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Phosphocholine Antagonizes Listeriolysin O-Induced Host Cell Responses of *Listeria monocytogenes*

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(See the Editorial Commentary by Tulkens, on pages 1425–7.)

Background. Bacterial toxins disrupt plasma membrane integrity with multitudinous effects on host cells. The secreted pore-forming toxin listeriolysin O (LLO) of the intracellular pathogen *Listeria monocytogenes* promotes egress of the bacteria from vacuolar compartments into the host cytosol often without overt destruction of the infected cell. Intracellular LLO activity is tightly controlled by host factors including compartmental pH, redox, proteolytic, and proteostatic factors, and inhibited by cholesterol.

Methods. Combining infection studies of *L. monocytogenes* wild type and isogenic mutants together with biochemical studies with purified phospholipases, we investigate the effect of their enzymatic activities on LLO.

Results. Here, we show that phosphocholine (ChoP), a reaction product of the phosphatidylcholine-specific phospholipase C (PC-PLC) of *L. monocytogenes*, is a potent inhibitor of intra- and extracellular LLO activities. Binding of ChoP to LLO is redox-independent and leads to the inhibition of LLO-dependent induction of calcium flux, mitochondrial damage, and apoptosis. ChoP also inhibits the hemolytic activities of the related cholesterol-dependent cytolysins (CDC), pneumolysin and streptolysin.

Conclusions. Our study uncovers a strategy used by *L. monocytogenes* to modulate cytotoxic LLO activity through the enzymatic activity of its PC-PLC. This mechanism appears to be widespread and also used by other CDC pore-forming toxin-producing bacteria.

Keywords. *Listeria monocytogenes*; listeriolysin O; pore-forming toxin; phospholipase C; PC-PLC; phosphocholine.

Pore-forming toxins (PFT) cause plasmalemmal injury and are potent virulence factors expressed by many pathogenic gram-positive microorganisms [1]. The cholesterol-dependent cytolysins (CDCs) are a widely occurring family of PFTs, produced by many gram-positive bacterial pathogens, including *Streptococcus pneumoniae* and *Listeria monocytogenes*. For *L. monocytogenes*, a foodborne pathogen that can cause serious infections in immunocompromised individuals and pregnant women, the CDC toxin listeriolysin O (LLO) represents a major virulence factor. The production of LLO in *L. monocytogenes* is required for perforation of membranes to enable escape of the bacteria from vacuolar compartments [2]. Hence, LLO-deficient mutants are unable to egress from the phagosome, do not replicate in host cells, and are essentially avirulent [3–7].

LLO also activates a diverse and disparate set of host cell activities, ranging from the modulation of signaling pathways and cellular processes involved in maintaining membrane integrity and organelle function to activities required for the activation of immune function [8]. Many of these activities have been attributed to the modulation of cytosolic calcium (Ca^{2+}) flux and/or potassium (K^+) efflux [9, 10]. Uncontrolled LLO activity would be cytotoxic to host cells and detrimental to intracellular replication of bacteria [11].

A wide range of host cell regulatory mechanisms affecting LLO-dependent pore formation during infection have been reported. These include pH-dependent modulation of LLO activity, the induction of proteolytic and proteostatic activities that affect the half-life of the toxin, induction of LLO activity by the γ-interferon–inducible lysosomal thiol reductase, inhibition of LLO activity by cholesterol loading, adaptor protein complex 2 subunit α-2–dependent removal of LLO from the plasma membrane, and inactivation by posttranslational *S*-glutathionylation modification of its conserved cysteine residue [12–15]. Also, apart from the transcription-dependent regulation of *hly*, the gene encoding LLO, by the positive regulatory factor A of virulence genes in *L. monocytogenes* [16–18], and indirect control of its secretion via upregulation of the Sec secretion system [19], no other bacteria-dependent regulatory activities are known.

In addition to LLO, *L. monocytogenes* also produces 2 membrane-damaging phospholipases that are specific phosphatidylinositol- and phosphatidylcholine-cleaving

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enzymes (PI-PLC and PC-PLC) [20, 21]. PI-PLC is a specialized phospholipase that cleaves only phosphatidylinositol [22] and has been reported to contribute to *L. monocytogenes* escape from primary vacuoles [23]. PC-PLC is a broad-range phospholipase that can cleave phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine (PS), and sphingomyeline, producing phosphocholine (ChoP), phosphoethanolamine, and phosphoserine [21, 24]. Its expression is associated with escape from secondary vacuoles, contributing to cell-to-cell spread to neighboring cells [25]. PC-PLC is produced as a preproenzyme and, following removal of its signal peptide, exists as a proenzyme with a 24-amino acid residue propeptide extension. Cleavage of the propeptide is dependent on Mpl, the metalloprotease of *L. monocytogenes* [26, 27].

Unexpectedly, a recombinant *L. monocytogenes* strain that constitutively produces mature active PC-PLC is attenuated for virulence in vivo and highly susceptible to killing by neutrophils [28]. Paradoxically, the constitutive production of PC-PLC also protects cells from LLO-induced mitochondrial fragmentation [28–30]. In addition, the ability of listerial phospholipases to trigger the microbicidal activities of phagocytic nicotinamide adenine dinucleotide phosphate oxidase is counteracted by the pore-forming activity of LLO [31]. Finally, phospholipase activity enables the release of bacteria from vesicular exofacial PS structures induced by LLO [32]. Thus PC-PLC appears to both promote LLO activity and/or inhibit membrane repair mechanisms.

As these effects reflect the reciprocal activities of PC-PLC and LLO, we hypothesized a modulatory effect of ChoP on cellular activities of LLO. Here we demonstrate that PC-PLC indeed blunts LLO-dependent host cell responses via the enzymatic production of ChoP. Using purified enzymes and their substrates we show that this is a consequence of a direct interaction between ChoP and LLO.

METHODS

Cell Culture

HeLa cells (human cervix adenocarcinoma) were maintained in Dulbecco's modified Eagle medium (DMEM; Life Technologies) and cultured in the presence of 10% fetal bovine serum (FBS; Biochrom) at 37°C in a humidified, 5% CO_2 atmosphere.

Cells were seeded out in cell culture dishes 1 day prior to the experiments. At 90%–100% confluency, they were rinsed with Hanks' Balanced Salt Solution (HBSS; Merck) and incubated in medium containing 10% FBS for 2 hours. For the infection assays, cells were washed twice with HBSS prior to bacterial addition. The infection was carried out in medium containing 0.5% FBS for indicated time points. For treatment with LLO, cells were washed 5 times with HBSS and LLO incubation was performed in DMEM without FBS at 37°C for indicated time points. For treatment with PI-PLC/PC-PLC, phospholipases were added simultaneously to LLO and incubated at 37°C for time points indicated.

Bacterial Strains, Growth Conditions, and Infection

Bacterial strains used in this study include *L. monocytogenes* EGD-e [33], *L. monocytogenes* Δ*hly* (a LLO-negative mutant) [34], *L. monocytogenes* Δ*PI-PLC* (a PI-PLC–negative mutant) [35], and *L. monocytogenes* Δ*PC-PLC* (a PC-PLC–negative mutant) [34]. For infections, *L. monocytogenes* was grown as previously described [36]. Infection was carried out at a multiplicity of infection of 10.

Protein Purification

Protocols for protein purification are given in the Supplementary Methods.

Measurement of Mitochondrial Reactive Oxygen Species, Mitochondrial Membrane Potential, and Caspase 3/7 Activation

Measurements of mitochondrial reactive oxygen species (ROS) production, mitochondrial membrane potential, and caspase 3/7 activation were performed with incubation of HeLa cells with MitoSox (Thermo Fischer Scientific), JC-1 (Thermo Fischer Scientific), and CellEvent Caspase-3/7 Green Detection Reagent (Thermo Fischer Scientific), respectively, according to manufacturer's instructions, and measured by flow cytometry.

Confocal Microscopy

Confocal microscopy is described in the Supplementary Methods.

Measuring Intracellular [Ca^{2+}]

HeLa cells were seeded out on glass coverslips. At approximately 80% confluency cells were used for determination of changes in cytoplasmic Ca^{2+} concentration by Fluo-4-AM (excitation/emission, 494/506; Life Technologies). To perform [Ca^{2+}] measurements, cells were loaded with 2 μM Fluo-4-AM in physiological solution (140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.5 mM glucose, 10 mM HEPES, pH 7.4) at 37°C for 30–90 minutes. Cells were washed with physiological solution for 10 minutes and the coverslips were mounted on an inverted confocal laser scanning microscope (Zeiss LSM 710). Cells were stimulated with LLO (150 ng/mL) in the presence or absence of PC-PLC (20 μg/mL). Changes in cytoplasmic Ca^{2+} were recorded by taking images every 2 seconds with the microscope. Each cell was independently tracked and the relative change in fluorescence intensity was plotted by arbitrarily setting the baseline fluorescence to 100%.

Isolation of Cytosolic and Mitochondrial Fraction

Preparation of cytosolic and mitochondrial fraction was done as described elsewhere [37].

Hemolysis Assay

Determination of hemolytic activity was performed as described previously [36]. Toxins were preincubated with phosphocholine

chloride calcium salt tetrahydrate (Sigma-Aldrich) where described at room temperature for 30 minutes. For determination of hemolysis in the presence of phospholipases, sheep erythrocytes (Acila) were preincubated with PC-PLC (20 μg/mL) or 10 U PLC from *Bacillus cereus* (Merck Millipore) at 37°C and 5% CO_2 for 1 hour. For determination of hemolysis under nonreducing conditions, 5 mM dithiothreitol (DTT) was removed from buffer.

Preparation of Large Unilamellar Vesicles

Large unilamellar vesicles (LUVs) were prepared as described previously [38].

Interaction Measurements

Interaction measurements are described in Supplementary Methods.

Immunoblot Analysis

Immunoblot analysis is described in Supplementary Methods.

Statistical Analysis

Statistical analysis of experiments was performed with SigmaPlot 11 (Systat Software). *P* values of ≤ .05, ≤ .01, and ≤ .001 were considered statistically significant. The number of individual experiments is indicated in the "Results" section.

RESULTS

Inhibition of LLO-Induced Mitochondrial ROS Generation by PC-PLC

We analyzed changes in mitochondrial dynamics induced by *L. monocytogenes* with wild-type (WT) bacteria and isogenic mutants Δ*hly*, Δ*PI-PLC*, and Δ*PC-PLC* lacking the hemolytic activity of LLO, and phospholipase activities of PI-PLC and PC-PLC, respectively. HeLa cells infected with *L. monocytogenes* WT and its isogenic derivatives were examined for their respective capacities to induce mitochondrial ROS production using flow cytometry. We first established that in-frame deletions did not affect protein expression of secreted and cell wall-located LLO, PI-PLC, and PC-PLC as well as hemolytic properties (Supplementary Figure 1A and 1B). Infection revealed that *L. monocytogenes* WT, Δ*PI-PLC*, and Δ*PC-PLC*, but not Δ*hly*, induced an increase in mitochondrial ROS generation as compared to the uninfected control. Generation of mitochondrial ROS in cells infected with the PC-PLC–negative mutant was, however, significantly higher than that of cells infected with either *L. monocytogenes* WT or Δ*PI-PLC* (Figure 1A). These results suggested that in the absence of PC-PLC, LLO-mediated induction of mitochondrial ROS in infected cells is enhanced.

We sought to isolate and understand the basis of this observation by employing purified PI-PLC and PC-PLC (Supplementary Figure 1C–1G). Purified phospholipases were used to confirm that PC-PLC indeed inhibits LLO-induced mitochondrial ROS production. In HeLa cells exposed to LLO, the addition of PC-PLC, but not purified PI-PLC, significantly reduced ROS production as compared to treatment with LLO alone (Figure 1B and 1C). This effect was concentration dependent, confirming the antagonistic effect of PC-PLC on LLO (Supplementary Figure 1G).

PC-PLC Inhibits Alteration of Mitochondrial Dynamics Induced by LLO

To investigate whether PC-PLC also antagonizes LLO-induced mitochondrial fragmentation, HeLa cells were infected with *L. monocytogenes* WT and the Δ*hly* or Δ*PC-PLC* mutants. Changes in mitochondrial membrane potential were measured as a surrogate marker of mitochondrial fragmentation. Cells treated with carbonyl cyanide 3-chlorophenylhydrazone were used as a positive control. Infection assays revealed that in cells infected with the PC-PLC–negative mutant a significant increase in mitochondrial fragmentation was observed as compared to *L. monocytogenes* WT (Figure 2A). Also, incubation with purified LLO induced mitochondrial fragmentation that was reduced significantly in the presence of PC-PLC (Figure 2B). To further examine changes in mitochondrial dynamics and morphology, confocal microscopy was performed. HeLa cells treated with LLO showed loss of mitochondrial tubular structures as compared to untreated cells. Simultaneous treatment with LLO and PC-PLC restored the tubular structure of mitochondria (Figure 2C), suggesting that PC-PLC has a protective effect on LLO-induced mitochondrial damage.

PC-PLC Alters LLO-Induced Ca^{2+} Flux

Because the induction of Ca^{2+} flux is a crucial step in altering mitochondrial dynamics in the presence of LLO [30], we next investigated whether PC-PLC interferes with LLO-induced Ca^{2+} flux. Treatment of HeLa cells with LLO alone induced an increase in fluorescence intensity, indicating Ca^{2+} influx. Extracellular Ca^{2+} was identified as major source of Ca^{2+} following LLO treatment, as this effect was abolished when using Ca^{2+}-free medium (Figure 3A). In this case the small increase in intracellular [Ca^{2+}] observed only after 240 seconds was probably due to release of Ca^{2+} from intracellular stores, for example, from the endoplasmic reticulum (ER). The increase in Ca^{2+} influx in the presence of PC-PLC was more modest than that observed with LLO alone and, in particular, the recovery to baseline [Ca^{2+}] levels was rapid and contrasted to that observed with LLO (Figure 3B).

PC-PLC Antagonizes LLO-Induced Apoptosis in a Cytochrome *c*- and Caspase 3/7-Dependent Manner

To verify the protective effect of PC-PLC on other LLO-induced host cell responses we also examined its role during LLO-induced apoptosis. HeLa cells infected with *L. monocytogenes* WT, and the mutants Δ*hly* and Δ*PC-PLC*, were incubated with fluorophore-coupled annexin V and monitored by flow cytometry. *L. monocytogenes* WT, but not Δ*hly*, induced an increase in annexin V-positive cells as compared to uninfected controls. However, annexin V staining in cells infected with the

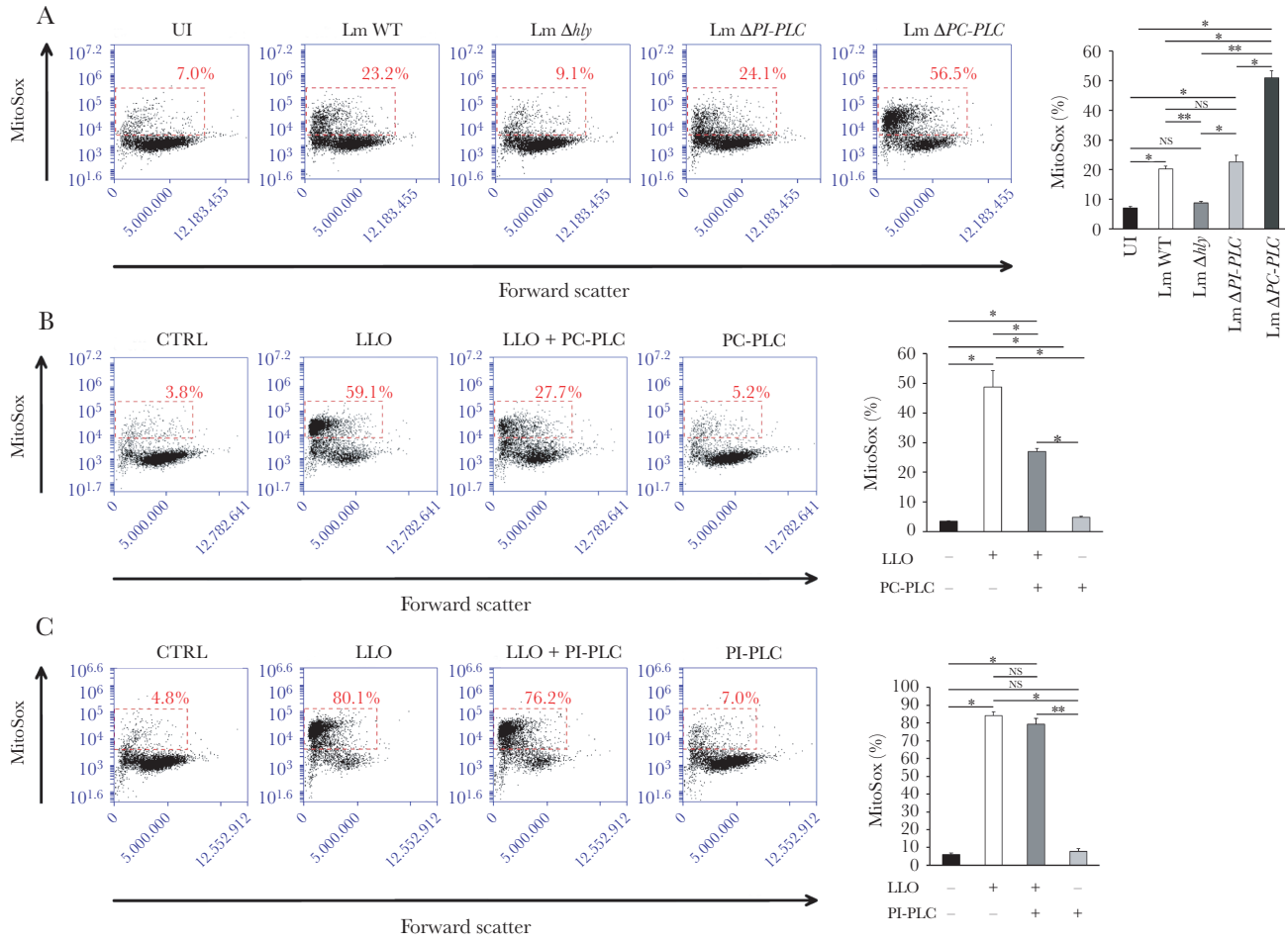

Figure 1. PC-PLC dampens LLO-mediated mitochondrial ROS generation. *A*, HeLa cells were infected with *Listeria monocytogenes* WT, Δ*hly*, Δ*PI-PLC*, Δ*PC-PLC*, or left UI. Cells were stained 4 hours postinfection with MitoSox at 37°C for 10 minutes. Samples were measured by flow cytometry. *L. monocytogenes* WT and Δ*PI-PLC* but not Δ*hly* increased mitochondrial ROS production. The level of mitochondrial ROS was even more increased after infection with *L. monocytogenes* Δ*PC-PLC*. Dead cells were excluded by gating. *B*, HeLa cells were treated with LLO (50 ng/mL) and PC-PLC (5 µg/mL) at 37°C for 10 minutes and stained with MitoSox at 37°C for 10 minutes. Samples were measured by flow cytometry. LLO-induced mitochondrial ROS generation was inhibited by PC-PLC. Dead cells were excluded by gating. *C*, HeLa cells were treated with LLO (50 ng/mL) and PI-PLC (5 µg/mL) at 37°C for 10 minutes and stained with MitoSox at 37°C for 10 minutes. Samples were measured by flow cytometry. PI-PLC had no significant effect on LLO-induced mitochondrial ROS generation. Dead cells were excluded by gating. *A*, *B*, and *C*, Mean values ± SEM are plotted from 3 independent experiments. * $P \leq .05$; ** $P \leq .01$; NS, not significant; Kruskal-Wallis test and Student-Newman-Keuls posthoc test. Abbreviations: CTRL, control; LLO, listeriolysin O; Lm, *Listeria monocytogenes*; PC-PLC, phosphatidylcholine-specific phospholipase C; PI-PLC, phosphatidylinositol-specific phospholipase C; ROS, reactive oxygen species; UI, uninfected; WT, wild type.

PC-PLC–negative mutant was significantly higher than that of cells infected with *L. monocytogenes* WT (Figure 4A). We also examined changes in cytochrome *c* localization within the cell. HeLa cells were infected with *L. monocytogenes* WT, Δ*hly*, and Δ*PC-PLC* respectively, and subjected to subcellular fractionation into cytosolic and mitochondrial fractions. Fraction purity was assessed using cytochrome-*c* oxidase (Cox IV) localization and glyceraldehyde-3-phosphate dehydrogenase as a loading control. Infection with *L. monocytogenes* WT, but not with Δ*hly*, led to a small increase in the amount of cytochrome *c* released into the cytosol. However, cells infected with *L. monocytogenes* Δ*PC-PLC* exhibited increased amounts of cytochrome *c* in the cytosolic fraction (Figure 4B), suggesting that PC-PLC alleviates *L. monocytogenes*-induced cytochrome *c*-dependent cell death. The suppressive effect of PC-PLC on LLO-induced host cell responses was also observed when analyzing the activation of caspase 3/7. Treatment with staurosporine was used as a positive control. Infection assays showed that WT and Δ*hly* displayed no significant levels of caspase 3/7 activation (Figure 4C). However, in cells infected with the Δ*PC-PLC* a strong increase in caspase 3/7 activation was observed, as compared to cells infected with WT (Figure 4C). This observation was also confirmed by monitoring changes in caspase 3/7 levels in cells treated with purified LLO both in the presence or absence of PC-PLC (Figure 4D). Treatment with LLO alone caused an increase in caspase 3/7 activity, which was reduced significantly by the addition of purified PC-PLC (Figure 4D). Collectively, these results show that extracellularly induced LLO-dependent apoptosis is alleviated by PC-PLC.

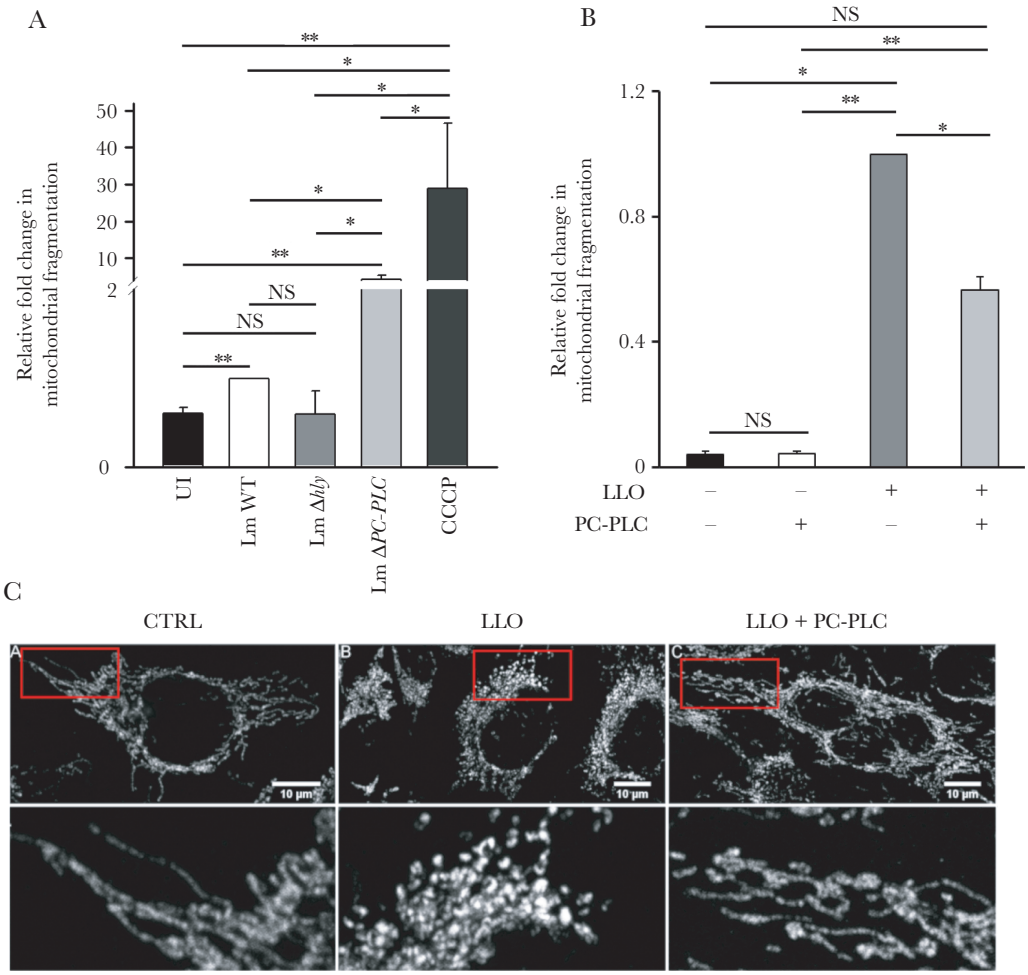

Figure 2. PC-PLC dampens LLO-mediated mitochondrial fragmentation. *A*, HeLa cells were infected with *Listeria monocytogenes* WT, Δ*hly*, Δ*PC-PLC*, left UI, or treated with CCCP, an inducer of mitochondrial fragmentation. At 4 hours postinfection, cells were stained with JC-1 at 37°C for 20 minutes and measured by flow cytometry. The ratio of green to red fluorescence was used to detect mitochondrial fragmentation. WT but not Δ*hly* induced mitochondrial fragmentation. Δ*PC-PLC* induced an even higher mitochondrial fragmentation. Dead cells were excluded by gating. *B*, HeLa cells were treated with LLO (50 ng/mL) and PC-PLC (5 μg/mL) at 37°C for 10 minutes and stained with JC-1 at 37°C for 20 minutes. Samples were measured by flow cytometry. The ratio of green to red fluorescence was used to detect mitochondrial fragmentation. LLO induced mitochondrial fragmentation, which was inhibited by PC-PLC. Dead cells were excluded by gating. *C*, HeLa cells were treated with LLO (50 ng/mL) and PC-PLC (5 μg/mL) at 37°C for 10 minutes and stained for mitochondria (Cox IV). Samples were imaged by confocal microscopy. Cells treated with LLO showed fragmentation of mitochondria compared to untreated cells. In cells treated with LLO and PC-PLC tubular structures were restored. *A* and *B*, Mean values ± SEM are plotted from 3 independent experiments. * $P \leq .05$; ** $P \leq .01$; NS, not significant; Kruskal-Wallis test and Student-Newman-Keuls posthoc test. Areas in boxes are expanded in images below. Abbreviations: CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CTRL, control; LLO, listeriolysin O; Lm, *Listeria monocytogenes*; PC-PLC, phosphatidylcholine-specific phospholipase C; UI, uninfected; WT, wild type.

ChoP Inhibits the Activity of LLO

We wondered whether the observations made were due to a direct interaction between ChoP, the catalytic reaction product of PC-PLC, and LLO. We first sought evidence for a direct interaction between ChoP and LLO by monitoring changes in mitochondrial ROS production. HeLa cells treated with LLO reacted with an increase in mitochondrial ROS production (Figure 5A). However, cells treated with LLO in the presence of increasing concentrations of either ChoP or PC-PLC exhibited decreased mitochondrial ROS production in a concentration-dependent manner (Figure 5A and Supplementary Figure 1H), suggesting that both active PC-PLC and ChoP antagonize the LLO activity.

To confirm this finding, we performed hemolytic titer assays wherein sheep erythrocytes were incubated with PC-PLC prior to addition of LLO. We observed a significant decrease in LLO-dependent hemolytic activity in the presence of PC-PLC (Figure 5B). This effect was also observed when preincubating sheep erythrocytes with a phospholipase C from *B. cereus*, suggesting that this is a more general effect of PC-specific phospholipases C (Supplementary Figure 2A). LLO-mediated hemolysis was also inhibited when LLO was preincubated with different concentrations of ChoP prior to the addition of sheep erythrocytes (Figure 5B). This effect was also observed for pneumolysin from *Streptococcus pneumoniae* (PLY) and streptolysin O from *Streptococcus pyogenes*

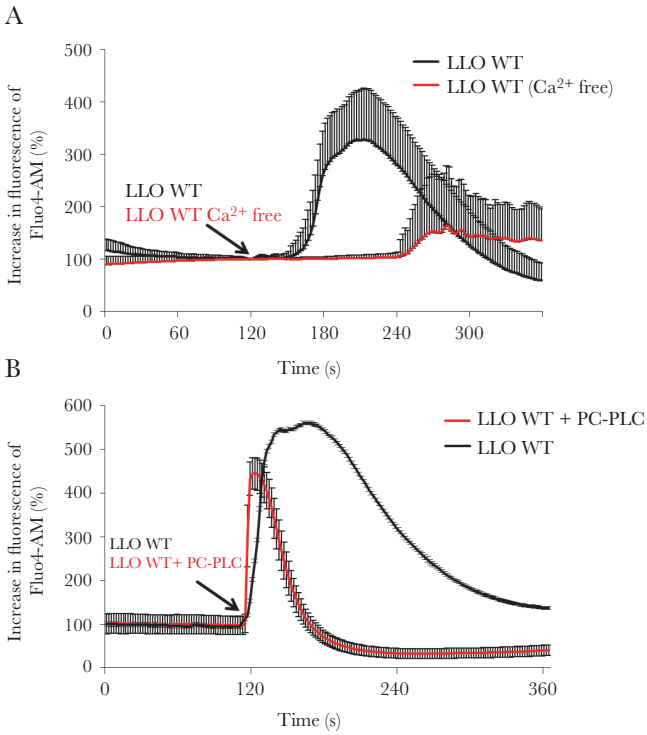

Figure 3. Modulation of LLO-promoted Ca^{2+} influx by PC-PLC. *A*, HeLa cells were grown on coverslips, loaded with Fluo4-AM, and treated with LLO (150 ng/mL) in the presence or absence of Ca^{2+}. Changes in cytoplasmic [Ca^{2+}] were recorded over time. [Ca^{2+}] flux increase was not observed when Ca^{2+} was not present in the medium. Mean values ± SD are plotted from 3 independent experiments with 51 (medium with Ca^{2+}) and 136 (medium without Ca^{2+}) cells analyzed. *B*, HeLa cells were grown on coverslips, loaded with Fluo4-AM, and treated with LLO (150 ng/mL) in the presence or absence of PC-PLC (20 µg/mL). Changes in cytoplasmic [Ca^{2+}] were recorded. Mean values ± SD are plotted from 4 independent experiments with 236 (LLO WT) and 164 (LLO WT + PC-PLC) cells analyzed. Abbreviations: LLO, listeriolysin O; PC-PLC, phosphatidylcholine-specific phospholipase C; WT, wild type.

(SLO), indicating a general inhibitory effect of ChoP on CDCs (Supplementary Figure 2B and 2C). As LLO activity is optimal under acidic conditions, we examined levels of antagonism at different pHs by monitoring for changes in hemolytic activity using both purified ChoP and PC-PLC. Inhibition of LLO activity was essentially pH independent for both assays (Supplementary Figure 2D and 2E). To exclude the possibility that ChoP-mediated inhibition is due to changes in the redox state of LLO, we performed hemolytic titer assays using an LLO variant harboring a mutation of the redox state-sensing cysteine (LLO C484S) [34]. No changes in ChoP-mediated inhibition of LLO-mediated lysis of sheep erythrocytes were detected when using LLO C484S as compared to LLO WT under both reducing and nonreducing conditions (with and without DTT) (Figure 5C), suggesting that ChoP-mediated inhibition of LLO is independent of its redox status.

ChoP Affects the Binding of LLO to Lipid Vesicles

As it is known that LLO binds to cholesterol resulting in cytolysis inhibition by impaired pore formation [14, 39], we analyzed whether ChoP also binds to LLO. Binding properties of LLO to LUVs in the absence or presence of ChoP were examined using surface plasmon resonance. LUVs immobilized on the sensor surface were incubated with LLO or with LLO preincubated with ChoP. ChoP-pretreated LLO bound to LUVs to a similar extent than untreated LLO (Figure 6A). However, the dissociation rate of ChoP-pretreated LLO was higher as compared to untreated LLO, suggesting that ChoP interacts with LLO to promote its dissociation from the plasma membrane. To confirm a direct interaction between ChoP and LLO, purified LLO was immobilized on the sensor surface and treated with increasing concentrations of ChoP. These results revealed that ChoP binds to LLO in a concentration-dependent manner (Figure 6B). Therefore, we assume that ChoP binding does not inhibit the interaction of LLO with the membrane, but instead increases its dissociation.

DISCUSSION

Nearly 30 years ago the broad-range phospholipase C (PC-PLC) of *L. monocytogenes* was identified as an essential virulence factor required for escape from the secondary vacuole formed after cell-to-cell spread, through its capacity to degrade host membrane phospholipids [25]. This view on the role of PC-PLC activity has been generally accepted because mutants lacking PC-PLC form small plaques when examined in assays for intercellular spread [25]. Nevertheless, mutant bacteria that constitutively produce mature active PC-PLC are also defective for plaque formation and, in particular, are attenuated 100-fold in a mouse assay of organ colonization [28]. Thus, it has been postulated that tight, compartment-specific regulation of transcriptional activities, together with posttranslational modification of virulence factors such as PC-PLC, are requisites for *L. monocytogenes*' success in infection.

We describe here a novel property associated with the cleavage product resulting from the enzymatic activity of PC-PLC on glycerophospholipids, the major structural lipids of eukaryotic membranes, that is ChoP. ChoP is an inhibitor of the hemolytic activity of LLO and other members of the CDC family of toxins. We note that while the presence of cholesterol is lowest in the ER membrane and highest in plasma membranes, ChoP concentrations are highest in the ER membrane and lowest in plasma membranes [40]. Thus *L. monocytogenes* exploits these compartmental differences to modulate LLO activity as the bacterium navigates its path through the infected cell. Our data provide an explanation as to why infected host cells are protected from overt toxicity despite the production of PFTs.

We demonstrate that inhibition of LLO activity also occurred at acidic pHs. This appears counterintuitive as LLO activity is essential for release of internalized bacteria within the phagosome to the host cytoplasm. Therefore, expression and production of PC-PLC must be highly regulated in order not to negate LLO-dependent activity. Indeed, expression of the *pc-plc* gene is exquisitely regulated and specifically activated when the bacterium is present in the host cell

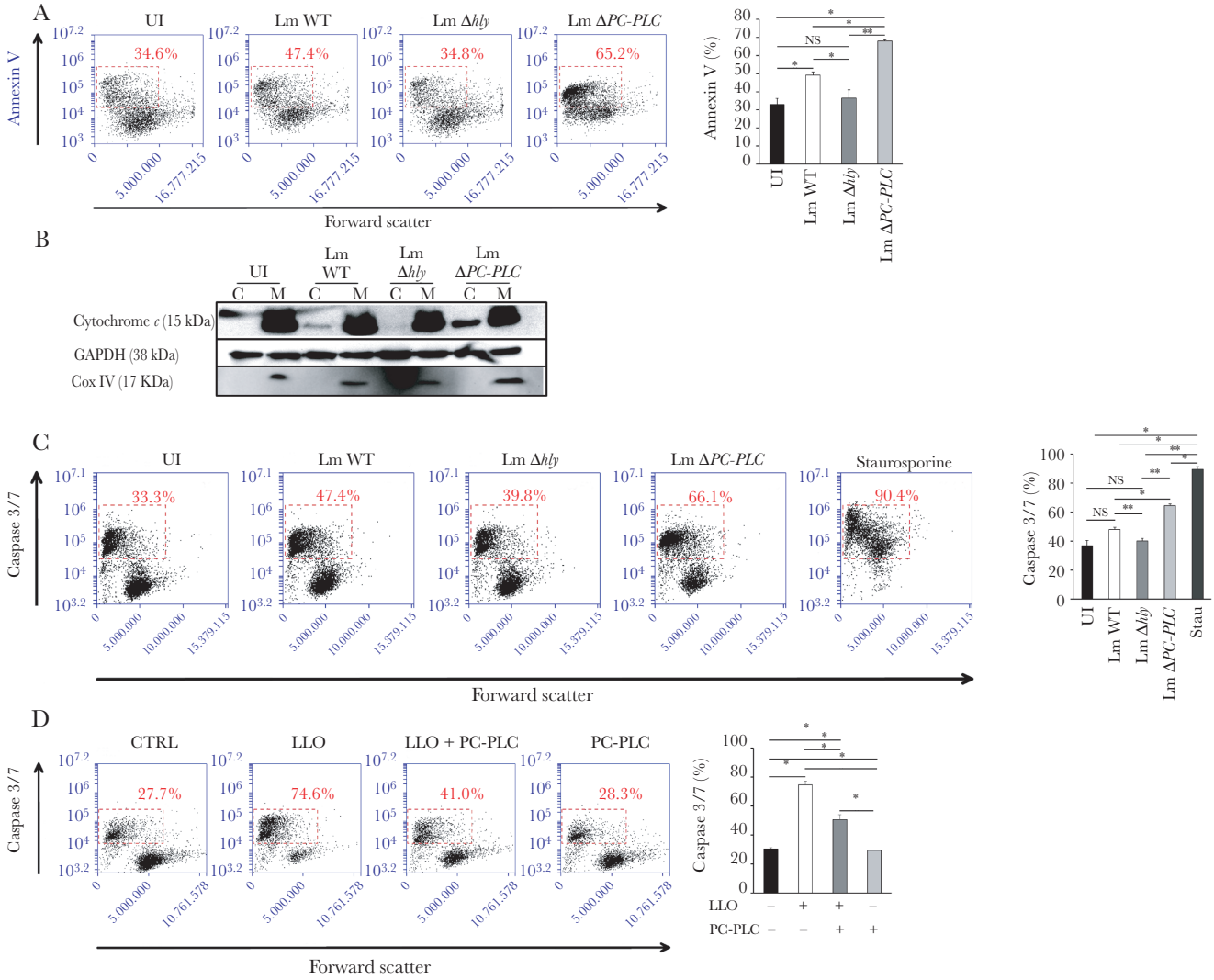

Figure 4. PC-PLC dampens LLO-induced cytochrome *c*-dependent cell death. *A*, HeLa cells were infected with *Listeria monocytogenes* WT, Δ*hly*, and Δ*PC-PLC* or left UI. At 4 hours postinfection, cells were stained with Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit at room temperature for 10 minutes. Samples were measured by flow cytometry. WT but not Δ*hly* showed an increase in apoptosis. Apoptosis was even more increased after infection with Δ*PC-PLC*. Dead cells were excluded by gating. *B*, Western blot analysis of HeLa cells infected with WT, Δ*hly*, Δ*PC-PLC*, or left UI. After infection, cells were fractionated in cytosolic and mitochondrial fractions. Infection with Δ*PC-PLC* induced a higher translocation of cytochrome *c* from mitochondria to cytosol as compared to WT. Infection with Δ*hly* did not induce cytochrome *c* release in the cytosolic fraction. Data from 1 of 3 experiments. *C*, HeLa cells were infected with WT, Δ*hly*, Δ*PC-PLC*, left UI, or treated with staurosporine, an inducer of apoptosis. At 4 hours postinfection, cells were stained with CellEvent caspase-3/7 at 37°C for 25 minutes and measured by flow cytometry. Neither WT nor Δ*hly* induced caspase 3/7 activation. However, the level of caspase 3/7 increased after infection with Δ*PC-PLC*. Dead cells were excluded by gating. *D*, HeLa cells were treated with LLO (50 ng/mL) and PC-PLC (5 µg/mL) at 37°C for 10 minutes and stained with CellEvent caspase-3/7 at 37°C for 25 minutes. Samples were measured by flow cytometry. LLO-induced caspase-3/7 activation was inhibited by PC-PLC. Dead cells were excluded by gating. *A*, *C*, and *D*, Mean values ± SEM are plotted from 3 independent experiments. * $P \leq .05$; ** $P \leq .01$; NS, not significant; Kruskal-Wallis test and Student-Newman-Keuls posthoc test. Abbreviations: C, cytosolic; LLO, listeriolysin O; Lm, *Listeria monocytogenes*; M, mitochondrial; PC-PLC, phosphatidylcholine-specific phospholipase C; Stau, staurosporine; UI, uninfected; WT, wild type.

cytoplasm [41–43]. Also, because PC-PLC is produced as an inactive precursor, expression of its phospholipase activity is subject to posttranslational modification, that is by proteolytic cleavage.

The enzymatic activity of PC-PLC directly antagonizes activities of LLO such as the induction of mitochondrial fragmentation and attendant ROS production [30]. Disruption of mitochondrial integrity and uncontrolled release of ROS is lethal for the infected cell and would negatively impact intracellular growth of bacteria. We demonstrate that the enzymatic activity of PC-PLC alleviates LLO-induced mitochondrial damage to promote cell survival.

The damage to host membranes by LLO is countered by induction of the caspase 3/7 pathway to promote membrane repair. Addition of PC-PLC to LLO-treated cells significantly decreased plasmalemmal injury as evidenced by the concentration-dependent decrease of caspase 3/7 complex

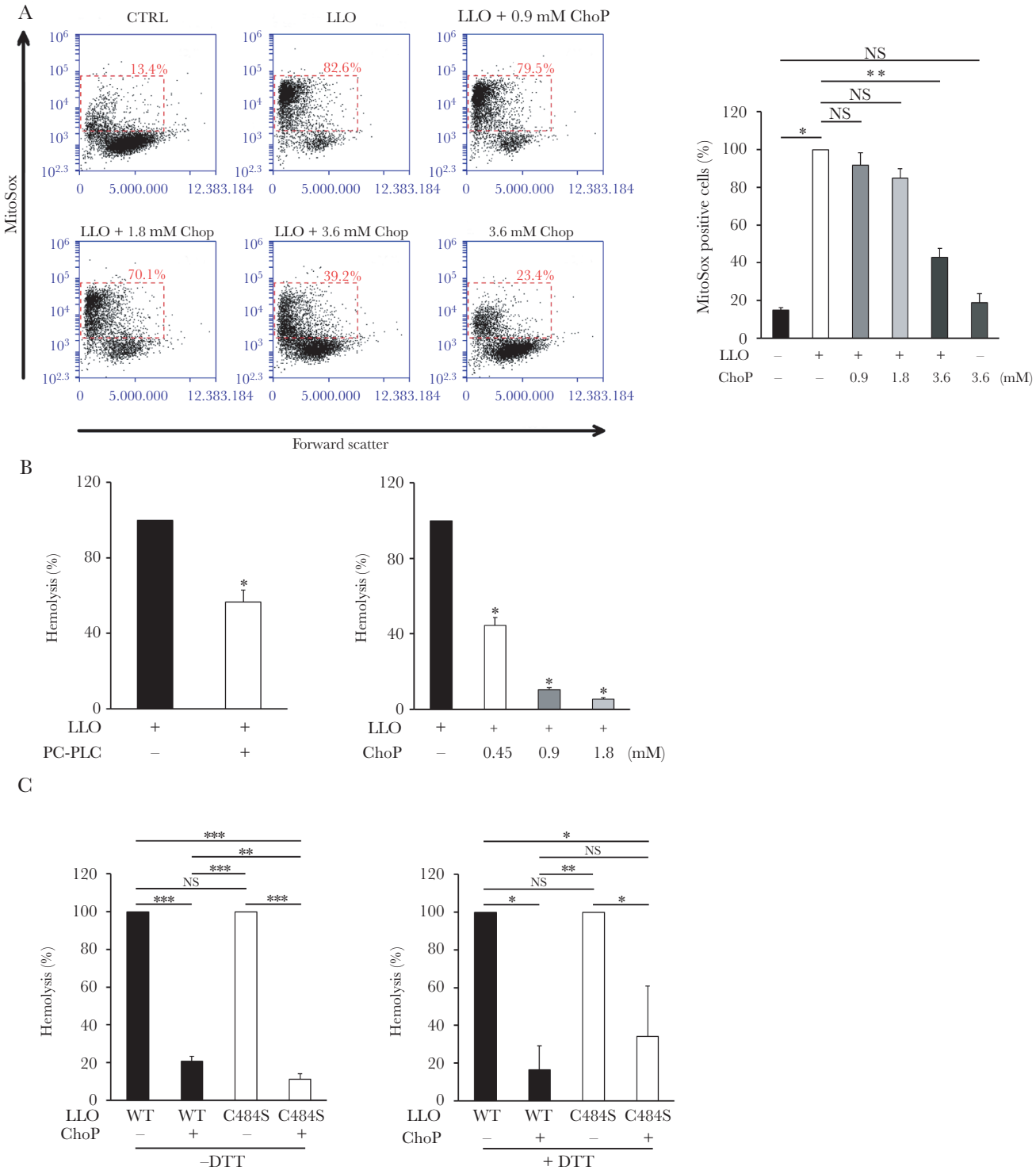

Figure 5. Phosphocholine inhibits mitochondrial ROS generation and modulates the hemolytic properties of LLO. *A*, HeLa cells were treated with LLO (50 ng/mL) and indicated concentrations of ChoP. Mitochondrial ROS generation was monitored after staining with MitoSox at 37°C for 10 minutes using flow cytometry. LLO-induced mitochondrial ROS generation was inhibited dose-dependently by ChoP. Dead cells were excluded by gating. Mean values ± SEM are plotted from 3 independent experiments, * $P \leq .05$; ** $P \leq .01$; NS, not significant; Kruskal-Wallis test and Student-Newman-Keuls posthoc test. *B*, Sheep erythrocytes were pretreated with PC-PLC (20 µg/mL) at 37°C for 1 hour and incubated in the presence of LLO (20 µg/mL) at 37°C for 1 hour or LLO (20 µg/mL) was pretreated with indicated concentrations of ChoP and incubated with sheep erythrocytes at 37°C for 1 hour. After centrifugation, the absorbance of the supernatant was measured at 405 nm. PC-PLC inhibited the lysis of sheep erythrocytes. Mean values ± SEM are plotted from 3 independent experiments. For PC-PLC pretreatment, * $P \leq .05$, different from LLO-treated cells, Mann-Whitney U test; for ChoP pretreatment, * $P \leq .05$, Kruskal-Wallis test and Student-Newman-Keuls posthoc test. *C*, LLO WT (1.25 µg/mL) and LLO C484S (20 µg/mL) were pretreated with 1.8 mM ChoP and incubated with sheep erythrocytes at 37°C for 1 hour under nonreducing (–DTT) or reducing (+DTT) conditions. After centrifugation, the absorbance of supernatant was measured at 405 nm. ChoP inhibited the lysis of erythrocytes independently of the redox status of LLO. Mean values ± SEM are plotted from 3 independent experiments. * $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$, NS, not significant; 1-way ANOVA and Student-Newman-Keuls posthoc test. Abbreviations: DTT, dithiothreitol; ChoP, phosphocholine; LLO, listeriolysin O; PC-PLC, phosphatidylcholine-specific phospholipase C; ROS, reactive oxygen species.

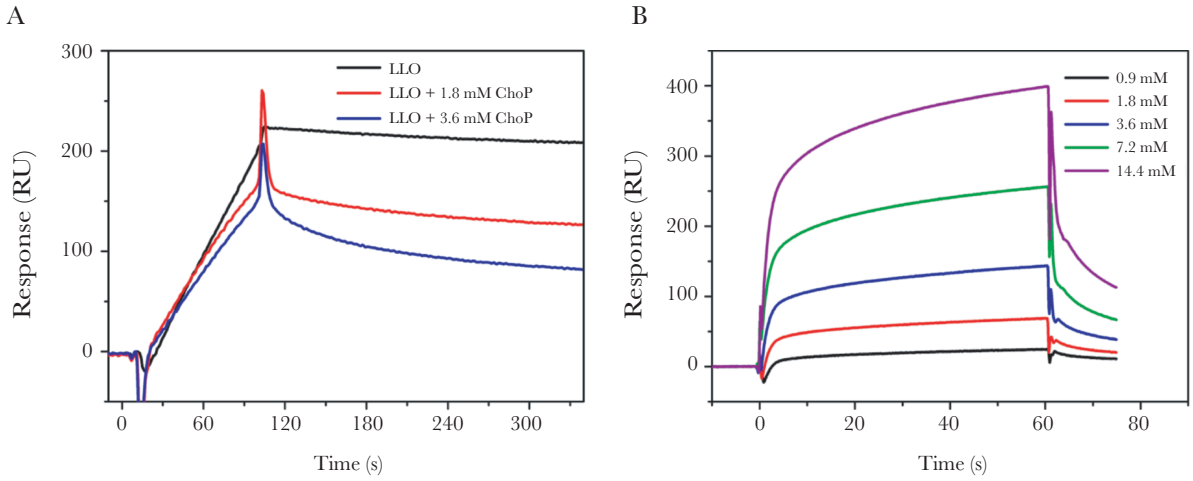

Figure 6. Phosphocholine increases the dissociation of LLO from the plasma membrane and binds directly to LLO. *A*, LLO preincubated with indicated concentrations of ChoP was injected across LUVs-immobilized surface. Binding of LLO was determined by SPR. Similar binding of LLO and ChoP-incubated LLO to LUVs was observed. However, ChoP-treated LLO dissociated faster from LUVs in a concentration-dependent manner as compared to untreated LLO. *B*, LLO immobilized to the sensor chip surface was incubated with ChoP. Dose-dependent binding of ChoP to LLO was observed. Abbreviations: ChoP, phosphocholine; LLO, listeriolysin O; LUV, large unilamellar vesicles; SPR, surface plasmon resonance.

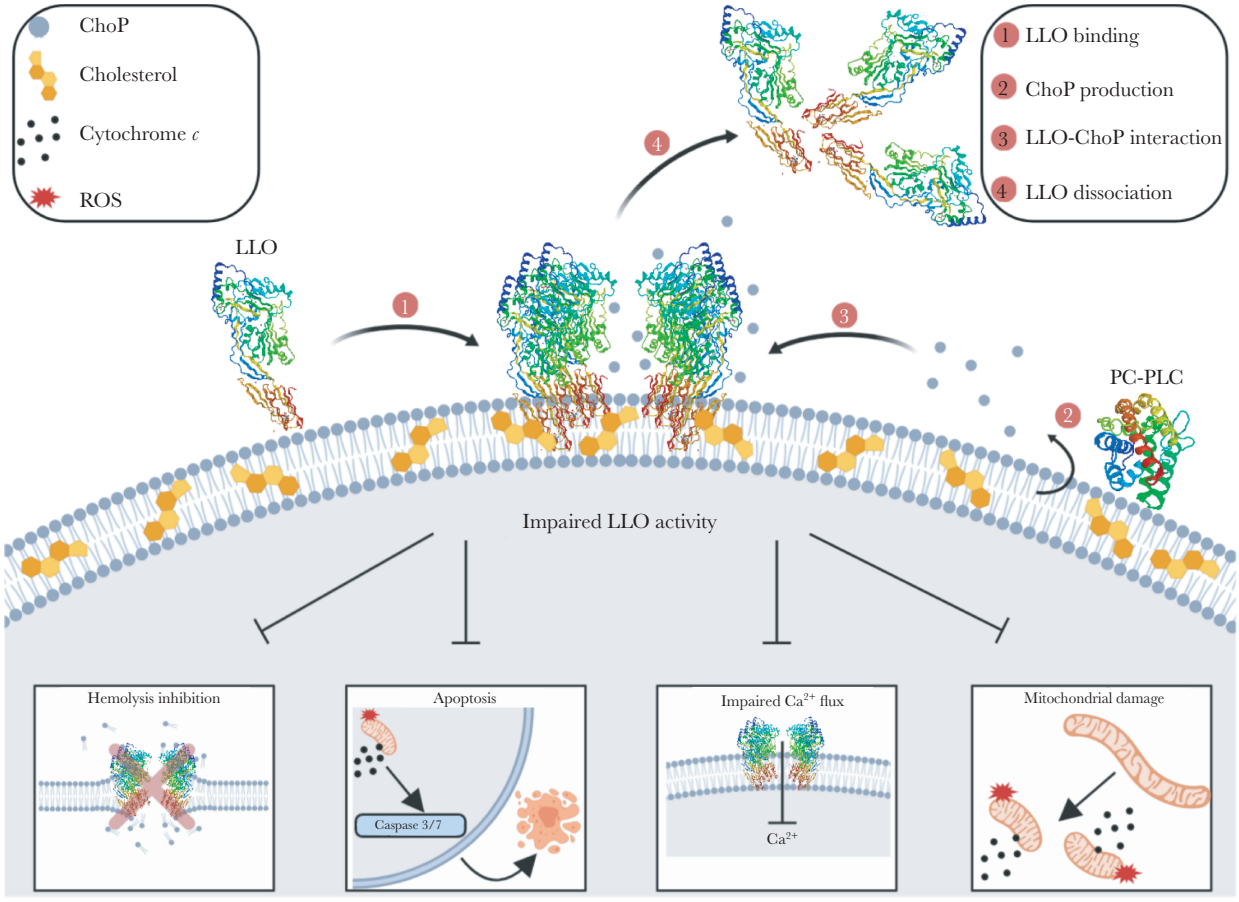

Figure 7. A model for phosphocholine-mediated suppression of LLO-induced host cell responses. *Listeria monocytogenes* secretes LLO, which binds to cholesterol-rich host plasma membrane leading to hemolysis inhibition, mitochondrial ROS production, mitochondrial fragmentation, and induction of apoptosis. Free ChoP or ChoP produced by PC-PLC binds directly to LLO, leading to enhanced dissociation of LLO pores from the plasma membrane, thus inhibiting LLO-mediated host cell responses. (Created with Biorender.com). Abbreviations: ChoP, phosphocholine; LLO, listeriolysin O; PC-PLC, phosphatidylcholine-specific phospholipase C; ROS, reactive oxygen species.

formation. Addition of PC-PLC together with LLO also reduced the extent and duration of Ca^{2+} influx, suggesting blunting of LLO activity at the plasma membrane.

Influx of extracellular Ca^{2+} is a powerful activator of the cellular repair machinery [44], and Ca^{2+}-dependent binding of annexin V to PS plays an important biological role by acting as local shields in the membrane repair response of damaged cells [45]. Here we demonstrate that a mutant lacking PC-PLC has higher levels of externalized PS, indicating increasing local lipid asymmetry. A previous study reported that infection of host cells using mutants strain lacking both listerial phospholipases exhibited an increase in PS-staining structures harboring bacteria that were subsequently taken up by efferocytosis [32]. Thus, we can speculate that the increase in PS-positive vesicles is probably due to the unrestricted plasmalemmal injury mediated by the PFT LLO.

ChoP modulates binding of LLO to both biological membranes as well as to artificially created LUVs by destabilizing the interaction of LLO with LUVs. This suggests that ChoP does not inhibit binding of LLO to host membranes but promotes its dissociation from membranes. This would also explain the more modest Ca^{2+} influx and the rapid recovery to baseline [Ca^{2+}] levels seen with intact cells.

We found that ChoP also inhibited the hemolytic activity of other members of the family of CDCs, that is PLY from *S. pneumoniae* and SLO from *S. pyogenes*, indicating that ChoP is also a general inhibitor of CDC activity. However, binding of ChoP to LLO is not linked to its redox state as hemolysis using a cysteine-free LLO variant was also inhibited. Conversely, PC-PLC from *B. cereus* also inhibited the hemolytic activity of LLO, suggesting that this interaction might be widespread among bacteria producing these membrane-targeting factors. Many gram-positive pathogens, including *B. anthracis, Clostridium perfringens, C. tetani,* and *C. botulinum,* express a CDC as well as a PC-PLC, suggesting that the regulation of toxin activity by PC-PLCs might be an evolutionary conserved process [46–49].

Our data suggest that *L. monocytogenes* uses the antagonistic activity of PC-PLC to mitigate cytotoxic effects of LLO on host cells during infection. Hence, ChoP serves as an important interaction partner for PFTs and showcases its significance as a general strategy used in microbial pathogenesis for evasion and suppression of host responses. Our model (Figure 7) suggests that ChoP (free or cleaved by PC-PLC) alters LLO-membrane association by enhancing the dissociation of LLO from the plasma membrane with modulation of LLO downstream host signaling responses and thus supports bacterial intracellular survival.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. L. L. P., H. P., and T. C. conceived the work and wrote the paper; L. L. P., M. H., B. B., S. A., V. H., G. L., A. R., and A. P. designed and performed the experiments; and L. L. P., H. P., M. A. M., V. H., G. L., G. A., and T. C. analyzed data.

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