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Inhibition of LtxA Toxicity by Blocking Cholesterol Binding with Peptides

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Abstract

The leukotoxin (LtxA) produced by Aggregatibacter actinomycetemcomitans kills host immune cells, allowing the bacterium to establish an ecological niche in the upper aerodigestive tract of its human host. The interaction of LtxA with human immune cells is both complex and multifaceted, involving membrane lipids as well as cell-surface proteins. In the initial encounter with the host cell, LtxA associates with lymphocyte function-associated antigen-1, a cell surface adhesion glycoprotein. However, we have also demonstrated that the toxin associates strongly with the plasma membrane lipids, specifically cholesterol. This association with cholesterol is regulated by a cholesterol recognition amino acid consensus (CRAC) motif, with a sequence of ^{334}LEEYSKR^{340}, in the N-terminal region of the toxin. Here, we have demonstrated that removal of cholesterol from the plasma membrane or mutation of the LtxA CRAC motif inhibits the activity of the toxin in THP-1 cells. To inhibit LtxA activity, we designed a short peptide corresponding to the CRAC^{336} motif of LtxA (CRAC^{336WT}). This peptide binds to cholesterol and thereby inhibits the toxicity of LtxA in THP-1 cells. Previously, we showed that this peptide inhibits LtxA toxicity against Jn.9 (Jurkat) cells, indicating that peptides derived from the cholesterol-binding site of LtxA may have a potential clinical applicability in controllinginfections of repeats-in-toxin-producing organisms. © 2016 John Wiley & Sons A/S.

Keywords

Aggregatibacter actinomycetemcomitans; Cholesterol recognition amino acid consensus motif; Cholesterol-binding; Leukotoxin

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Inhibition of LtxA toxicity by blocking cholesterol binding with peptides

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SUMMARY

The leukotoxin (LtxA) produced by *Aggregatibacter actinomycetemcomitans* kills host immune cells, allowing the bacterium to establish an ecological niche in the upper aerodigestive tract of its human host. The interaction of LtxA with human immune cells is both complex and multifaceted, involving membrane lipids as well as cell-surface proteins. In the initial encounter with the host cell, LtxA associates with lymphocyte function-associated antigen-1, a cell surface adhesion glycoprotein. However, we have also demonstrated that the toxin associates strongly with the plasma membrane lipids, specifically cholesterol. This association with cholesterol is regulated by a cholesterol recognition amino acid consensus (CRAC) motif, with a sequence of ^{334}LEEYSKR^{340}, in the N-terminal region of the toxin. Here, we have demonstrated that removal of cholesterol from the plasma membrane or mutation of the LtxA CRAC motif inhibits the activity of the toxin in THP-1 cells. To inhibit LtxA activity, we designed a short peptide corresponding to the CRAC^{336} motif of LtxA (CRAC^{336WT}). This peptide binds to cholesterol and thereby inhibits the toxicity of LtxA in THP-1 cells. Previously, we showed that this peptide inhibits LtxA toxicity against Jn.9 (Jurkat) cells, indicating that peptides derived from the cholesterol-binding site of LtxA may have a potential clinical applicability in controlling infections of repeats-in-toxin-producing organisms.

INTRODUCTION

The pathogenicity of *Aggregatibacter actinomycetemcomitans* is regulated by a number of virulence factors (Fives-Taylor *et al.*, 1999), including a leukotoxin (LtxA) that selectively kills human immune cells (Taichman *et al.*, 1987), allowing the organism to colonize the host. As a member of the repeats-in-toxin (RTX) family of protein toxins (Welch, 1991), LtxA shares with this family the common RTX operon organization, which includes five genes that regulate the translation and secretion of the toxin. The leukotoxin operon contains four genes, *ltxCABD*, where *ltxA* encodes the inactive protein (proLtxA), *ltxC* encodes an acyltransferase that post-translationally activates the protoxin (Hardie *et al.*, 1991; Issartel *et al.*, 1991 Hackett *et al.*, 1994; Balashova *et al.*, 2009; Fong *et al.*, 2011), and *ltxB* and *ltxD*, along with a fifth gene, *tdeA* (Crosby & Kachlany, 2007), located 572 kb downstream of the *ltx* operon, produce proteins involved in the secretion of the activated toxin in a Type I secretion system (Kanonenberg *et al.*, 2013).

The mechanism by which LtxA kills cells is congruent with a wide variety of bacterial protein toxins, whereby target cell recognition initiates a multi-step process that culminates in cell death (Van Rie *et al.*, 1989; Parker *et al.*, 1990; London, 1992; Isberg & Tran Van, 1994; Merritt & Hol, 1995). The RTX toxins vary in their target cell specificity, and have historically been classified as either hemolysins or leukotoxins. The RTX hemolysins, such as *Escherichia coli* α-hemolysin (Cavalieri *et al.*, 1984), and *Actinobacillus pleuropneumoniae* ApxIA (Frey *et al.*, 1991) are broadly toxic to a wide variety of cell types derived from many species, whereas the leukotoxins are much more restrictive in their toxicity. As an RTX leukotoxin, the *A. actinomycetemcomitans* LtxA specifically kills immune cells from man, the great apes, and Old World monkeys (Taichman *et al.*, 1984, 1987) that express the β_2 integrin, lymphocyte function-associated antigen-1 (LFA-1) (Lally *et al.*, 1997; Kieba *et al.*, 2007). Other RTX toxins, including *Bordetella pertussis* adenylate cyclase toxin and the *Mannheimia haemolytica* LktA, have been found to bind to β_2 integrins as well (Atapattu & Czuprynski, 2007; Bumba *et al.*, 2010). The exclusive association of these bacteria with their respective hosts has possibly been driven by parallel evolution of their respective *rtxA* genes, with the result being the high degree of target cell specificity that we now observe.

In addition to these specific receptor interactions, LtxA and the RTX cytotoxins are highly membrane-active, with a demonstrated effect on the packing of the plasma membrane lipids (Martin *et al.*, 2004; Brown *et al.*, 2012; Barcena-Uribarri *et al.*, 2015) and a clustering of the toxin and their integrin receptor in cholesterol-enriched lipid rafts (Fong *et al.*, 2006; Atapattu & Czuprynski, 2007; Bumba *et al.*, 2010).

Examination of the deduced amino acid sequences of LtxA and several RTX toxins revealed that they contain cholesterol recognition amino acid consensus (CRAC) motifs (Brown *et al.*, 2013). We demonstrated that in LtxA, one of these motifs, CRAC^{336}, is responsible for the cholesterol binding by LtxA that regulates the lipid raft clustering. In the characterization of the CRAC motif in LtxA, we discovered that a peptide derived from the CRAC sequence of LtxA is able to bind to cholesterol and inhibit LtxA binding to cholesterol. Recently, using molecular dynamics simulations, we have demonstrated that this CRAC peptide interacts strongly with membranes containing 40% cholesterol and that this association results in a decrease in secondary structure in the peptide, which is not observed in the absence of cholesterol (Miller *et al.*, 2014).

In the current work, we have explored the possibility of inhibiting LtxA binding to cholesterol as a means of inhibiting activity. We found that the interaction between LtxA and cholesterol is highly specific, requiring both an intact CRAC sequence and a specific sterol structure, and disruption of this interaction in a number of different ways is sufficient to inhibit LtxA toxicity. We inhibited the association of LtxA with cholesterol in the target cell plasma membrane by removing cholesterol with methyl-β-cyclodextrin (MβCD), preincubating LtxA with cholesterol-containing liposomes, and blocking plasma membrane cholesterol with a cholesterol-binding peptide. All three methods significantly reduced the ability of LtxA to kill THP-1 cells, demonstrating the potential therapeutic use of inhibition of cholesterol-binding by LtxA to minimize cytotoxicity.

METHODS

Chemicals

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Chol), MβCD, and methyl-β-cyclodextrin-cholesterol (MβCD-Chol) were purchased from Sigma-Aldrich (St. Louis, MO), and ergosterol (Ergo) was purchased from MP Biomedicals (Solon, OH). Peptides were synthesized and purified (98% purity) by Biomatik (Wilmington, DE).

LtxA purification

Aggregatibacter actinomycetemcomitans strain JP2 was grown overnight in AAGM broth (Fine *et al.*, 1999) supplemented with 12.5 $\mu g\ ml^{-1}$ vancomycin and 75 $\mu g\ ml^{-1}$ bacitracin. LtxA was purified as described previously (Kachlany *et al.*, 2002). The purity of the toxin was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting, and the activity was confirmed using a cytotoxicity assay. To inactivate LtxA, the toxin was heated at 65°C for 30 min.

Production of the CRAC mutant, $\text{LtxA}^{\text{Y336P}}$, was accomplished in *E. coli* using a pSHH plasmid containing the *ltxA*-promoter region, *ltx*C, and the mutated *ltx*A genes (Brown *et al*., 2013). The CRAC mutant was constructed by substituting proline for tyrosine at amino acid position 336 through site-directed mutagenesis, performed using a QuikChange® site-directed mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA), according to the manufacturer's instructions. The primers containing the substitutions were designed using the OligoPerfect™ Designer, as shown previously (Brown *et al*., 2013). The reactions were performed on an automated thermal cycler with an initial step of 30 s at 95°C, followed by polymerase chain reaction amplification for 16 cycles of 30 s at 95°C, 1 min at 55°C, and 3 min at 68°C. The polymerase chain reaction products obtained were transformed into DH5α-T1 cells, and the mutant clones were selected on Luria–Bertani agar plates with 50 μg ml^{-1} of ampicillin. The $LtxA^{Y336P}$ mutant is constitutively expressed in the cytosol. To collect this protein, 200 ml of Luria–Bertani agar supplemented with ampicillin (50 μg ml^{-1}) was inoculated with overnight cultures of the *E. coli* DH5α-T1 mutant clones and then grown to an optical density at 600 nm of approximately 0.4. The cultures were centrifuged and resuspended in 6 ml of buffer (20 mM Tris–HCl, 250 mM NaCl, 0.2 mM $CaCl_2$, pH 6.8), sonicated (six times for 45 s, on ice), and centrifuged to remove the cell debris (12,000 ***g***, 15 min, 4°C).

Liposome preparation

Liposomes composed of POPC and cholesterol were prepared using the lipid film technique. Stock solutions of POPC and cholesterol (both 25 mg ml^{-1}) were prepared in chloroform and then added to a glass vial in the required amounts. The chloroform was evaporated under a stream of nitrogen, and the residual chloroform was removed under vacuum to create a thin lipid film on the glass surface. Multilamellar liposomes were created by hydrating the lipid film with phosphate-buffered saline (PBS). Liposomes composed of POPC and ergosterol were formed using the rapid solvent exchange technique (Buboltz & Feigenson, 1999). Stock solutions of POPC and ergosterol were added to a glass vial, PBS was added directly, and the chloroform was evaporated while the solution was vortexed. Large unilamellar vesicles were formed from all multilamellar liposome solutions by extrusion (MacDonald *et al*., 1991) through a 100-nm polycarbonate Whatman membrane (GE Healthcare Bio-Sciences, Pittsburgh, PA) 35 times with a LiposoFast® extruder (AVESTIN Inc., Ottawa, ON).

Cell culture

THP-1 cells obtained from the American Type Culture Collection (Manassas, VA) were maintained in RPMI-1640 medium containing 10% fetal bovine serum and 0.05 mM 2-mercaptoethanol at 37°C under 5% CO_2.

Immunofluorescence

LtxA was labeled with the DyLight® 647 Amine-Reactive dye (ThermoScientific, Waltham, MA), according to the manufacturer's instructions with some modifications. Specifically, the toxin was purified from an excess of the dye using a 40k MWCO Zeba Spin Desalting column (ThermoScientific).

For the LtxA binding studies, 0.5×10^6 THP-1 cells ml^{-1} were washed with live cell imaging solution (ThermoScientific), and the cell nuclei were stained with 5 μM Hoescht 33342 for 15 min (ThermoScientific). The cells were then placed for 20 min in ibiTreat 60 μ-dishes (Ibidi, Martinsried, Germany) coated with poly-L-lysine (Sigma-Aldrich). The attached cells were covered with 1 ml live cell imaging solution and were examined using a Nikon A1R laser scanning confocal microscope with a 60 × water objective (NA 1.2). After the dish was adjusted on the microscope stage, the initial image was collected, and then 20 ng of LtxA was carefully added to the cells and images of the same area were collected for 30 min. The images were processed using Nikon's Elements software 4.1. Approximately 50 cells per image were analyzed in each experiment to identify the mean fluorescence intensities by sorting cell-associated areas in three combined Z-planes collected for each image.

Depletion and replenishment of plasma membrane cholesterol levels

THP-1 cells, maintained in cell culture media, were depleted of cholesterol through incubation with 10 mM MβCD for 15 min at 37°C and 5% CO_2. After the incubation, the cells were washed with cell culture media to remove any excess MβCD and were used in the cytotoxicity assay immediately. To replenish cholesterol, some of the MβCD-treated cells were subjected to an additional incubation with 1 mM MβCD-Chol for 1 h at 37°C and 5% CO_2. These cholesterol-replenished cells were then washed and used immediately. The concentration of cholesterol in the THP-1 cell membranes before depletion, after depletion, and after replenishment was measured with an Amplex® Red Cholesterol Assay Kit ThermoScientific (Waltham, MA). Intensity measurements were performed with an Infinite 200 Pro plate reader (Tecan Group Ltd., Männedorf, Switzerland) with an excitation wavelength of 555 nm and an emission wavelength of 580 nm.

Cytotoxicity assays

For the cytotoxicity tests, LtxA-containing samples were added to THP-1 cells (1×10^6 cells ml^{-1}) and incubated for 3 h. The cell membrane permeability was determined with a trypan blue assay using an automated cell counter. Each experiment was performed three times, independently. Untreated cells were used as a control. (a) To determine the role of cholesterol in toxicity, cholesterol-depleted and cholesterol-replenished cells were incubated with 2 μg of LtxA for 3 h. Untreated cells, as well as cholesterol-depleted and cholesterol-replenished cells that had not been treated with LtxA, were used as controls. (b) In the case of the LtxA CRAC mutant, 50 μg of total protein in the *E. coli* cytosolic fraction was added to the THP-1 cells and incubated for 3 h. An *E. coli* DH5α-T1 cytosolic fraction that did not contain pSHH served as a control. (c) To measure the protective effect of cholesterol-containing liposomes, the THP-1 cells were incubated with (i) LtxA, (ii) LtxA + 100% POPC liposomes, (iii) LtxA + 60% POPC/40%Chol liposomes, or (iv) LtxA + 60%POPC/40%Ergo liposomes. The mass of LtxA in each sample was 2 μg, and all liposome concentrations were 9.0×10^{-7} M. Controls included PBS, 100% POPC liposomes alone, 60%POPC/40%Chol liposomes alone, and 60%POPC/40%Ergo liposomes alone. (d) To measure the protective effect of the CRAC peptide, THP-1 cells were incubated with protein samples containing (i) LtxA, (ii) LtxA + CRAC^{336WT} or (iii) LtxA + CRAC^{336SCR}. The mass of LtxA in each sample was 2 μg, and the molar LtxA : peptide ratio was 1 : 100. Controls included PBS, CRAC^{336WT} alone, and CRAC^{336SCR} alone.

The percentage of cells alive after each treatment was calculated using the following equation:

$$\%\text{viability} = \frac{N_3}{N_0}$$

where N_0 is the number of cells before treatment, and N_3 is the number of cells after 3 h of treatment.

Peptide binding assay

To measure the binding of the CRAC^{336WT} and CRAC^{336SCR} peptides to cholesterol, a centrifugation assay was performed (Sophianopoulos *et al.*, 1978). The peptides (7.0×10^{-5} M) were incubated with 100% POPC or 60%POPC/40%Chol liposomes at a lipid : protein ratio of 100 : 1 for 30 min, then added to a centrifugal filter (Amicon® 30k MWCO; EMD Millipore, Billerica, MA) and centrifuged for 1 h at 6000 ***g*** (Voglino *et al.*, 1998, 1999). The unbound peptide concentrations were determined by comparing the intrinsic fluorescence of the eluate at 305 nm to a set of standards of the same peptide with known concentrations. The fluorescence measurements were recorded on a Quantamaster® 400 spectrofluorometer (PTI Horiba, Edison, NJ) using an excitation wavelength of 281 nm. The bound peptide concentrations were then calculated from the total and free concentrations of peptide.

Statistical analysis

Statistical analysis of the data was performed using OriginPro® 2015 (OriginLab, Northampton, MA) using one-way analysis of variance, followed by a Tukey test. In cases where $P > 0.05$, we reported no statistically significant difference between the two data sets in question.

RESULTS

LtxA associates with the THP-1 cell surface

We investigated the process of LtxA binding to THP-1 cells, which express active LFA-1 on their surface (DiFranco *et al.*, 2012) and are highly susceptible to LtxA (Kachlany *et al.*, 2010). Active and heat-inactivated LtxA was labeled with DyLight® 647 for confocal imaging. When active DyLight® 647-LtxA (20 ng) was added to THP-1 cells in a time-lapse live confocal imaging experiment, binding of the toxin to the surface of most of the cells was observed within 5 min (Fig. 1A), as indicated by blue fluorescent dots accumulating on the cell membrane and presented as mean fluorescence intensity (Fig. 1B). In contrast, significantly less binding was detected when the same amount of the heat-inactivated DyLight® 647-LtxA was applied to THP-1 cells, even after a 30-min incubation, suggesting that heat-inactivated LtxA was inhibited in its ability to interact with the membrane.

LtxA toxicity is dependent on the presence of cholesterol

The association of LtxA with the membrane suggests that the toxin may interact with the cell plasma membrane lipids. Previously, we found that LtxA must bind to cholesterol on the Jurkat (Jn.9) cell plasma membrane to kill the cells (Brown *et al.*, 2013). To investigate whether LtxA binding to the THP-1 membrane is likewise regulated by the presence of cholesterol, we extracted cholesterol from the THP-1 plasma membrane using MβCD and found that the toxicity of LtxA was significantly diminished in the absence of cholesterol (Fig. 2). When the plasma membrane was replenished with cholesterol, using MβCD followed by MβCD-Chol, the cells again became susceptible to LtxA, indicating that the interaction of LtxA with cholesterol on the THP-1 plasma membrane is an essential element of the toxin's mechanism of action. One-way analysis of variance followed by a Tukey test indicated that the cholesterol-dependence of LtxA activity is statistically significant (Table 2).

Neither treatment with MβCD nor treatment with MβCD followed by MβCD-Chol was toxic over the time–course of the experiment (data not shown). The actual cholesterol concentrations in the cell membrane before and after MβCD treatment were determined using an Amplex® Red Cholesterol Assay. Untreated cells had a cholesterol concentration of 112.01 ± 1.87 μM, and after treatment with MβCD, the cholesterol concentration decreased 67.6% to 36.38 ± 1.34 μM. Replenishment of cholesterol with MβCD-Chol restored the cholesterol concentration to near original levels, 104.88 ± 1.34 μM.

LtxA toxicity is regulated by an intact cholesterol-binding site

Previously, we demonstrated that a CRAC motif regulates the binding of LtxA to Jn.9 cells and the resulting toxicity. To confirm that this same $CRAC^{336}$ motif regulates cholesterol binding in THP-1 cells as well, we induced a point mutation ($Y^{336} \rightarrow P^{336}$) in the *ltxA* $CRAC^{336}$ site using site-directed mutagenesis. The wild-type and mutant *ltxA* genes were cloned into

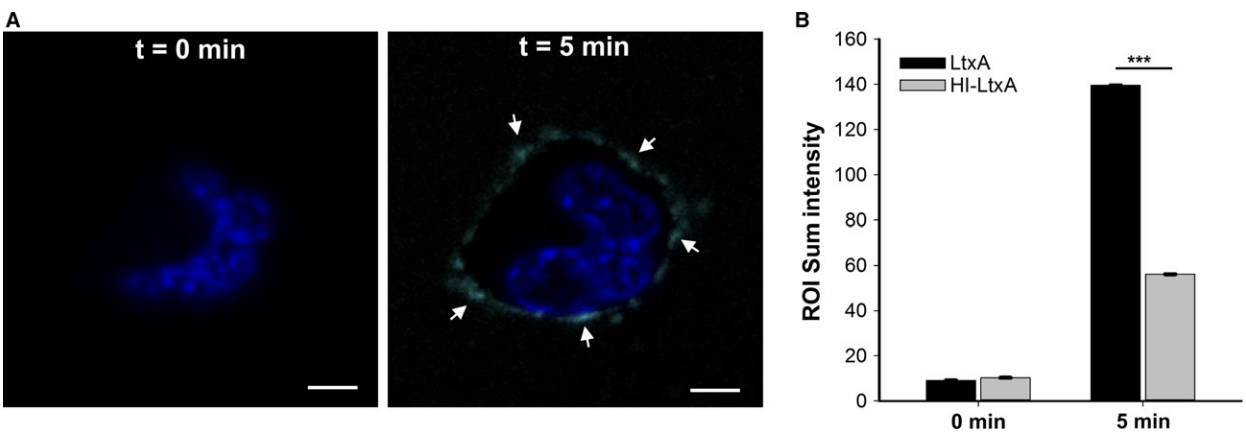

Figure 1 Association of LtxA with THP-1 cells. (A) Confocal images showing the interaction between leukotoxin (LtxA) and THP-1 cells. The left panel demonstrates the cell before treatment and the right panel demonstrates the same cell 5 min after 20 nM DyLight® 647-LtxA (light blue) was added. Cell nuclei were stained with Hoescht 33342 (dark blue). Arrows demonstrate some areas of toxin binding on the plasma membrane. Representative images are shown. Scale bar = 5 μm. (B) Quantitative fluorescence intensity analysis of confocal microscopy images indicating association of LtxA and heat-inactivated (HI)-LtxA with THP-1 cells. Each bar represents the summed intensity of 55 regions of interest (ROI). A *t*-test indicates that there is a significant difference in the binding of LtxA and HI-LtxA after 5 min. ***$P \leq 0.001$.

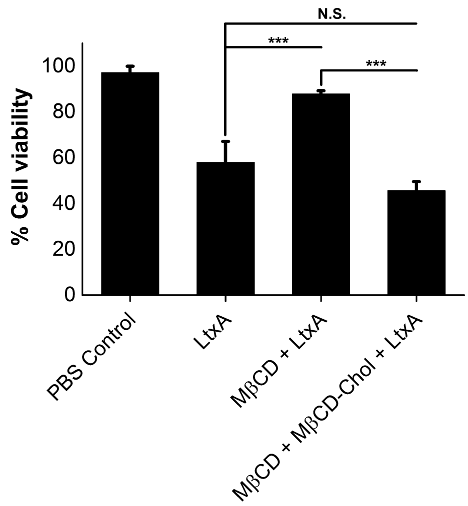

Figure 2 Cytotoxicity of leukotoxin (LtxA) after cholesterol extraction from THP-1 cells. The toxicity of LtxA was measured in THP-1 cells as a function of cholesterol composition. THP-1 cells were either untreated, treated with methyl-β-cyclodextrin (MβCD) for 15 min to extract cholesterol, or treated with MβCD for 15 min followed by MβCD-Chol for 1 h to replenish cholesterol. Cells with reduced cholesterol compositions were significantly less susceptible to LtxA than were those with wild-type cholesterol levels. Replenishment of cholesterol restored susceptibility to LtxA. The data represent the averages of three independent experiments, and the error bars represent the standard deviation. A one-way analysis of variance followed by a Tukey test was used to determine the level of significance between each experiment. ***$P \leq 0.001$; N.S., not significant.

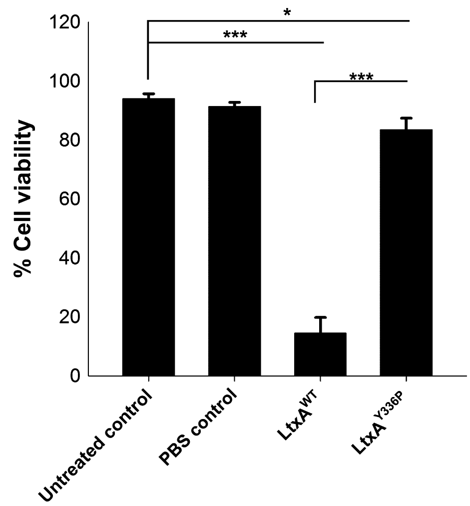

Figure 3 Mutation of the cholesterol-binding site inhibits leukotoxin (LtxA) cytotoxicity. Point mutations were induced in *ltx*A by substituting proline for tyrosine at amino acid position 336 using site-directed mutagenesis. The wild-type *ltxA* gene and mutant gene were cloned into pSHH and expressed in tandem with *ltx*C under the control of the native LtxA promoter. Overnight cultures of LtxA and LtxA^{Y336P} were constitutively expressed in the *Escherichia coli* DH5α cytosol. *Escherichia coli* cytosolic fractions containing 50 μg of total protein were incubated with THP-1 cells for 3 h. The THP-1 cells were highly susceptible to LtxA^{WT} sonicates but not to LtxA^{Y336P}. A sonicate from *E. coli* DH5α containing the pSHH empty vector served as the negative control. Cell death was measured with a trypan blue assay. ***$P \leq 0.001$; *$P \leq 0.05$.

pSHH and expressed in tandem with the *ltxC* gene, under the control of the wild-type promoter. The proteins were constitutively expressed in the cytosol of *E. coli*. The bacterial suspensions were sonicated, and the supernatants were collected and normalized by the total protein concentration. These normalized supernatants were then incubated with THP-1 cells, and the cytotoxicity was measured using a trypan blue assay. We have previously demonstrated that LtxA^{CRACY336P} is stable and produced at comparable levels to that of LtxA^{WT} (Brown *et al.*, 2013).

As shown in Fig. 3, after 24 h of exposure to the *E. coli* supernatants, cells exposed to LtxA^{WT} had a very low viability relative to those exposed to a blank control. Cells exposed to the LtxA^{CRACY336P} mutant remained viable during the time-scale of the experiment, indicating that, as in Jn.9 cells, the CRAC^{336} site is essential for the toxicity of LtxA in THP-1 cells. This result suggests that LtxA binds to cholesterol on the THP-1 plasma membrane during its initial interaction with the cell.

Inhibition of binding to cholesterol inhibits LtxA toxicity

We investigated the possibility of blocking the binding of LtxA to cholesterol on the target cell plasma membrane as a means to inhibit the toxin's activity by preincubating the toxin with liposomes composed of POPC and 40% cholesterol. First, we incubated LtxA with cholesterol-containing liposomes before incubating the mixture with THP-1 cells, with the idea that the LtxA would bind to cholesterol on the liposome and therefore be unable to bind to cholesterol on the cell membrane. Figure 4 demonstrates that this approach was successful. THP-1 cells were susceptible to free LtxA; however, when the LtxA was preincubated with POPC/Chol liposomes, the cells remained viable throughout the experiment.

To determine the specificity of this inhibition, we repeated the experiment using two types of liposomes that did not contain cholesterol, 100% POPC liposomes and 60%POPC/40%Ergo liposomes. Ergo is a sterol found in yeast and other fungal membranes that differs in structure from cholesterol in both the ring and tail domains. As shown in Fig. 4, neither type of liposome was able to inhibit LtxA toxicity, demonstrating that the binding of LtxA to cholesterol is specific to this particular lipid and suggesting that inhibiting the binding of LtxA to cholesterol could be an effective approach to alter LtxA activity. The specificity of this interaction was statistically significant as determined by a one-way analysis of variance followed by a Tukey test (Table 2).

Cholesterol-binding peptides inhibit LtxA toxicity

We next investigated the possibility of inhibiting LtxA activity using a cholesterol-binding peptide derived from the CRAC^{336} site of LtxA. A cholesterol-binding peptide (CRAC^{336WT}), consisting of the CRAC336 sequence of LtxA with six flanking residues on either side, and a scrambled control (CRAC^{336SCR}), in which the CRAC motif was scrambled, were synthesized for this purpose. The sequences of the two peptides are shown in Table 1. An analytical centrifugation assay was used to demonstrate that the CRAC^{336WT} peptide binds more effectively to liposomes containing 40% cholesterol than it does to those without cholesterol (Fig. 5), indicating that this peptide binds specifically to cholesterol in the liposome. Additionally, the CRAC^{336WT} peptide bound to a greater extent to the 40% cholesterol liposomes than did the CRAC^{336SCR} peptide, demonstrating that, as in the full-length toxin, the intact CRAC sequence is essential for this binding. The results of a statistical analysis of these data are included in Table 2.

Table 1 Cholesterol recognition amino acid consensus (CRAC) peptide sequences

Name	Sequence
CRAC^{336WT}	FDRARM**LEEYSKR**FKKFGY
CRAC^{336SCR}	FDRARM**YEKLERS**FKKFGY

The CRAC motif is underlined and in bold type. Each peptide was acetylated at the N-terminus and amidated at the C-terminus.

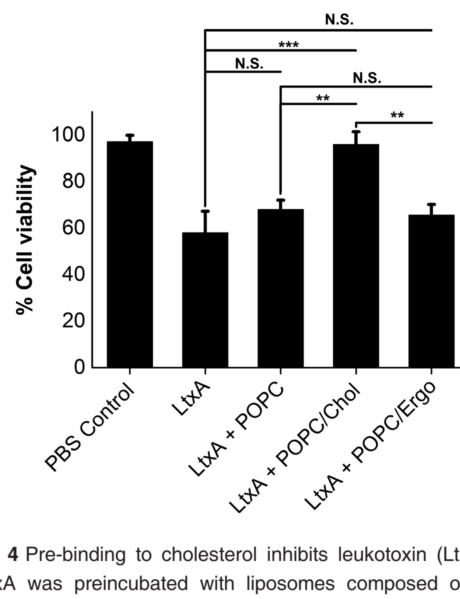

Figure 4 Pre-binding to cholesterol inhibits leukotoxin (LtxA) toxicity. LtxA was preincubated with liposomes composed of 60% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 40% cholesterol (Chol) for 15 min before incubation with THP-1 cells. Preincubation of LtxA with these cholesterol-containing liposomes completely inhibited the toxicity of LtxA. Preincubation of LtxA with liposomes without cholesterol, composed of either 100% POPC or 60% POPC/40% ergosterol (Ergo), did not inhibit LtxA toxicity. A one-way analysis of variance followed by a Tukey test was used to determine the level of significance between each experiment. ***$P \leq 0.001$; **$P \leq 0.01$; ns, not significant.

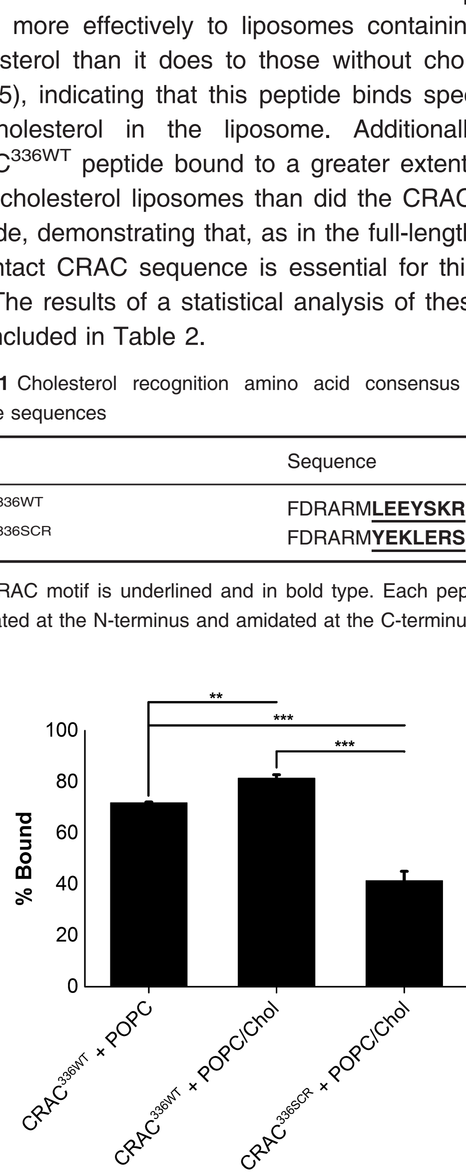

Figure 5 CRAC^{336WT} peptide has a specific affinity for cholesterol. A peptide corresponding to the cholesterol-binding motif in leukotoxin (LtxA) (CRAC^{336WT}) was synthesized along with a control peptide in which the cholesterol-binding sequence was scrambled (CRAC^{336SCR}). The peptides were incubated with liposomes composed of either 100% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or 60%POPC/40% cholesterol (Chol) for 30 min. Unbound peptide was separated from the liposome–peptide complexes using a centrifugal filter, and the concentration of unbound peptide was determined by comparing the fluorescence intensity of the eluate to a set of standards. CRAC^{336WT} bound significantly more to liposomes containing cholesterol than to those without cholesterol. CRAC^{336SCR} bound with a lower affinity to the POPC/Chol liposomes than did CRAC336^{WT}. A one-way analysis of variance followed by a Tukey test was used to determine the level of significance between each experiment. **$P \leq 0.001$; **$P \leq 0.01$.

This binding experiment was repeated with multiple liposome concentrations and the half maximal effective liposome concentration (EC_{50}) and the liposome saturation limit of CRAC^{336WT} binding to POPC/Chol liposomes were determined using a sigmoidal fit of the data. The results of this fit predict an EC_{50} of 3.2 μM and a saturation limit, representing 100% binding of the peptide to the liposomes, of 73.5 mM.

To inhibit LtxA binding to cholesterol and the resulting toxicity, we incubated THP-1 cells with LtxA alone or in combination with the CRAC^{336WT} peptide or the CRAC^{336SCR} peptide. As shown in Fig. 6, The CRAC^{336WT} peptide, but not the CRAC^{336SCR} peptide, inhibited the activity of LtxA almost completely. A statistical analysis of these results is included in Table 2. Neither peptide was toxic to the cells at the concentrations used over the time course of the experiment.

To determine the half maximal peptide inhibitory concentration (IC_{50}) and peptide saturation limit of the CRAC^{336WT} peptide, the experiment was repeated with several peptide concentrations, and the data were fitted to a sigmoidal curve. The results of this fit predict an IC_{50} of 6.1 μM and a saturation limit, representing the peptide concentration resulting in 100% viability of the cells, of 0.0341 mM for the CRAC^{336WT} peptide.

Previously, we demonstrated that the CRAC^{336WT} peptide inhibits LtxA toxicity against Jn.9 cells (Brown *et al.*, 2013). Therefore, this result demonstrates that an LtxA-derived cholesterol-binding peptide can be used to specifically alter the binding and subsequent toxicity of LtxA against several cell types, suggesting that the approach may have broad applicability in the treatment of *A. actinomycetemcomitans* infections.

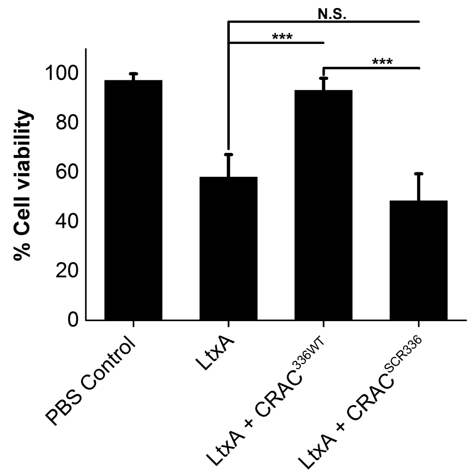

Figure 6 CRAC^{336WT} peptide inhibits leukotoxin (LtxA) toxicity. LtxA and either CRAC^{336WT} or CRAC^{336SCR} were incubated with THP-1 cells for 3 h, and the viability of the cells was measured using a trypan blue assay. The CRAC^{336WT} peptide, which binds to cholesterol, inhibited the toxicity of LtxA, but the CRAC^{336SCR} peptide, which does not bind to cholesterol, did not inhibit LtxA toxicity. A one-way analysis of variance followed by a Tukey test was used to determine the level of significance between each experiment. ***$P \leq 0.001$; ns, not significant.

Table 2 Summary of statistical comparisons of data

Comparison			*P* value	Significantly different
LtxA	vs.	MβCD + LtxA	0.00008	Yes
LtxA	vs.	MβCD + MβCD-Chol + LtxA	0.07240	No
MβCD + LtxA	vs.	MβCD + MβCD-Chol + LtxA	0.00003	Yes
LtxA	vs.	LtxA + POPC	0.24400	No
LtxA	vs.	LtxA + POPC/Chol	0.00000	Yes
LtxA	vs.	LtxA + POPC/Ergo	0.46400	No
LtxA + POPC	vs.	LtxA + POPC/Chol	0.00225	Yes
LtxA + POPC	vs.	LtxA + POPC/Ergo	0.98300	No
LtxA + POPC/Chol	vs.	LtxA + POPC/Ergo	0.00103	Yes
CRAC^{336WT} + POPC	vs.	CRAC^{336WT} + POPC/Chol	0.00502	Yes
CRAC^{336WT} + POPC	vs.	CRAC^{336SCR} + POPC/Chol	0.00001	Yes
CRAC^{336WT} + POPC/Chol	vs.	CRAC^{336SCR} + POPC/Chol	0.00000	Yes
LtxA	vs.	LtxA + CRAC^{336WT}	0.00000	Yes
LtxA	vs.	LtxA + CRAC^{SCR336}	0.24800	No
LtxA + CRAC^{336WT}	vs.	LtxA + CRAC^{SCR336}	0.00001	Yes

One-way analysis of variance was performed using the Tukey comparisons test within OriginPro 2015.

Chol, cholesterol; CRAC, cholesterol recognition amino acid consensus; Ergo, ergosterol; LtxA, leukotoxin; MβCD, methyl-β-cyclodextrin; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

DISCUSSION

More than 90% of cellular cholesterol resides at the plasma membrane and is essential for cell viability and proliferation (Yeagle, 1991; van Meer *et al.*, 2008). Cholesterol is not uniformly dispersed throughout biological membranes; rather, it is sequestered in membrane microdomains known as lipid rafts (Simons & Ikonen, 1997), along with sphingolipids and specialized proteins, such as glycosylphosphatidylinositol-anchored proteins, heterotrimeric G protein-coupled receptors, and Src family kinases (Varma & Mayor, 1998; Simons & Toomre, 2000; Liang *et al.*, 2001; Chini & Parenti, 2004). Many pathogens and their virulence factors have therefore developed the ability to recognize and bind to lipid raft cholesterol on the surface of host cells. For example, cholesterol is required for the uptake of mycobacteria by host cells (Gatfield & Pieters, 2000) and *Leishmania* (Pucadyil & Chattopadhyay, 2007) and allows these intracellular pathogens to avoid degradation by inhibiting lysosomal–phagosomal fusion (Pieters & Gatfield, 2002). In addition, the activity of a number of bacterial toxins depends on the presence of cholesterol in the target membrane (Orlandi & Fishman, 1998; Patel *et al.*, 2002; Schraw *et al.*, 2002; Giddings *et al.*, 2004; Boesze-Battaglia *et al.*, 2006, 2009; Fong *et al.*, 2006; Atapattu & Czuprynski, 2007; Bumba *et al.*, 2010; Farrand *et al.*, 2010; Zhao *et al.*, 2012; Brown *et al.*, 2013; Lai *et al.*, 2013). Influenza (Sun & Whittaker, 2003), human immunodeficiency virus type 1 (Guyader *et al.*, 2002; Liao *et al.*, 2004) and the Ebola virus (Bavari *et al.*, 2002) also require cholesterol in the host membrane for binding to and/or exit from the cell.

Previously, we reported that the binding of LtxA to cholesterol occurs with high affinity (Brown *et al.*, 2013), suggesting the involvement of a lipid-binding site within the toxin. Several proteins that interact with cholesterol have an amino acid sequence in the juxta-membrane region conforming to the pattern -L/V-($X_{1–5}$)-Y-($X_{1–5}$)-R/K-, in which ($X_{1–5}$) represents between one and five residues of any amino acid (Li & Papadopoulos, 1998). The stringency of this motif is flexible, and therefore, not all predicted CRAC sites bind cholesterol or possess an *in vivo* function. In the deduced amino acid sequence of LtxA, $CRAC^{336}$ (^{334}LEEYSKR^{339}) and $CRAC^{503}$ (^{502}VDYLKK^{505}) were identified as potential cholesterol binding sites. Whereas both sequences are juxtaposed to hydrophobic domains in LtxA, only $CRAC^{336}$ was found to bind to cholesterol and regulate the toxicity of LtxA (Brown *et al.*, 2013). $CRAC^{336}$ is highly conserved among other RTX toxins, suggesting a common cholesterol-binding pathogenesis among the members of the RTX family, although additional studies to substantiate this are necessary.

In the current study, we used THP-1, a human monocytic leukemia cell line, which expresses LFA-1 (DiFranco *et al.*, 2012), to investigate the role of cholesterol binding by LtxA on its toxicity. Although we and others have shown that LtxA cytotoxicity requires the expression of LFA-1 by the host cell (Lally *et al.*, 1997; Kieba *et al.*, 2007; Kachlany *et al.*, 2010), we have also shown that LtxA, like other RTX toxins, is strongly membrane-active (Brown *et al.*, 2012, 2013; Walters *et al.*, 2013) and has a particularly strong affinity (10^{-12} M) for membranes containing 40% cholesterol (Brown *et al.*, 2013). This membrane activity is correlated with subtle conformational changes in the entire protein structure (Lear *et al.*, 2000; Walters *et al.*, 2013), but a significant decrease in helicity within the cholesterol-binding domain upon association with cholesterol (Miller *et al.*, 2014), which allows the protein to move from a water-soluble to a membrane-active state.

This work demonstrates the requirement of cholesterol binding by LtxA in cytotoxicity. Based on this and our previous results, we can conclude that LtxA requires both LFA-1 and cholesterol to be present on the membrane for the toxin to kill the target cells. In addition, this work suggests that a possible approach to the inhibition of LtxA activity may be the blocking of cholesterol binding by the toxin. We are currently investigating the long-term toxicity of this cholesterol-binding peptide to study its possible clinical use as an alternative to current, toxic approaches to inhibit the activity of cholesterol-binding proteins and pathogens. Future work will focus on refining the CRAC motif in the peptide to enhance cholesterol binding and improve inhibition of toxin/pathogen binding.

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