and RL B cells (right) were pretreated with 10 mM 3-MA for 1 h and then various concentrations of LtxA for 24 h. Results represent the average of three independent experiments. Error bars show S.D. B. Jurkat and RL cells were treated with various concentrations of LtxA for 3 h, stained with LysoTracker Green, and analyzed by flow cytometry.
Leukotoxin Causes Lysosomal Mediated Cell Death

FIGURE 9. Proposed model for LtxA-mediated cell death in monocytes. LtxA binds to active LFA-1 and is taken into the cell via receptor-mediated endocytosis. Vesicle formation activates caspases associated with the plasma membrane and transports LtxA to the lysosome where it causes lysosomal membrane disruption and leakage of lysosomal contents. This leakage also leads to the activation of downstream caspases.

ins, such as Escherichia coli α-hemolysin, B. pertussis adenylate cyclase, Actinobacillus pleuropneumoniae Apx toxins, and Mannheimia hemolytica leukotoxin intoxicate cells via a similar mechanism.

The model that we propose to explain LtxA-mediated cell death in monocytes is shown in Fig. 9. In the model, LtxA binds active membrane-bound LFA-1, which triggers vesicle formation and endocytosis. In support of this, Fong et al. found that LtxA causes clustering of LFA-1 into lipid rafts (10). This disruption in the membrane may also cause activation of caspases. LFA-1 then serves as a shuttle to deliver LtxA to the lysosome. Once in the lysosome, LtxA may change conformation and disrupt the lysosomal membrane, possibly by direct insertion and pore formation. Repeats in toxins have been described as pore-forming toxins (35), and perhaps it is in the lysosomal compartment where they exhibit this property. Disruption of the lysosomal membrane causes leakage of toxic components, which can cause cell death directly, or induction of apoptosis through activation of caspases via a cathepsin-mediated pathway (31).

Although lysosomes play a major role in LtxA-mediated cell death of monocytes, preventing the vesicular-trafficking pathway in lymphocytes with 3-MA did not prevent killing by LtxA. It is possible that LtxA-mediated cell death occurs through a different pathway in lymphocytes or that lymphocytes die via apoptosis prior to activation of a lysosomal pathway. Indeed several reports demonstrate LtxA-induced apoptosis in lymphocytes (10, 11).

Interestingly, therapeutic type II monoclonal antibodies, such as anti-CD20 tositumomab and GA101, have been shown to cause direct cell death by inducing the release of lysosomal contents into the cytosol, a process termed lysosomal membrane permeabilization (31, 36). Compared with other mechanisms of monoclonal antibody-induced cell death such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, lysosomal membrane permeabilization is considered to be a more efficient and rapid way to cause cell death. Along these lines, LtxA is being developed as a novel targeting agent for the treatment of hematological malignancies and autoimmune/inflammatory diseases (5, 6). Because these diseases are characterized by chronic activation of WBCs bearing high levels of active LFA-1, LtxA is an ideal agent that would target specifically the subset of WBCs involved in disease. Furthermore, because other healthy tissues of the body do not express LFA-1, LtxA should exhibit minimal adverse effects. Although monoclonal antibody therapies, such as efalizumab, have been used for targeting LFA-1-expressing cells in disease, it is important to highlight that Anti-LFA-1-specific mAbs bind to cells but do not kill them. Anti-LFA-1 mAb prevents interactions between WBCs and vascular endothelial cells, and patients who were treated with efalizumab actually exhibit marked leukocytosis because the drug prevents WBC migration out of the blood (17). In contrast, LtxA binds active LFA-1-expressing cells and kills them. Elimination of the diseased cells, rather than transiently blocking their migration, represents a more robust strategy for treatment of WBC diseases.

In addition, as we have shown here, LtxA induces multiple pathways of cell death, a property that should minimize the chance of developing resistance against the therapy. In conclusion, LtxA represents a first-in-class biologic that induces lysosomal mediated cell death through interaction with an activated integrin.

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