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Aggregatibacter actinomycetemcomitans leukotoxin induces cytosol acidification in LFA-1 expressing immune cells

N. Balashova
University of Pennsylvania

A. Dhingra
University of Pennsylvania

K. Boesze-Battaglia
University of Pennsylvania

E. T. Lally
University of Pennsylvania

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Abstract

Studies have suggested that Aggregatibacter actinomycetemcomitans leukotoxin (LtxA) kills human lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18)-bearing immune cells through a lysosomal-mediated mechanism. Lysosomes are membrane-bound cellular organelles that contain an array of acid hydrolases that are capable of breaking down biomolecules. The lysosomal membrane bilayer confines the pH-sensitive enzymes within an optimal acidic (pH 4.8) environment thereby protecting the slightly basic cytosol (pH 6.8-7.5). In the current study, we have probed the effect of LtxA-induced cytolysis on lysosomal integrity in two different K562 erythroleukemia cell lines. K562-puro/LFA-1 cells were stably transfected with CD11a and CD18 cDNA to express LFA-1 on the cell surface while K562-puro, which does not express LFA-1, served as a control. Following treatment with 100 ng ml^{-1} LtxA cells were analyzed by live cell imaging in conjunction with time-lapse confocal microscopy and by flow cytometry. Using a pH-sensitive indicator (pHrodo®) we demonstrated that the toxin causes a decrease in the intracellular pH in K562-puro/LFA-1 cells that is noticeable within the first 15 min of treatment. This process correlated with the disappearance of lysosomes in the cytosol as determined by both acridine orange and LysoTracker® Red DND-99 staining. These changes were not observed in K562-puro cells or when heat inactivated toxin was added to K562-puro/LFA-1. Our results suggest that LtxA induces lysosomal damage, cytosol acidification, which is followed by cell death in K562-puro/LFA-1 cells. © 2016 John Wiley & Sons A/S.

Keywords

Bacterial pathogenesis; Cytosol acidification; Cytosolic pH; Lysosome; RTX toxin

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N. Balashova[1], A. Dhingra[2], K. Boesze-Battaglia[2] and E.T. Lally[1]

1 Department of Pathology, University of Pennsylvania School of Dental Medicine, Philadelphia, PA, USA
2 Department of Biochemistry, University of Pennsylvania School of Dental Medicine, Philadelphia, PA, USA

Correspondence: Edward T. Lally, Department of Pathology, University of Pennsylvania, School of Dental Medicine, 240 S. 40th Street, 317 Levy Building, Philadelphia, PA 19104, USA Tel.: +1 215 898 5913; fax: +1 215 573 2050; E-mail: lally@dental.upenn.edu

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SUMMARY

Studies have suggested that *Aggregatibacter actinomycetemcomitans* leukotoxin (LtxA) kills human lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18)-bearing immune cells through a lysosomal-mediated mechanism. Lysosomes are membrane-bound cellular organelles that contain an array of acid hydrolases that are capable of breaking down biomolecules. The lysosomal membrane bilayer confines the pH-sensitive enzymes within an optimal acidic (pH 4.8) environment thereby protecting the slightly basic cytosol (pH 6.8–7.5). In the current study, we have probed the effect of LtxA-induced cytolysis on lysosomal integrity in two different K562 erythroleukemia cell lines. K562-puro/LFA-1 cells were stably transfected with CD11a and CD18 cDNA to express LFA-1 on the cell surface while K562-puro, which does not express LFA-1, served as a control. Following treatment with 100 ng ml^{-1} LtxA cells were analyzed by live cell imaging in conjunction with time-lapse confocal microscopy and by flow cytometry. Using a pH-sensitive indicator (pHrodo®) we demonstrated that the toxin causes a decrease in the intracellular pH in K562-puro/LFA-1 cells that is noticeable within the first 15 min of treatment. This process correlated with the disappearance of lysosomes in the cytosol as determined by both acridine orange and LysoTracker® Red DND-99 staining. These changes were not observed in K562-puro cells or when heat inactivated toxin was added to K562-puro/LFA-1. Our results suggest that LtxA induces lysosomal damage, cytosol acidification, which is followed by cell death in K562-puro/LFA-1 cells.

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a pioneer colonizer of the upper aerodigestive tract of both humans and non-human primates. In both man (Lamell *et al.*, 2000) and captive *Macaca fascicularis* (Beighton *et al.*, 1989) colonization of the tongue and buccal mucosa begins at 3–6 months of age. *Aggregatibacter actinomycetemcomitans*, a HACEK organism, is part of the group of causative organisms associated with gram-negative endocarditis that are not linked to illicit drug use (Meyer, 1989; Das *et al.*, 1997). However, the most-studied infection of *A. actinomycetemcomitans* is localized juvenile periodontitis (Zambon, 1985), which has been subsequently renamed localized aggressive periodontitis (Armitage, 1999). Localized aggressive periodontitis is a destructive inflammatory process of the periodontal ligament that affects the first molars and central incisors and results in rapid destruction of alveolar bone and subsequent tooth loss.

The organism has several virulence determinants including a 114.5 kDa leukotoxin (LtxA) that is a member of the RTX (Repeats in ToXin) family of cytolytic proteins (Welch, 1991). The interaction of LtxA with human immune cells is both complex and multifaceted with interactions that involve membrane hydrocarbons as well as cell-surface proteins. LtxA selectively kills white blood cells bearing human β_2-integrin lymphocyte function antigen-1 (LFA-1) (Lally *et al.*, 1997). Although the binding of LtxA to LFA-1 on the surface of leukocytes is well established, our knowledge of the mechanisms by which LtxA causes intracellular changes leading to host cell death is only beginning to be understood. The ability of the toxin to destroy THP-1 lysosomes has recently been demonstrated (DiFranco *et al.*, 2012).

This work is aimed to further understanding of the intracellular events leading to LtxA-induced cytolysis. Here we report LtxA-induced cytosolic acidification in K562 cells expressing LFA-1, which occurred concurrently with lysosomal rupture in these cells.

METHODS

LtxA purification

Aggregatibacter actinomycetemcomitans strain JP2 (Tsai *et al.*, 1984) was grown in *A. actinomycetemcomitans* growth medium (AAGM) (Fine *et al.*, 1999). The bacteria were grown on solid AAGM for 48 h at 37°C in the presence of 10% CO_2. Colonies were inoculated into AAGM broth and were incubated for 24 h. LtxA was purified by ammonium sulfate precipitation from the bacterial culture supernatants as described previously (Diaz *et al.*, 2006). Purified protein was resolved on a 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualized by staining with Coomassie Brilliant Blue G-250 protein stain (Bio-Rad Laboratories, Hercules, CA). The protein concentration was measured with a nanodrop (Thermo Fisher Scientific, Waltham, MA).

Cell culture

Human erythroleukemia K562 cells obtained from the American Type Culture Collection (Manassas, VA) were grown in RPMI-1640 medium containing 10% fetal bovine serum. The K562-derived cell line expressing CD11a and CD18 (K562-puro/LFA-1) was provided by Dr. Timothy A. Springer (Lu and Springer, 1997). To generate control K562-puro cell line, pEF1-puro/hpb1 (Addgene, Cambridge, MA) was digested by *Bam*HI and *Pme*I, and the 6-kilobase pEF1puro fragment was eluted from gel. Two micrograms of linearized pEF1-puro fragment was applied to electroporate 0.5×10^6 K562 cells at 155 V and 960 μF in 0.4-cm path-length cuvettes using a GenePulser X cell instrument (Bio-Rad Laboratories, Hercules, CA). The stable cell lines K562-puro and K562-puro/LFA-1 were maintained in the complete medium containing 3 μg ml^{-1} puromycin.

Cytotoxicity assay

For toxicity tests, 2–500 ng ml^{-1} purified LtxA was added to 1×10^6 erythroleukemia cells in RPMI-1640 with 10% fetal bovine serum and incubated for 3 or 24 h. The cell membrane permeability was determined with Trypan blue assay using Vi-cell Cell Viability Analyzer (Beckman Coulter, Brea, CA). All reactions were run in technical duplicate; the assay was performed three times independently. The controls included LtxA inactivated at 65°C for 30 min and untreated cells.

Confocal microscopy

For pHrodo staining 1×10^6 erythroleukemia cells were washed twice with Live Cell Imaging Solution (LCIS) (Life Technologies, Carlsbad, CA). The cells were incubated with 100 ng ml^{-1} LtxA for different periods of time and washed. The cells were then treated with a mix containing 5 mM of pHrodo™ Red AM, 10 μl of PowerLoad™ (Life Technologies, Carlsbad, CA) in 1 ml of LCIS for 37°C for 30 min. After the treatment the cells were washed twice with LCIS and imaged immediately. The pH calibration was performed using an Intracellular pH Calibration Buffer Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Briefly, the cell loading solution was prepared by diluting the valinomycin and nigericin stock solution in the desired cellular pH calibration buffer to a concentration of 10 μM each. The cell loading solution was then added to pHrodo™ Red AM-labeled cells, incubated at 37°C for 5 min, and the cells were imaged. To analyze the effect of LtxA on cytosolic pH, K562 cells were labeled with pHrodo Red, treated with 100 ng ml^{-1} LtxA, and subjected to

confocal imaging at different time points (0, 15, and 30 min).

For lysosomal staining, the cells were treated with 1 μM acridine orange (Sigma, St Louis, MO) for 15 min or 100 nM LysoTracker® Red DND-99 (Life Technologies) for 2 h at 37°C. Then the cells were washed with LCIS and the cell nuclei were stained 1 μM Hoechst 33342 for 15 min (Image-iT™ LIVE Plasma Membrane and Nuclear Labeling Kit; Life Technologies, Carlsbad, CA). The cells were washed with LCIS and treated with 100 ng ml^{-1} LtxA for different time intervals at 37°C. Treatment with 1 mM Leu-Leu methyl ester (Leu-Leu-OMe) (Sigma, St. Louis, MO) for 30 min at 37°C was used to induce lysosomal rupture. The cells were then washed with LCIS and placed to attach for 20 min in ibiTreat 60 μ-dishes (Ibidi, Madison, WI) coated with poly-L-lysine (Sigma St. Louis, MO.), then they were washed again and covered with LCIS. The cells were examined using a Nikon A1R laser scanning confocal microscope with a 60× water objective (NA 1.2). The images were processed using Nikon's ELEMENTS software 4.1 (Nikon, Inc. Morville, NY). Approximately 50 cells per image were analyzed in each experiment to identify the mean and maximum fluorescent intensity per cell by sorting non-saturated areas in three combined Z planes collected for each image. For live time lapse confocal microscopy images were collected every 2 min for 1 h.

Flow cytometry

To test LFA-1 expression, erythroleukemia cells (0.5×10^6 cells per run) were incubated with anti-human CD11a Alexa Fluor 594 clone HI111 (BioLegend, San Diego, CA) or anti-human CD11a fluorescein isothiocyanate clone TS1/18 (BioLegend, San Diego, CA) (1 : 1000) for 1 h at 25°C and washed with phosphate-buffered saline. Fluorescein isothiocyanate mouse immunoglobulin G1 and Alexa Fluor 647 mouse immunoglobulin G1 (BioLegend, San Diego, CA) served as isotype controls. To analyze cytosolic pH, K562 cells were labeled with pHrodo Green and treated with LtxA as for confocal imaging. At different periods of time (0, 15, and 30 min) the cells were analyzed by flow cytometry (509/533 nm). The fluorescence measurements were made with a BD LSR II flow cytometer (BD Biosciences, San Jose, CA). Ten thousand events were recorded per sample, and mean fluorescence intensities (MFI) were determined using WINLIST software, version 5.0 (Verity Software House Inc., Topsham, ME).

Statistical analysis

The data were analyzed using an unpaired Student's *t*-test, with $P < 0.05$ considered to be statistically significant. The analysis was performed using SIGMAPLOT version 11.0 software (Systat Software, Inc., San Jose, CA).

RESULTS

K562 cells as a model for study of LtxA-mediated cell death

K562 erythroleukemia cells can spontaneously develop characteristics similar to early-stage erythrocytes, granulocytes, and monocytes (Lozzio and Lozzio, 1975; Lozzio and Lozzio, 1979). K562 cell lines transfected with different β_2-integrins cDNA were used previously to study the function of the integrin proteins (Lu and Springer, 1997; Lub *et al.*, 1997; Sigal *et al.*, 2000) and for the identification of the receptors for RTX leukotoxins (Fett *et al.*, 2008; Reinholdt *et al.*, 2013; Munksgaard *et al.*, 2014). In our study of LtxA-mediated cytolysis, we used a K562 cell line stably transfected with CD11a/CD18 cDNA (K562-puro/LFA-1) (Lu and Springer, 1997). We generated a K562 cell line stably transected with pEF1-puro DNA (K562-puro) to be used as a negative control. The expression of LFA-1 subunits (CD11a and CD18) on the surface of K562-puro/LFA-1 and their absence from the surface of K562-puro were confirmed by flow cytometry analysis (Fig. 1A). We determined the sensitivity of these two cell lines to LtxA in dose-dependent manner after 24 h of treatment. The dose-dependent killing curves for toxin concentrations from 2 to 500 ng ml^{-1} applied to both K562-puro/LFA-1 and K562-puro are provided in Fig. 1(B). We selected 100 ng ml^{-1} LtxA for our experiments because it was a sub-maximal dose at which the effect of LtxA on K562-puro/LFA-1 was pronounced; however, K562-puro control cells were not significantly affected. This concentration was compatible with imaging studies. At the concentration of 100 ng ml^{-1} LtxA caused about 10% K562-puro/LFA-1 cell death in 3 h and more than 30% in 24 h whereas K562-puro cells were not significantly

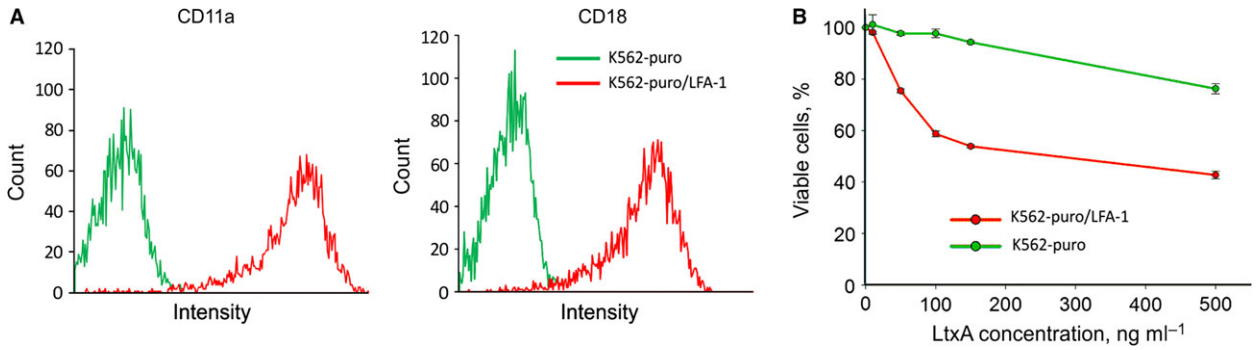

Figure 1 (A) Flow cytometry analysis of erythroleukemia cell lines K562-puro (green) and K562-puro/LFA-1 (red) with anti-human CD11a (HI111) and anti-human CD18 (TS1/18) antibody. The experiment was performed three times independently. Representative images are shown. (B) Dose-dependent determination of leukotoxin (LtxA) cytoxicity for K562 cell lines. For toxicity tests, 2–500 ng ml^{-1} LtxA was added to 1 × 10^6 K562 cells in RPMI-1640 with 10% fetal bovine serum. Toxicity of LtxA on K562 cell lines was measured using Trypan blue assay after 24 h. Untreated cells and cells treated with the toxin that was heat inactivated (HI LtxA) at 65°C for 30 min served as controls. Average results of three independent experiments are displayed. Error bars indicate ±SEM.

affected (Fig. 1B). Heat-inactivated toxin did not cause any K562-puro/LFA-1 cell lysis (data not shown). Hence, as expected, LFA-1 expression on the surface of K562-puro/LFA-1 significantly increased its susceptibility to LtxA.

LtxA causes intracellular acidification in K562-puro/LFA-1

Cytosolic pH plays an important role in maintenance of normal cell function. This parameter is tightly regulated within cells and varies depending on cell type (pH 6.8–7.5) (Deutsch *et al.*, 1982). Hence, we sought to determine if LtxA could change cytosolic pH in host leukocytes. We used pHrodo™ Red/Green AM fluorogenic probes (pKa 6.5) to measure cytosolic pH in live K562 cell lines. These membrane-permeable dyes are weakly fluorescent at neutral pH, but increase in fluorescence with a decrease of pH as the surrounding environment becomes more acidic. Using the valinomycin/nigericin system, which equilibrates extracellular and cytosolic pH, the intracellular fluorescence intensity in pHrodoRed-labeled K562-puro/LFA-1 cells was analyzed using confocal microscopy in the pH range (5.5–7.5) as shown in Fig. 2 (A, left panel). A steady increase in the fluorescence intensity in K562-puro/LFA-1 cells as the pH is decreased from 7.5 to 5.5. Due to the low pH of the endocytic vesicles and lysosomes, these cellular compartments were intensively stained with pHrodo, however, we observed acidification not only in acidic organelles but throughout the intracellular milieu. The fluorescence intensity values in pHrodo Green-labeled K562-puro/LFA-1 cells were quantified by flow cytometry analysis (indicated as MFI; Fig. 2A, right panel).

Hence this technique enabled the quantification of cytosolic pH in K562-puro/LFA-1 cells, we then used pHrodo dyes to monitor this parameter in K562 cell lines after treatment with 100 ng ml^{-1} LtxA. We observed cytosolic pH decrease approximately 15 min after LtxA treatment of K562-puro/LFA-1 cells pre-stained with pHrodo Red using confocal microscopy (Fig. 2B, left panel), which reached a plateau by 30 min of the treatment with LtxA. Some loss of fluorescence signal was occasionally observed at later time points (after 30 min) of the experiment. This could be because pHrodo dye might leak out of the cells or be spontaneously released from dead cells, in addition, it might be bleached by exposure to light during the time lapse confocal experiment. To confirm the confocal result, pHrodo-labeled K562-puro/LFA-1 cells were subsequently treated with 100 ng ml^{-1} LtxA and analyzed by flow cytometry (Fig 2B, right panel). Thus, both methods clearly demonstrated that the acidification occurred after the exposure to LtxA. Treatment of K562-puro cells did not result in significant changes in cytosolic pH of these cells, as was observed by both confocal microscopy (Fig. 2C, left panel) and flow cytometry (Fig. 2C, right panel); in addition, no changes in cytosolic pH were detected when K562-puro/LFA-1 cells were treated with heat inactivated LtxA (data not shown).

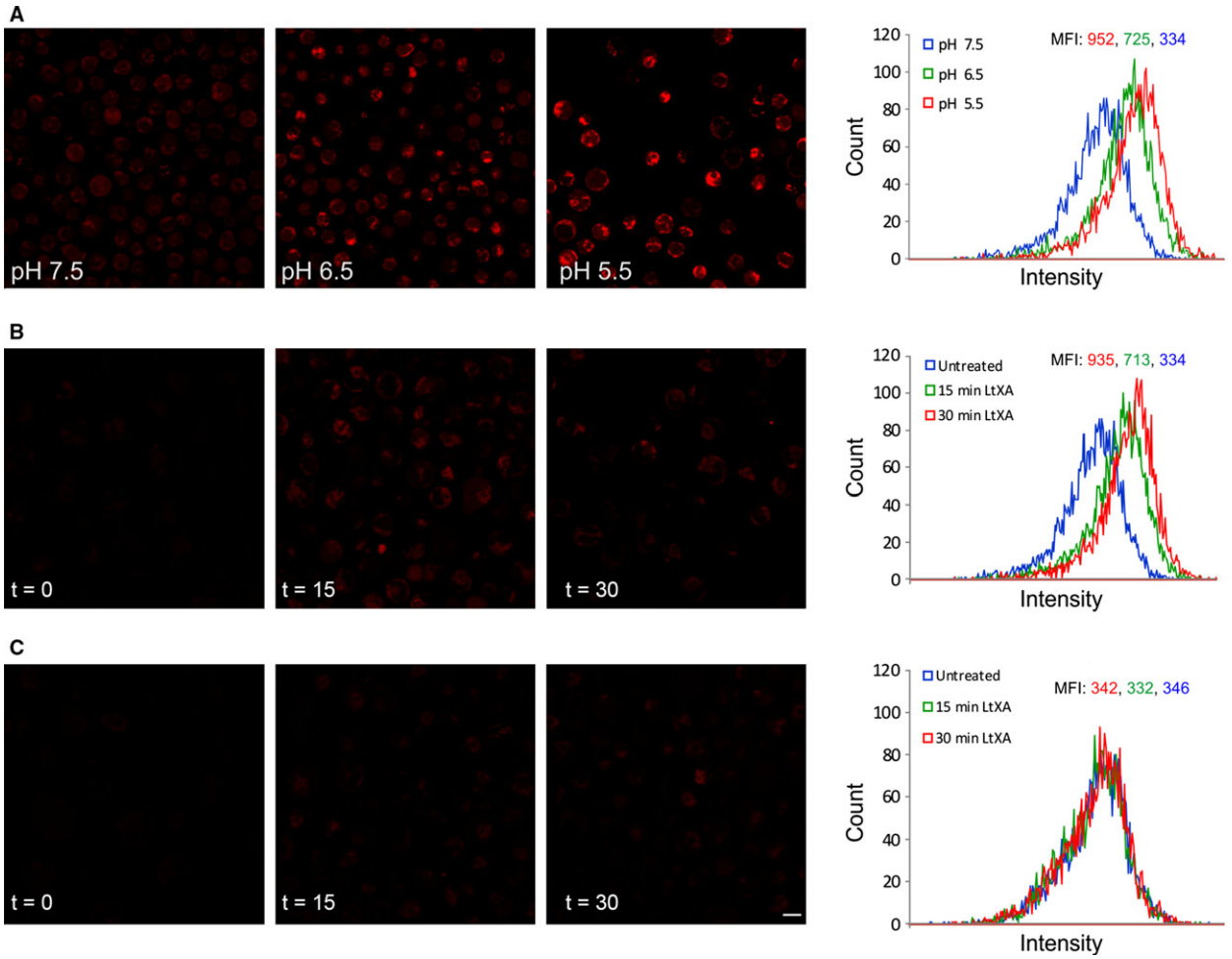

Figure 2 (A) Left panel: confocal images of K562 cells incubated with pHrodo™ Red AM and clamped with intracellular pH calibration buffer. K562 cells were incubated with 5 mM pHrodo™ Red AM for 30 min at room temperature. Calibration Buffer Kit (Life Technologies) was used to clamp the intracellular pH with extracellular buffer at pH 5.5, 6.5 and 7.5. Right panel: flow cytometry analysis of cytosolic pH calibration. K562-puro/LFA-1 cells were stained pHrodo™ Green AM. Calibration Buffer Kit was used to clamp the intracellular pH with extracellular buffer at pH 7.5 (blue), pH 6.5 (green), and pH 5.5 (red). MFI value for each experimental condition. The experiment was performed three times independently. (B) Left panel: confocal images of K562-puro/LFA-1 cells stained with pHrodo™ Red AM (red). The mages demonstrate cytosolic acidification in K562-puro/LFA-1 cells after 0, 15, and 30 min treatment with 100 ng ml^{-1} LtxA. Right panel: flow cytometry analysis of K562-puro/LFA-1 cells stained with pHrodo Green AM after treatment with 100 ng ml^{-1} LtxA for 0, 15, and 30 min. (C) The experiment as described in (B) performed on K562-puro cells. MFI value for each experimental condition is shown. The experiment was performed on three independent occasions. Average lysosomal fluorescence intensities per cell calculated in 50 cells at different time points are shown on the right. Representative images are shown and are the results of three independent experiments. Scale bar = 10 μm.

LtxA causes lysosomal rupture in K562-puro/LFA-1

We hypothesized that the fast cytosol acidification observed in K562-puro/LFA-1 cells upon LtxA treatment could be due to the toxin-induced lysosomal membrane damage and the release of the highly acidic lysosomal content into the intracellular space. Therefore, we wanted to see if LtxA was able to cause lysosomal damage or rupture in K562-puro/LFA-1 cells. To test this hypothesis we used acridine orange, a cell-permeant metachromatic fluorescent dye that stains cellular acidic organelles red. Additionally, acridine orange is a nucleic acid binding dye that emits green fluorescence when bound to double-stranded DNA and red fluorescence when bound to single-stranded DNA or RNA. As a positive control we used a lysosomotropic agent Leu-Leu-OMe, which is known to induce apoptosis of certain immune cells by causing rupture of the lysosomes and DNA fragmentation (Uchimoto *et al.*, 1999). When acridine

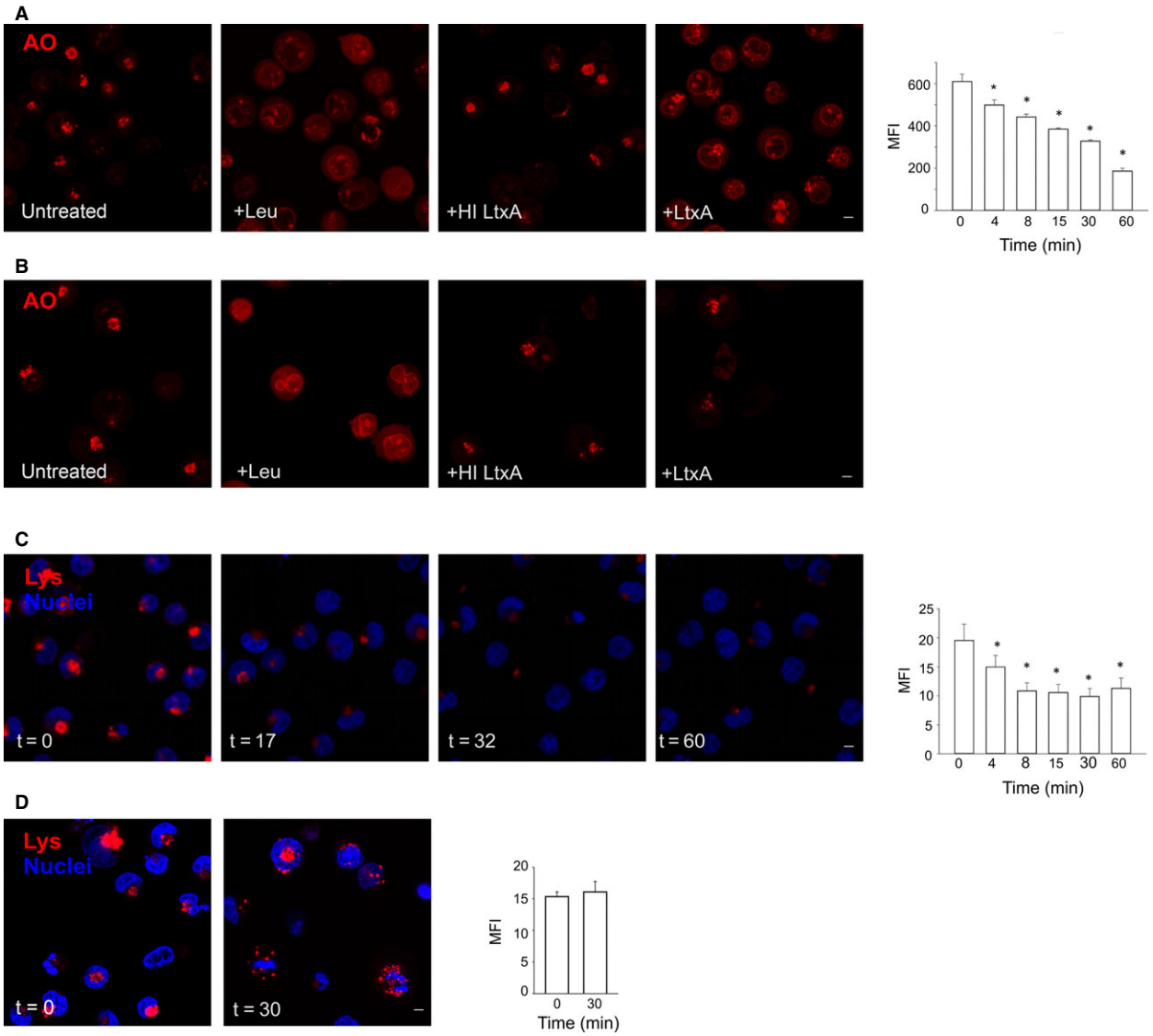

Figure 3 (A) The confocal images of live K562-puro/LFA-1 cells treated with acridine orange which stains cellular lysosomes red. The cells stained with acridine orange (AO) were untreated, treated with 100 ng ml^{-1} leukotoxin (LtxA), 1 mM Leu-Leu-OMe, or 100 ng ml^{-1} heat-inactivated (HI-) LtxA for 30 min and imaged. The lysosomal staining intensity was observed in K562-puro/LFA-1 cells during 60 min of treatment with 100 ng ml^{-1} LtxA using the time-lapse confocal microscopy. Average lysosomal fluorescence intensities per cell calculated in 50 cells at different time points are shown on the right. (B) The confocal images of live K562-puro cells stained with acridine orange and treated as described above. (C) Lysosomes in K562-puro/LFA-1 cells were stained with LysoTracker® Red DND-99 (red) and nuclei were stained with Hoescht 33342 (blue). The lysosomal integrity was observed in the cells during 60 min after treatment with 100 ng ml^{-1} LtxA using the time lapse confocal microscopy. The images collected at 0, 17, 32, and 60 min of the treatment with LtxA are shown. (D) The experiment as described in Fig. 3(B) was performed on K562-puro cells. The images at 0 and 30 min of treatment with LtxA are shown. Average red fluorescence intensity per cell calculated in 50 cells are shown on the right. Error bars indicate ±SEM. *$P \leq 0.05$ compared with untreated cells ($t = 0$) lysosomal intensity. Representative images are shown and are the results of three independent experiments. Scale bar = 5 μm.

orange-stained cells were used to monitor lysosomal membrane integrity, red granular fluorescence was seen in untreated cells (Fig. 3A,B). In contrast, 1 mM Leu-Leu-OMe-treated K562 cells showed significant change in red fluorescence intensity and distribution throughout the cytoplasm, suggesting that Leu-Leu-OMe induced lysosomal rupture. Treatment of K562-puro/LFA-1 cells with 100 ng ml^{-1} LtxA also caused changes in red fluorescence similar to those observed after Leu-Leu-OMe exposure. These changes were

tracked over time in live cell imaging experiments and the decrease in lysosomal fluorescence was detected as early as 4 min after toxin addition (Fig. 3A) indicating that LtxA is able to cause lysosomal degradation in K562-puro/LFA-1 cells. By contrast, no significant changes in fluorescence intensity were observed when the heat-inactivated toxin was applied to K562-puro/LFA-1 (Fig. 3B). Using another lysosomal dye, LysoTracker® Red DND-99, we next followed changes in the lysosomal properties of K562-puro/LFA-1 cells over time using live imaging in conjunction with time-lapse confocal microscopy. We detected a decrease in red fluorescence intensity within first 5 min of treatment and continued treatments resulted in further lysosomal degradation as indicated by the decrease in red fluorescence intensity (Fig. 3C). The loss of the fluorescence intensity was not observed by both lysosomal staining methods when K562-puro cells were used (Fig. 3D), suggesting that the expression of LFA-1 is a critical component required for LtxA to initiate cytosolic changes in host cells.

DISCUSSION

Cellular homeostasis is dependent upon proper regulation of cytosolic pH. There are numerous mechanisms that can cause change in cytosolic pH, including metabolic acid production, leakage of acid through plasma and organelle membranes, and membrane transport processes. Imbalance in cytosolic pH affects many cellular functions, including ionic homeostasis (Park & Brown, 1995), apoptosis (Park, 1995), cell cycle, cell volume regulation (Ritter *et al.*, 1992), muscular contraction (Hao *et al.*, 1999), vascular function (Schulz & Munzel, 2011), concentration of intracellular messengers such as Ca^{2+} and cAMP (Brokaw, 1987; Speake & Elliott, 1998), and cytoskeletal interactions (Edmonds *et al.*, 1995).

Techniques to measure intracellular pH include H^+-permeable microelectrodes, nuclear magnetic resonance analysis of cellular metabolites whose resonance frequency is influenced by pH, and use of weak acid fluorescent dyes, such as pHrodo™ AM (Wray, 1988; Kotyk & Slavik, 1989). Here we present a quantitative methodology to assess cytosolic acidification in K562 cells in response to LtxA treatment by both confocal microscopy and flow cytometry. A shift in the fluorescence signal intensity in pHrodo-labeled K562-puro/LFA-1 cells indicates cytosol acidification in these cells. An evaluation of both lysosomal rupture and cytosolic acidification using pHrodo in a single confocal experiment will result in induction of a technical artifact (pHrodo photobleaching). However, cytosolic acidification measured by pHrodo staining and changes in lysosomal structure/intensity detected using LysoTracker® Red DND-99 were observed in a very close time interval. Cytosol acidification becomes visible at approximately 15 min after an addition of LtxA. We routinely observe the decrease in lysosomal intensity as early as 4 min after addition of LtxA (see Fig. 3) after staining with both LysoTracker Red® and acridine orange. Given that the decrease in cytosolic pH coincides with reduced lysosomal fluorescence intensity rupture could be the major reason for cytosolic acidification in these cells. Combination of cell imaging techniques with flow cytometry allowed us to analyze the effect of LtxA on both a whole population of cells and individual cells at the same time.

Lysosomes are fundamental for cell growth, and so inhibition of the lysosomal function often leads to cell death. Besides that, lysosomal membrane and the subsequent leakage of the highly acidic content into the cytosol may lead to the cell death. The lysosomal cell death may have necrotic and apoptotic features depending on the nature of the stimuli and the extent of the leakage (Aits & Jaattela, 2013). Extensive lysosomal damage may lead to inevitable necrotic cell death, whereas less extensive detriment of lysosomes may induce several apoptotic pathways, which can be attenuated by inhibition of lysosomal proteases (cathepsins) (Kagedal *et al.*, 2001a,b; Kirkegaard & Jaattela, 2009). A number of organic and non-organic agents are known to cause lysosomal damage. These are lysosomotropic detergents such as imidazole, ciprofloxacin, Leu-Leu-OMe (de Duve *et al.*, 1974); reactive oxygen species (Kurz *et al.*, 2008a,b); some viral proteins (Lozach *et al.*, 2011); bacterial, fungal, and snake toxins (Feofanov *et al.*, 2005; Newman *et al.*, 2009; DiFranco *et al.*, 2012; Ivanova *et al.*, 2012); and other regulators (Aits & Jaattela, 2013).

Recent data indicate that LtxA was detected in lysosomes and could trigger lysosome-mediated cell death in THP-1 monocytes (DiFranco *et al.*, 2012). Our study shows that the effect of LtxA on lysosomes in K562-puro/lFA-1 is similar to the effect induced in THP-1 cells. Additionally, our results demonstrate

that a decrease in cytosolic pH of K562-puro/LFA-1 correlates well to the loss of lysosomal membrane integrity in these cells and is dependent upon expression of LFA-1. The killing rate of K562-puro/LFA-1 engineered cells is relatively low, compared with THP-1 cells, in which cell death of most of the cells was detected within the first 3 h. This feature of K562-puro/LFA-1 gives us an advantage in imaging of the toxin during trafficking inside the host cells. K562-puro cells provide an excellent negative control to be used in this study. Moving forward the role of different integrins in the toxin trafficking will be investigated using this recombinant system.

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