Aggregatibacter actinomycetemcomitans leukotoxin (LtxA; Leukothera) induces cofilin dephosphorylation and actin depolymerization during killing of malignant monocytes

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INTRODUCTION

Leukotoxin (LtxA; Leukothera) is a protein produced by the oral bacterium Aggregatibacter actinomycetemcomitans, the causative agent of localized aggressive periodontitis in adolescents (Fine et al., 2007; Newman et al., 1976; Slots & Ting, 1999; Zambon, 1985). In the oral cavity, LtxA binds and kills localized white blood cells (WBCs); thus, allowing the bacterium to evade host immune responses (Kachlany, 2010). Evasion of the immune response allows A. actinomycetemcomitans to proliferate and cause periodontitis that is characterized by bone and tooth loss. LtxA shows targeted specificity towards lymphocyte function associated antigen-1 (LFA-1), and causes rapid death of the WBC (Kachlany et al., 2010; Lally et al., 1997; Taichman et al., 1991). LFA-1 is a β2 integrin expressed only on WBCs. The integrin is a heterodimer of CD11a and CD18, and becomes activated under certain immune stimulating conditions, such as infection and injury. Integrin activation involves changes in the conformational state and clustering of the integrin on the cell surface (Carman & Springer, 2003; Evans et al., 2009; Kinashi, 2005; van Kooyk et al., 1994; Yauch et al., 1997). Activated LFA-1 binds intercellular adhesion molecule-1 (ICAM-1) on vascular endothelial cells to mediate cell adhesion and migration into the surrounding tissue (Carman & Springer, 2003; Hogg et al., 2004; Kinashi, 2005). LFA-1 acts both as an adhesion molecule and as a signalling receptor that signals rearrangement of the F-actin cytoskeleton during cell adhesion and migration (Porter et al., 2002). We have shown that LtxA binds preferentially to the active form of LFA-1 and minimally affects cells that express resting-state LFA-1 (DiFranco et al., 2012; Hioe et al., 2011; Stenderup et al., 2011). The mechanism of cellular killing by LtxA has been studied in several cell types. Studies indicate that in HL-60 monocytes, LtxA causes necrosis at high doses by forming pores in the host cell membrane, while at low doses LtxA induces apoptosis (Korostoff et al., 1998, 2000). In Jurkat T cells, LtxA-mediated apoptosis

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Leukotoxin (LtxA; Leukothera), a protein toxin secreted by the oral bacterium Aggregatibacter actinomycetemcomitans, specifically kills white blood cells (WBCs). LtxA binds to the receptor known as lymphocyte function associated antigen-1 (LFA-1), a β2 integrin expressed only on the surface of WBCs. LtxA is being studied as a virulence factor that helps A. actinomycetemcomitans evade host defences and as a potential therapeutic agent for the treatment of WBC diseases. LtxA-mediated cell death in monocytes involves both caspases and lysosomes; however, the signalling proteins that regulate and mediate cell death remain largely unknown. We used a 2D-gel proteomics approach to analyse the global protein expression changes that occur in response to LtxA. This approach identified the protein cofilin, which underwent dephosphorylation upon LtxA treatment. Cofilin is a ubiquitous actin-binding protein known to regulate actin dynamics and is regulated by LIM kinase (LIMK)-mediated phosphorylation. LtxA-mediated cofilin dephosphorylation was dependent on LFA-1 and cofilin dephosphorylation did not occur when LFA-1 bound to its natural ligand, ICAM-1. Treatment of cells with an inhibitor of LIMK (LIMKi) also led to cofilin dephosphorylation and enhanced killing by LtxA. This enhanced sensitivity to LtxA coincided with an increase in lysosomal disruption, and an increase in LFA-1 surface expression and clustering. Both LIMKi and LtxA treatment also induced actin depolymerization, which could play a role in trafficking and surface distribution of LFA-1. We propose a model in which LtxA-mediated cofilin dephosphorylation leads to actin depolymerization, LFA-1 overexpression/clustering, and enhanced lysosomal-mediated cell death.

Abbreviations: DIGE, 2D-difference gel electrophoresis; HRP, horse-radish peroxidase; PE, phycoerythrin; WBC, white blood cell.
involves modification of the actin cytoskeleton and clustering of LFA-1 in lipid rafts. Initially, the non-specific interaction of LtxA with Jurkat T cells leads to an increase in intracellular calcium levels, followed by calpain activation. Activated calpain then cleaves talin, a protein that tethers LFA-1 to the cytoskeleton. In the absence of talin, LFA-1 mobilizes and clusters into lipid rafts, and finally activates an integrin signalling apoptotic pathway, the components of which remain unknown (Fong et al., 2006). In JY B cells, LtxA-induced apoptosis involves a decrease in 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 (Lally et al., 1999; Yamaguchi et al., 2001). In THP-1 monocytes, LtxA triggers a lysosomal-mediated cell-death pathway and activates several caspases. The LtxA/LFA-1 complex gets internalized and transported to lysosomes causing disruption of the lysosomal membrane, and release of LAMP1 and cathepsin D from the lysosome into the cytosol, leading to cell death (DiFranco et al., 2012). In addition, Kelk et al. (2011) showed that LtxA activates the inflammasome in human monocytes, resulting in the release of IL-1β and IL-18 ultimately resulting in pro-inflammatory cell death. Overall, the current literature suggests that LtxA triggers different cell-death pathways in different cell types.

LtxA is also being studied as an experimental therapeutic agent (Leukothera) for the treatment of WBC diseases. We have demonstrated significant therapeutic efficacy for LtxA in animal models for leukemia (Kachlany et al., 2010), lymphoma (unpublished), psoriasis (Stenderup et al., 2011) and allergic asthma (unpublished). The specificity of LtxA for active LFA-1 makes LtxA a promising therapeutic for various haematological malignancies and autoimmune/inflammatory diseases. The WBCs that are involved in these diseases have increased LFA-1 expression and activation (Bechter et al., 1999; Mengarelli et al., 2001; Pinto et al., 1993; Reuss-Borst et al., 1995), which make them more sensitive to LtxA killing. In addition, LtxA has shown synergy with various standard chemotherapeutic agents; thus, indicating its potential use as a combination therapy to reduce the incidence of chemotoxicity and resistance (Gupta et al., 2011).

In this study, we attempted to identify potential proteins that are either upregulated or downregulated in response to LtxA treatment. Our studies identified two proteins, cofilin and actin, to be significantly affected by LtxA treatment. We report here the impact of pharmacological modulation of these proteins on LtxA-mediated cytotoxicity. The elucidation of signalling proteins impacting LtxA cytotoxicity is critical for understanding how LtxA functions as a virulence factor and may be used as a novel therapeutic agent.

**METHODS**

**Cell lines and growth conditions.** THP-1 and HL-60 (human monocytic leukaemia) cells and K562 (human erythromyeloblastoid leukaemia) cells were obtained from the American Type Culture Collection and were maintained in RPMI 1640 media supplemented with 10% FBS (Invitrogen) at 37 °C, 5% CO₂.

**Purification of LtxA.** Leukotoxin (LtxA) was purified from culture supernatants of A. actinomycetemcomitans strain NJ4500 as previously described (Diaz et al., 2006). LPS is not detectable in these purified LtxA preparations.

**Inhibitors, antibodies and reagents.** The inhibitor used was LIM kinase (LIMK) inhibitor (LIMKi) (Calbiochem). Anti-cofilin (cytoskeleton), anti-p-cofilin (hSer3) (Santa Cruz Biotechnology) and anti-GAPDH (Biolgend) primary antibodies were used for Western blot analysis. Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Pierce) was used as a secondary antibody. Anti-CD11a-PE (phycoerythrin) clone HI111 (BioLegend) was used for flow cytometric analysis of LFA1 levels. Cell death was measured using annexin V-FITC and 7-aminoactinomycin (7-AAD) (Biolgend). Acti-stain 488 fluorescent phalloidin (cytoskeleton) was used to stain filamentous actin (F-actin). Recombinant human ICAM-1 (R&D systems) was used for the cell adhesion assay.

**Cellular viability assays.** THP-1 cells at 0.7–1.0 × 10⁶ cells ml⁻¹ were incubated with LtxA at 37 °C, 5% CO₂, for the indicated time. A trypan blue exclusion assay was used to stain dead cells with trypan blue, followed by counting the number of unstained viable cells using a Vi-CELL cell viability analyser (Beckman Coulter). The annexin staining assay involved washing cells with annexin binding buffer followed by 15 min incubation with annexin V–FITC and 7-AAD at 4 °C, and analysing using a FACSCalibur flow cytometer (BD Biosciences). Ten thousand cells were analysed for every sample.

**Flow cytometric analysis of CD11a and F-actin.** Surface CD11a staining of THP-1 cells was performed by incubating the cells with PE-labelled mAb to CD11a for 30 min at 4 °C prior to analysis. Phalloidin staining of cells first involved fixing the cells with 4% formaldehyde, followed by permeabilization using 0.5% Tween 20 for 15 min. The cells were washed and stained with 100 nM Acti-stain 488 phalloidin for 30 min at room temperature, followed by flow cytometric analysis. Ten thousand cells were analysed per sample with a FACSCalibur flow cytometer and the data were analysed using FlowJo software.

**Imaging flow cytometry for LFA-1 clustering.** THP-1 cells (5 × 10⁵) resuspended in PBS were incubated with DMSO alone or 30 µM LIMKi for 1 h at 37 °C. Cells were then stained with FITC anti-human CD11a antibody for 30 min on ice. Subsequently, the cells were incubated for 10 min at 37 °C, washed twice with cold PBS and fixed using 2% formaldehyde. Cells were imaged with the Amnis ImageStream 100 instrument and the data were analysed with Amnis IDEAS 6 software. Five thousand events were acquired per sample. Cells were analysed for single cells by gating the area of the nuclear stain compared to the aspect ratio intensity of the nuclear stain. LFA-1 positive cells were gated by plotting the intensity of the FITC stain (LFA-1) and selecting the positive population. Focused cells were then selected by gating the gradient root mean square (RMS) of the nuclear stain. Finally, focused cells were then analysed for clustering by measuring the bright detail intensity R3 on a membrane mask off of the LFA-1 mask. Gating for clustering was verified by visual inspection of bins of the histogram.

**Western blot analysis.** Cells (2 × 10⁶) were lysed using M-PER (mammalian protein extraction reagent) buffer supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). Protein extracts were mixed with SDS loading dye and equal amounts of protein were resolved through an SDS-PAGE gel. Proteins were then transferred from the gel to a nitrocellulose membrane using the iBlot dry blotting transfer system (Invitrogen). The membrane was
incubation at 37 °C for 1 h, followed by 15 min incubation on ice and then a 1 h incubation at 37 °C and 5% CO₂ to allow adherence of cells to ICAM-1- or LtxA-coated wells. For determination of relative cell adherence, the cells that did not attach were washed out using serum-free media and adherent cells were measured using the CellTiter Glo-luminescent ATP viability assay (Promega). For Western blot analysis, the cell extracts of adhered cells were prepared and analysed as described above.

## RESULTS

### Protein expression changes in response to LtxA

To assess the global protein expression changes that occur inside mammalian cells in response to LtxA treatment, 2D-difference gel electrophoresis (DIGE) analysis was carried out. HL-60 cells were treated with LtxA or buffer alone (negative control) for 24 h and then total cellular protein was labelled with fluorescent dyes and separated by 2D-gel electrophoresis. Overall, 2174 protein spots were analysed and 2131 spots (98%) were found to be similarly expressed between the two samples. The spots that showed greater than a 1.4-fold change in expression were considered significant. Compared to the control, the LtxA-treated sample had 20 (0.9%) spots that decreased and 23 (1.1%) spots that increased in abundance after treatment. We were particularly interested in proteins that changed isolectric points in response to LtxA, which would indicate that post-translational modification was occurring. One protein that met this criterion was identified by MALDI-MS as cofilin 1. Cofilin showed changes in phosphorylation state with increased in abundance after treatment. We were able to identify at least 20 spots that increased and 23 spots that decreased in abundance. A significant percentage did not meet this criterion was identified by MALDI-MS as cofilin 1.

### LtxA causes cofilin dephosphorylation

To confirm the dephosphorylation of cofilin in response to LtxA, Western blot analysis of HL-60 and THP-1 cell extracts was performed using an antibody against p-cofilin. Cofilin dephosphorylation was observed after 1 h in the immunoblot assay in the HL-60 cells (Fig. 1). The time-dependent dephosphorylation in response to LtxA treatment was even more rapid in THP-1 cells (Fig. 1) compared to HL-60 cells. In THP-1 cells, significant cofilin dephosphorylation was observed as early as 5 min after LtxA treatment. In addition, no significant changes in total cofilin expression were observed (Fig. 1).

### Deophosphorylation of cofilin increases susceptibility to LtxA killing by enhancing lysosomal destabilization

To assess the role of cofilin phosphorylation in LtxA killing, LIMKi was used to inhibit LIMK (Ross-Macdonald et al., 2008), a kinase that phosphorylates cofilin and, thus, deactivates it. Therefore, in the presence of LIMKi, cofilin is expected to show increased dephosphorylation resulting in increased activation (Scott et al., 2010). We found that LIMKi treatment of THP-1 cells for 1 h indeed led to cofilin dephosphorylation (Fig. 3a). We next determined the effect of LIMKi-mediated cofilin dephosphorylation on sensitivity to LtxA. Cells were treated with LIMKi for 1 h to induce cofilin dephosphorylation. These preconditioned cells were then treated with LtxA. In THP-1 cells, LIMKi treatment increased LtxA-mediated cell death by approximately 30% (Fig. 3b).

We have previously shown that LtxA-mediated death of monocytes proceeds via a lysosomal mechanism (DiFranco et al.

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**Fig. 1.** Cofilin dephosphorylation in response to LtxA. HL-60 and THP-1 cells were treated with 0.5 μg LtxA ml⁻¹ for the times indicated; this was followed by immunoblot analysis of the protein using anti-p-cofilin and anti-cofilin antibodies.

**Cofilin dephosphorylation requires LtxA/LFA-1 interaction**

To confirm that LtxA-triggered cofilin dephosphorylation resulted from a specific interaction with LFA-1, the receptor for LtxA, K562 cells (an LFA-1 deficient cell line) were treated with LtxA for different lengths of time, and this was followed by protein extraction and Western blot analysis with antibody to p-cofilin. No change in cofilin phosphorylation was observed after 1 h (Fig. 2a), suggesting that cofilin activation is LFA-1 dependent. Since cofilin dephosphorylation was found to be LFA-1 dependent, we next tested whether ICAM-1 (a natural ligand of LFA-1) could trigger the same effect as LtxA. To explore this, an ICAM-1 cell adhesion assay was performed using THP-1 cells. Cells were allowed to adhere to ICAM-1- or LtxA-coated wells, and this was followed by cell lysis preparation and Western blot analysis. Unlike LtxA, the ICAM-1/LFA1 interaction did not result in cofilin dephosphorylation (Fig. 2b), even though ICAM-1 was able to mediate adhesion to the dish (Fig. 2c).
et al., 2012). Specifically, treatment of monocytes with LtxA causes rapid loss of lysosomal membrane potential and an incidental rise in the lysosomal pH. We therefore asked whether the increase in killing that we observed with LIMKi was due to an effect on the lysosome. We treated cells with LIMKi alone, LtxA alone or the combination of the two, and then determined the pH of the lysosomes using LysoTracker stain and flow cytometry. LIMKi alone did not affect the pH of the lysosomes (Fig. 4a). After 30 min, the combination of LtxA and LIMKi caused a significantly greater increase in lysosomal pH than LtxA alone (Fig. 4b). For the sample that was treated with LtxA alone, the shift in lysosomal pH continued through 90 min, while the sample that was treated with the combination showed no greater shift at this later time point (Fig. 4c). These results suggest that treatment with LIMKi and LtxA in combination results in more rapid disruption of the lysosomal membrane than with LtxA alone.

LIMKi treatment leads to increased LFA-1 surface expression and clustering

We wished to understand how cofilin dephosphorylation could affect lysosomal disruption and cell death in the presence of LtxA. It is known that levels and cell surface distribution of LFA-1 can affect sensitivity to LtxA-mediated cell death (Fong et al., 2006; Kachlany et al., 2010). We thus proposed that cofilin dephosphorylation could lead to an increase in lysosomal destabilization and killing by LtxA by increasing surface levels and/or clustering of LFA-1. To test this hypothesis, we treated cells with LIMKi or vehicle control (DMSO) and then analysed the levels of LFA-1 on the surface using flow cytometry. We found that treatment with LIMKi resulted in a reproducible increase (46 ± 7 %) in LFA-1 surface levels. Next, we determined whether clustering of LFA-1 was affected by LIMKi treatment. Cells were treated with DMSO or LIMKi and then analysed for the surface distribution of LFA-1 on cells using imaging flow cytometry (Fig. 5a). We found that LIMKi enhanced clustering of LFA-1 (Fig. 5b and Fig. 5c). Therefore, LIMKi treatment of cells results in enhanced expression of surface LFA-1 and greater clustering.

LIMKi treatment leads to actin depolymerization

We wished to understand how cofilin dephosphorylation can lead to increased surface expression and clustering of LFA-1. It is known that dephosphorylated cofilin can bind actin and mediate actin depolymerization (Hayden et al., 1993; Lappalainen & Drubin, 1997; Moon & Drubin, 1995). Reorganization of the actin cytoskeleton is known to facilitate the transport of integrins to the surface and clustering of the integrin (Calderwood et al., 2000; Margadant et al., 2011). Therefore, to determine whether LIMKi-induced dephosphorylation of cofilin also translated to an increase in

![Image]
actin depolymerization, THP-1 cells were treated with LIMKi for 4 h and this was followed by phalloidin staining of actin. A decrease in phalloidin staining of F-actin was observed for THP-1 cells (Fig. 6a); thus, indicating depolymerization of actin.

As shown in Fig. 1, LtxA is able to cause cofilin dephosphorylation. We thus determined whether LtxA, similar to LIMKi, could also cause depolymerization of actin. THP-1 cells were treated with LtxA and stained with phalloidin. A significant decrease in phalloidin staining of F-actin was observed for both THP-1 and HL-60 cells (Fig. 6b); thus, indicating depolymerization of actin.

**DISCUSSION**

We used a global approach of DIGE combined with MS to identify cofilin, a protein whose phosphorylation state decreased in response to LtxA. Lee et al. (2006) similarly used 2D-gel electrophoresis and MS to identify cofilin dephosphorylation during H2O2-induced apoptosis in smooth muscle cells. Cofilin is an actin-binding protein known to regulate actin dynamics through changes in its phosphorylation state via LIMK and phosphatases slingshot and chronophin (Arber et al., 1998; Bamburg, 1999; Bamburg et al., 2010). LIMK-mediated cofilin phosphorylation at Ser-3 inactivates the actin depolymerizing activity of cofilin, whereas dephosphorylation renders cofilin active.

LtxA-mediated killing of THP-1 cells is known to involve caspases and lysosomes; however, other protein partners in the apoptotic signalling pathway remain unknown. We showed here that LtxA treatment of HL-60 and THP-1 cells led to cofilin dephosphorylation in a time-dependent manner. We showed that LtxA did not induce cofilin dephosphorylation in LFA-1-deficient K562 cells and dephosphorylation was not detected in THP-1 cells adhering to ICAM-1, the natural ligand for LFA-1. This suggests that cofilin dephosphorylation requires the specific interaction between LFA-1 and LtxA in THP-1 monocytic cells. The involvement of cofilin during LtxA cytotoxicity has not yet been reported and, to our knowledge, this is the first report of cofilin dephosphorylation being associated with LFA-1 signalling.

In 2000, Johansson and colleagues (Johansson et al., 2000) reported that LtxA induces rapid degranulation of polymorphonuclear leukocytes prior to lysis of cells. Degranulation is a process that requires actin polymerization. Interestingly, these authors found that degranulation occurred even when LFA-1 was blocked with antibody that prevented cell death. Thus, some effects of LtxA do not require the interaction between toxin and LFA-1, while others, such as the dephosphorylation of cofilin and cell death, require LFA-1 to be present. To understand the functional significance of cofilin dephosphorylation during LtxA-mediated killing of cells, a LIMKi was used. LIMK is an upstream inhibitor of cofilin activity and so LIMKi is expected to inhibit cofilin phosphorylation and, thus, lead to cofilin activation. LIMKi treatment resulted in cofilin dephosphorylation as well as increased LtxA killing of THP-1 cells. This result suggests that cofilin dephosphorylation in response to LtxA is triggered as part of the cell-death mechanism. This observation can be supported by a study from Yang et al. (2003) who demonstrated that over-expression of WT LIMK1 increased resistance to apoptosis induced by serum withdrawal. Further, dephosphorylated cofilin has been shown to localize in apoptotic blebs during etoposide-induced apoptosis (Mannherz et al., 2005).

During LtxA-mediated cell death of monocytes, LtxA gets internalized into the lysosome causing its rapid destabilization, which results in a rise in lysosomal pH and release of lysosomal proteins into the cytosol (DiFranco et al., 2012). We determined that LtxA caused lysosomal destabilization (increase in pH) more rapidly in the presence of LIMKi. This more rapid destabilization and cell death may be due to...
to the involvement of the actin cytoskeleton and its effect on LFA-1 trafficking. Indeed, we found that treatment of cells with LIMKi resulted in actin depolymerization and a greater amount of LFA-1 on the surface of cells, as well as an increase in LFA-1 clustering. Higher levels of LFA-1 and increased clustering would make cells more susceptible to LtxA-mediated cell death. In support of this proposal, Fong et al. (2006) have shown that after LtxA interacts with cells, LFA-1 clusters into lipid rafts, and disruption of the rafts with an inhibitor leads to resistance to LtxA. When these investigators caused disassembly of the actin cytoskeleton, they observed an increase in LFA-clustering. Furthermore, clustering of LFA-1 is associated with activation of the integrin (Bazzoni & Hemler, 1998; Cambi et al., 2006; Kinashi, 2005), and we have previously reported that LtxA preferentially targets cells expressing active LFA-1. LIMKi
treatment of cells results in greater LFA-1 surface expression, clustering and possibly activation, all of which LtxA can exploit to kill cells more efficiently. Interestingly, Kelk et al. (2011) found that pre-stimulation of human monocytes resulted in a significantly enhanced pro-inflammatory response and this effect may be due to activation of LFA-1. In support of our hypothesis, it has been shown that LIMK and cofilin signalling can regulate trafficking and clustering of acetylcholine receptors in neurons (Lee et al., 2009) and EGFR receptors in tumour cells (Nishimura et al., 2006). Thus, through its effect on actin dynamics, cofilin may be a key player in the trafficking and distribution of numerous cell surface receptors. A model to explain the role of cofilin and actin in LtxA-mediated cell death is presented in Fig. 7.

Recently, Reinholdt et al. (2013) reported that LtxA may be able to interact with two other β2 integrins as well (CD11b/CD18 and CD11c/CD18). This is consistent with other reports that LtxA binds to the CD18 chain of LFA-1 (Dileepan et al., 2007). Thus, the effects that are observed with LtxA could be due to its interaction with other β2 integrins, as well as with certain WBCs. Future studies will decipher whether all β2 integrins act in the same way and result in the same cellular outcome.

In conclusion, we have identified a protein that has previously not been associated with LFA-1 signalling or the mechanism of LtxA-mediated cell death of myeloid cells. We showed that perturbation of the actin cytoskeleton through cofilin can affect properties of LFA-1 and susceptibility to LtxA-mediated cytotoxicity. The knowledge that cofilin dephosphorylation enhances LtxA-mediated cytotoxicity of myeloid cells could be exploited to increase the therapeutic efficacy of LtxA in treating haematological malignancies and inflammatory diseases. Further dissection of the signalling pathways that are activated by LtxA treatment will help us better understand how this fascinating protein functions.

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