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# Listeriolysin O: from bazooka to Swiss army knife

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*Listeria monocytogenes* (*Lm*) is a Gram-positive facultative intracellular pathogen. Infections in humans can lead to listeriosis, a systemic disease with a high mortality rate. One important mechanism of *Lm* dissemination involves cell-to-cell spread after bacteria have entered the cytosol of host cells. Listeriolysin O (LLO; encoded by the *hly* gene) is a virulence factor present in *Lm* that plays a central role in the cell-to-cell spread process. LLO is a member of the cholesterol-dependent cytolysin (CDC) family of toxins that were initially thought to promote disease largely by inducing cell death and tissue destruction—essentially acting like a 'bazooka'. This view was supported by structural studies showing CDCs can form large pores in membranes. However, it is now appreciated that LLO has many subtle activities during *Lm* infection of host cells, and many of these likely do not involve large pores, but rather small membrane perforations. It is also appreciated that membrane repair pathways of host cells play a major role in limiting membrane damage by LLO and other toxins. LLO is now thought to represent a 'Swiss army knife', a versatile tool that allows *Lm* to induce many membrane alterations and cellular responses that promote bacterial dissemination during infection.

This article is part of the themed issue 'Membrane pores: from structure and assembly, to medicine and technology'.

## 1. The many faces of listeriolysin O function

As our understanding of listeriolysin O (LLO) expands, so too does the number of functions ascribed to this toxin. LLO was previously thought to be 'phagosome-specific lysin', since it plays a key role in phagosome escape by bacteria [1]. However, it is now appreciated that LLO can impact host cells from several locations: the extracellular medium, the phagosome and the cytosol. In all environments, LLO has important functions that are thought to promote infection of the host by *Listeria monocytogenes* (*Lm*).

### (a) Extracellular listeriolysin O activities

#### (i) Bacterial internalization

Automated fluorescence-based assays revealed that LLO was sufficient to direct bacterial internalization into HepG2 cells. Coating non-invasive bacteria or polystyrene beads with LLO promoted their internalization into phagosomes, which rapidly acquired the early endosome marker EEA1 [2]. Calcium and potassium fluxes across the plasma membrane were shown to be required for this LLO-dependent internalization [3,4].

#### (ii) Activation of host signalling pathways

The transient cytosolic calcium elevation following LLO pore formation is responsible for the induction of multiple signalling pathways in the host cell [5–7]. To date these include activation of ERK-1, ERK-2, p38, c-Jun and Raf-MEK-MAP kinases pathways [8–10], phosphatidylinositol metabolism [11,12], nuclear translocation of NF-κB and secretion of the proinflammatory cytokines IL-6,



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IL-8, GM-CSF and IL-1α [5–7,13]. Activation of tyrosine kinases was shown to promote internalization of bacteria via LLO [2].

#### (iii) Apoptosis

*Lm* has been shown to induce apoptosis of several cell types during infection [14–16]. Relevant to the immune response, LLO was found to mediate rapid apoptosis of lymphocytes both *in vitro* and *in vivo* [17]. Treatment with subnanomolar doses of LLO was sufficient (in the absence of bacteria) to induce apoptosis by caspase-dependent and -independent pathways. LLO-mediated apoptosis increases susceptibility to *Lm* infection due to upregulation of IL-10, an anti-inflammatory cytokine [18].

### (b) Listeriolysin O functions in the phagosome

*Lm* pathogenesis requires escape from the phagosome/vacuole (these terms are used interchangeably) and entry into the host cell cytosol. Early electron microscopy (EM) studies demonstrated that *Lm* mutants lacking LLO (Δ*hly*) were restricted to the vacuole and avirulent *in vivo* [19–23]. Since then, phagosome escape has become the most well-established function of LLO. It is now clear that phagosome escape is a dynamic process accompanied by multiple host cell responses and is accomplished by only a minority of internalized bacteria (estimated 14%) [24]. During cell-to-cell spread, LLO also plays a role in escape from double-membrane compartments, referred to as spreading vacuoles, in neighbouring cells.

#### (i) Graded perforations of the phagosomal membrane

The previous model for LLO function was based on its homology and predicted structural similarity to other cholesterol-dependent cytolysin (CDC) toxins that generated 23–26 nm pores [25]. However, live cell imaging studies by Joel Swanson and colleagues showed that LLO can induce a graded series of much smaller membrane perforations in the phagosomal membrane during infection [26–28]. In these studies, small fluorescent molecules were internalized passively during *Lm* phagocytosis by macrophages. Leakage of the fluorescent molecules from phagosomes into the cytosol occurred in a size-dependent manner (e.g. leakage of Lucifer Yellow (522 Da) preceded Dextran Texas Red (10 000 Da) during phagosome maturation). Remarkably, leakage of protons and calcium was observed under conditions where small molecules were retained in phagosomes. Since proton and calcium accumulation in phagosomes is required for their fusion with lysosomes, Dr Swanson's group proposed a model whereby small perforations of the phagosome (with channel-like activity) create a 'window of opportunity' for other bacterial and host factors to promote phagosome escape.

#### (ii) Managing reactive oxygen species

Production of reactive oxygen species (ROS) by the phagocyte NOX2 NADPH oxidase is a well-established anti-microbial defence system against *Lm*. The virulence attenuation seen in Δ*hly* infections can be partially recovered in a NADPH oxidase knockout (*Cybb*/*NOX2*$^{-/-}$) background [29], demonstrating a role for LLO in managing ROS. In agreement, Δ*hly* mutants had elevated levels of intracellular ROS localized to the phagosome [29]. It is unclear how LLO pore formation limits ROS production in *Lm*-containing phagosomes.

#### (iii) Growth in phagosomes and establishment of chronic infection

*Lm* was shown to induce a chronic infection in severe combined immunodeficiency (SCID) mice [30]. At 28 days post-infection, the host was able to contain the bacteria within spacious vacuolar structures, limiting their cytosolic growth and cell-to-cell spread in liver phagocytes. This landmark study by Unanue and colleagues established that *Lm*, previously considered a 'cytosol-adapted pathogen', could also colonize vacuoles during infection of host cells *in vivo*.

Using SCID mice and macrophage cell lines *in vitro*, we characterized the population of *Lm* that can grow in spacious *Listeria*-containing phagosomes (SLAPs) [31]. We observed that SLAPs are large, non-degradative compartments with a neutral pH and a single delimiting membrane that stains positively for several markers, including LAMP1, LC3-B and the proton ATPase. SLAPs contain multiple *Lm*, and bacteria were found to grow in these compartments with a doubling time of approximately 8 h (versus 40 min for *Lm* in the cytosol).

LLO production was both necessary and sufficient for the formation of SLAPs during infection. We found that a bacterial mutant expressing lower amounts of LLO (approximately one-third of the normal haemolytic activity) did not escape phagosomes but were able to grow in SLAPs over a delayed time course [31]. The mechanism by which LLO facilitates SLAP formation requires further investigation. Other pathogens may also use pore-forming toxins to colonize phagosomes during infection of host cells.

#### (iv) Inducing autophagy

Damaged cellular compartments can act as intracellular danger signals and trigger autophagy [32,33]. It is known that components of the autophagy pathway can limit *Lm* infection *in vivo* in both mice and flies [34,35] and that autophagy is activated during *Lm* infection of host cells *in vitro* under some conditions [36]. LLO is sufficient for lipidation of the autophagy protein LC3 and its recruitment to *Lm* [37]. Thus, LLO-mediated damage of phagosomes may be sufficient to induce an autophagic response. Despite this, autophagy does not seem to impact bacterial growth in host cells, and several strategies for bacterial evasion of autophagic killing have been described [38–42]. The relationship between *Lm* and autophagy continues to be explored. Moving forward, it must be borne in mind that *Lm* interactions with host autophagy are strain-specific [43].

### (c) Listeriolysin O functions in the cytosol of host cells

#### (i) Mitochondrial fragmentation

Infection with LLO-competent *Lm* leads to a transient calcium-dependent burst of mitochondrial network fragmentation [44]. This corresponded to a drop in respiration and cellular ATP levels. Disrupting mitochondrial fission or fusion was found to inhibit intracellular growth of *Lm* [44]. The mechanism for how mitochondrial fission and fusion events impact *Lm* pathogenesis remains to be explored.

#### (ii) Endoplasmic reticulum stress and the unfolded protein response

LLO-mediated damage to the endoplasmic reticulum (ER), the main site for intracellular calcium storage, was shown to be a source of calcium elevation during infection [45]. The presence of LLO also led to induction of the unfolded protein response

(UPR) [46]. Activation of the UPR does not benefit *Lm*, and artificial induction of ER stress reduced bacterial intracellular growth [46].

#### (iii) Protein degradation

Through unknown mechanisms, LLO pore formation promotes degradation of several host proteins [47–49]. During *Lm* infection there is a reduction in the number of proteins undergoing SUMOlyation; the reversible addition of SUMO, a ubiquitin-like polypeptide [49]. This was due to LLO-dependent degradation of Ubc9, a key enzyme in the SUMO pathway. Although Ubc9 degradation was not calcium dependent, degradation of the DNA breaks sensor Mre11 and human telomerase reverse transcriptase (hTERT) were [47,48]. It is likely that other yet unidentified host proteins are targeted for degradation by LLO-dependent mechanisms.

#### (iv) Inflammasome activation

*Lm* infection activates the NLRP3, AIM2, NALP3, IPAF and NLRC4 inflammasomes leading to activation of caspase-1, maturation of IL-1β and IL-18, and pyroptosis [50–54]. It is unclear whether the reduced caspase-1 activity observed with Δ*hly* infection is the result of pore formation or the absence of *Lm* in the cytosol [50,54,55]. In support of the latter possibility, the presence of *Lm* DNA and flagellin in the cytosol triggers caspase-1 activation [56] as does cytosolic bacterial lysis [57]. Both models could exist simultaneously as purified LLO and cytosolic *Lm* could separately activate the NLRP3 inflammasome in human peripheral blood mononuclear cells [53].

#### (v) Cell-to-cell spread

*Lm* that enter the cytosol express ActA, a cell-surface protein that interacts with actin-regulatory factors from the host cell to promote actin-based motility [58]. Motile bacteria can induce cell surface filopodia-like structures (called protrusions) that can lead to subsequent spread to neighbouring cells. We recently showed that LLO activity in protrusions can cause localized plasma membrane damage, visualized by the exofacial exposure of phosphatidylserine (PS); normally localized exclusively to the inner leaflet of the plasma membrane [59]. In macrophages, this loss of membrane asymmetry promoted association of protrusions with neighbouring cells through the PS-binding receptor TIM-4 and enhanced cell-to-cell spread by bacteria. TIM-4 plays an important role in efferocytosis, the clearance of dead/dying cells in tissues, and was linked to innate immunity to *Mycobacterium tuberculosis* [60]. Therefore, *Lm* can exploit efferocytosis through LLO-mediated plasma membrane damage to promote its own cell-to-cell spread.

### (d) Pore-independent functions of listeriolysin O

Most of the functions attributed to LLO appear to be by-products of either pore formation or entry of *Lm* into the cytosol. However, there are some functions of LLO that seem to be independent of its role in pore formation.

#### (i) Listeriolysin O as an immune antigen

LLO acts as an immune antigen: LLO-specific $CD8^+$ T cells are protective against *Lm* infection [61,62]. In support of this activity being distinct from its role in pore formation, mutations that render LLO non-haemolytic do not affect its antigenicity [63]. In fact, non-haemolytic forms of LLO have proved useful as adjuvants in tumour immunotherapy [64].

#### (ii) Histone modification

*Lm* infection causes dephosphorylation of histone H3 and deacetylation of histone H4, impacting the expression of 146 genes [65]. The effect was dependent on LLO membrane binding but not pore formation. Other CDCs, but not membrane permeabilizing detergents, could similarly induce histone modifications, indicating the effect is specific to CDC–membrane interactions.

### (e) Summary of listeriolysin O functions

The diversity of LLO-dependent effects on the host cell is remarkable and highlights the importance of this virulence factor to *Lm* pathogenesis. Many of LLO's biological impacts stem from its ability to drive an influx of calcium across the plasma membrane and/or release of calcium from intracellular stores during *Lm* infection [45]. LLO-dependent vacuolar escape elicits activation of immune and host signalling pathways as bacteria move from one intracellular niche to another. As described above (§1a–d), LLO also has functions that are not linked to its pore-forming activity. Importantly, LLO activity is not unrestricted—the 'bazooka' does not just kill everything in its vicinity. Otherwise, host cells would not be able to survive infection by hundreds of bacteria, which is routinely observed. Instead, LLO activity is highly regulated by both *Lm* and host cellular processes.

## 2. Regulation of listeriolysin O activity during infection

### (a) Regulation of listeriolysin O by *Listeria monocytogenes*

#### (i) Transcription of *hly*

Expression of *hly* was quantified in bacteria trapped at each stage of infection: the primary vacuole (using a Δ*hly* mutant), the cytosol (using a non-spreading Δ*actA* mutant), and in spreading vacuoles (using a Δ*plcB* mutant that cannot escape spreading vacuoles) [66]. This and other studies revealed robust *hly* expression regardless of the infection stage [66–69]. However, these studies are limited in that they use population-based measurements. Infection of J774 and Caco2 cells revealed heterogeneous *hly* expression that was not seen with *actA*, *iap* or *inlC* reporters [66], indicating that *Lm* may exploit heterogeneous expression of LLO during its interaction with host cells.

PrfA is the most well-characterized transcription factor required for *hly* expression [70,71]. *prfA* is transcribed from three promoters (P1–3). Basal transcription from P1 and P2 appears sufficient to drive primary vacuole escape [72]. Cell adherence is sufficient to induce *prfA* expression which is further amplified once intracellular [73,74]. P3, a bi-cistronic *plcA-prfA* promoter, is part of a positive auto-regulatory loop that increases PrfA levels in the host cytosol [72]. The PrfA regulon is most strongly activated following entry into the host cell cytosol [66].

Environmental cues regulating *prfA* (and by extension *hly*) expression include ROS [75], pH [76], sugar availability [72,77] and branched chain amino acids [78]. PrfA translation is thermally regulated. At non-permissive temperatures (30°C), the

*prfA* 5′ untranslated region (UTR) forms a non-permissive secondary structure that is relieved following a shift to 37°C [79–81]. The UTR is also constrained by two *S*-adenosylmethionine (SAM) riboswitches SreA and SreB [82].

PrfA exists in two functional states: a state of low activity and of high activity following interaction with an unidentified cofactor [83]. Bacterial and host glutathione were recently shown to activate PrfA activity during infection of host cells [84]. A PrfA mutant (PrfA*) locked in the high activity state was sufficient to bypass the requirement of glutathione during infection. PrfA* mutants do not cause a virulence defect and, when used *in vitro*, more closely resemble expression seen *in vivo* [72]. Using a cell-wall binding domain fluorescent probe and Rab7 localization, it was shown that PrfA* did not affect phagosome escape for up to 8 h post-infection in J774 cells [85]. Overall, these findings demonstrate that expression of *hly* is not limited to the vacuole and, given its membership in the PrfA regulon, is actively transcribed in the cytosol.

### (ii) Secretion of listeriolysin O

LLO is transported across the bacterial membrane by the Sec secretion system. In line with the need for LLO activity during phagosome escape, phagosome trapped (Δ*hly*) *Lm* showed high expression of Sec secretion system components [86]. The Sec accessory proteins SecD and SecF and the post-translational secretion chaperone PrsA2 are required for both proper secretion and activity of LLO [86–89]. Proper LLO secretion and function also rely on cleavage of the Sec secretion signal after translocation by the signal peptidase SipZ. Expression of *sipZ* increases in the phagosome and, albeit at lower levels, continues to be expressed in the cytosol [90]. Deletion of *sipZ* decreased LLO secretion and reduced haemolytic activity by fivefold [91]. Secretion of LLO during cell-to-cell spread has yet to be investigated.

## (b) Regulation of listeriolysin O by the host cell

### (i) Reduction/oxidation of listeriolysin O

LLO requires reduction at Cys485 for activation *in vitro* [92]. The thiol oxidoreductase GILT has been shown to reduce LLO in the phagosome, promoting its activity and the subsequent entry of *Lm* into the cytosol [93]. *Lm* has a reduced ability to escape the phagosome in GILT$^{-/-}$ bone marrow-derived macrophages [93]. To our knowledge, no thiol oxidoreductases in the cytosol have been identified as regulating LLO activity, though the naturally reducing environment provided by the glutaredoxin and thioredoxin systems of the mammalian cytosol may negate the need for additional regulators [94,95].

Oxidation of LLO may also limit its activity in the phagosome. ROS (produced by the NOX2 NADPH oxidase) and reactive nitrogen species (produced by iNOS) have been proposed to inactivate LLO, thereby limiting phagosome escape by *Lm* [96]. Whether LLO is oxidized by host cellular factors in the cytosol has not been investigated (figure 1).

### (ii) Chloride promotes listeriolysin O oligomerization

LLO oligomerization depends on chloride availability [97–100]. Although cellular chloride levels vary from cell to cell, chloride levels are higher in phagosomes relative to the cytosol, suggesting a tendency to form higher-order LLO oligomers in the vacuole. In neutrophils, it was shown that there is a chloride influx into the phagosome lumen such that the concentration approaches 70 mM in contrast to the 40–50 mM seen in the cytosol [101,102]. Inhibition of the chloride transporter cystic fibrosis transmembrane conductance regulator (CFTR) decreased *Lm* vacuole escape [100]. The role of other chloride transporters in *Lm* pathogenesis has not been explored.

### (iii) Proteolytic degradation of listeriolysin O in the phagosome

Cathepsin D was found to cleave LLO during *Lm* infection of fibroblasts and macrophages [103,104]. In neutrophils, LLO can also be degraded prior to phagosome closure at the plasma membrane by the matrix metalloproteinase-8 [105]. Also, some α-defenins appear to limit CDC activity in the phagosome [106] though their expression is cell-type dependent.

### (iv) The proline, glutamic acid, serine and threonine sequence and listeriolysin O degradation by the ubiquitin–proteasome system

LLO stability in the host cell cytosol is impacted by proteolytic degradation mechanisms which impact the ability of *Lm* to cause infection. While sufficient levels of LLO are required to infect host cells and promote cell-to-cell spread, abnormally high levels of LLO (caused by loss of intrinsic regulatory mechanisms described below in §2b(v–vii)) are linked to cellular toxicity and clearance of extracellular bacteria by innate immune mechanisms, including killing by neutrophils and inflammatory monocytes.

The N-terminus of LLO, unlike other CDCs, is rich in proline, glutamate, serine and threonine (PEST) residues [1,107]. Deletion of this PEST sequence does not affect LLO haemolytic activity but does attenuate *Lm* virulence [108,109]. Although there are conflicting reports on whether ΔPEST mutants can escape the phagosome [108,109], studies agree that ΔPEST mutants have greater cytotoxicity. Changes to the PEST nucleotide sequence in LLO (but not the amino acid sequence) were found to increase LLO expression, potentially contributing to this greater toxicity [110].

The LLO PEST domain contains three putative mitogen-activated protein kinase phosphorylation sites of which mutation of Ser44 phenocopies ΔPEST (increased cytosolic toxicity) [108,111]. Inhibition of the proteasome leads to an accumulation of LLO, indicating that LLO is degraded in the host cell. This accumulation was reversed with phosphatase treatment, suggesting phosphorylation was linked to degradation [111]. However, neither the PEST sequence nor Ser44 were necessary for proteasomal degradation of LLO. Immunoprecipitation experiments have further demonstrated that LLO is ubiquitinated, which may contribute to protein turnover [111]. Although it is clear that the PEST sequence plays a role in limiting LLO activity in the phagosome, our understanding of the mechanism remains incomplete. Structural studies indicated that the PEST sequence interacts with the adjacent symmetry-related molecule in the crystal lattice which could point to a role in oligomerization [112]. Transmission EM showed that wild-type and ΔPEST mutant pores looked different with an increase in the number of incomplete arcs in crowded rows in the latter [112].

LLO has been shown to be degraded by the N-end rule pathway. Mutation of the N-terminal Lys in LLO led to increased cellular toxicity in J774 macrophages, but overall had only a minor impact on virulence [113].

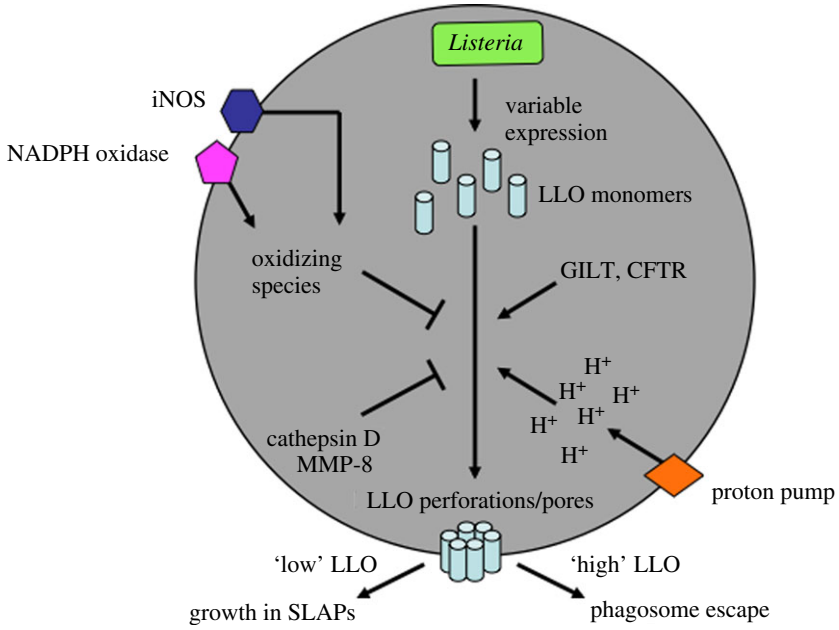


**Figure 1.** Factors impacting LLO activity in the phagosome. Individual *Listeria monocytogenes* produce and secrete variable amounts of LLO monomers, depending on many intrinsic and extrinsic factors they encounter prior to uptake by host cells. Thus, in the lumen of the phagosome, variable amounts of LLO have the capability of binding to cholesterol (and possibly other factors) on the luminal face of the phagosomal membrane. Before that binding can occur, many factors in the phagosome impact LLO. Reduction of LLO by GILT, chloride delivery to the phagosome by CFTR and acidification of the phagosome by the vacuolar ATPase (proton pump) all promote LLO activity. In contrast, oxidizing species generated by iNOS and the NOX2 NADPH oxidase and proteolytic cleavage of LLO by cathepsin D and MMP-8 all inhibit LLO activity in the phagosome. Together, all of these factors impact the amount (and likely also the type) of LLO activity at the phagosomal membrane. Relatively 'high' activity of LLO is thought to promote escape of bacteria from the phagosome, in combination with other bacterial and host factors (e.g. phospholipases). In contrast, 'low' activity of LLO has been shown to allow bacterial growth in phagosomes (SLAPs).

### (v) pH dependence

The role of pH in limiting LLO pore formation is the best recognized form of LLO activity modulation [1,97,114]. Blocking acidification of the phagosome has been shown to completely block phagosome escape by *Lm* [26]. Structural studies have revealed an inhibition of LLO activity at neutral pH. In addition to the acidic residues E247, D208 and D320 previously identified as the pH sensor, the structure of LLO revealed multiple pH-sensitive clusters that interact both directly and indirectly through bound $Na^+$ and $H_2O$ [97,112]. Deprotonation of these critical residues leads to charge repulsion, unfolding and aggregation of regions within D3, a region involved in oligomerization and ring formation [112,115]. However, pH regulation of LLO appears more complex than originally thought and many groups do report LLO membrane binding at neutral pH [2,26,116]. Haemolytic activity of LLO is also normal after a rapid shift from acidic to neutral pH, albeit this activity is short-lived as the protein begins to aggregate [116–119]. These findings are consistent with the ability of LLO to impact cellular membranes in the cytosol of host cell (discussed in §1), but in a limited manner that typically does not cause host cell rupture.

### (vi) Monomer diffusion

LLO activity requires assembly of monomers into oligomeric structures on membranes. In the confines of a phagosome, this process would be relatively efficient since monomers are released into a single compartment with high access to their target membrane. In the cytosol, monomer diffusion is expected to be higher in the larger volume of this gel-like, expansive compartment and potentially limited further by movement of the bacteria through actin-based motility. While this is not a specific host regulation of LLO, it is nonetheless a factor impacting LLO activity in the cytosol that must be considered.

### (vii) Plasma membrane repair pathways

A number of cellular pathways promote integrity of the plasma membrane and mediate resistance to bacterial toxins [120,121]. LLO mediates damage to the plasma membrane during *Lm* infection, and this damage was shown to be limited by caspase-7 activity [122]. Macrophages deficient in caspase-7 had increased plasma membrane permeability and deficient intracellular growth. Additionally, members of the annexin family of membrane repair proteins were found to limit plasma membrane damage by LLO during *Lm* infection of HeLa cells [59].

## 3. Structural insights into listeriolysin O activity

*Lm* pathogenicity relies heavily on LLO pore formation and the aforementioned host responses to this toxin. For many years our mechanistic understanding of LLO relied on extrapolation from related CDCs but recent structural and atomic force microscopy (AFM) analysis has provided new insight into LLO function as it relates to membrane perforation.

### (a) Structure of listeriolysin O

X-Ray crystallography studies revealed that the LLO monomer is an elongated, four domain structure (D1–D4) with strong structural resemblance to other CDCs [123–126]. D1, D2 and D3 form the LLO core, whereas D4 extends away from the core. D4 contains the highly conserved undecapeptide sequence (ECTGLAWEWWR) and loops (L1–L3) and is required for cholesterol recognition and membrane binding [112]. Despite strong conservation of D4, LLO carries more polar residues in

L2 and a more neutral charge, compared to the negatively charged PFO. The transmembrane pore constitutes a β-barrel where each monomer contributes two transmembrane β-hairpins derived from α-helices in D3 [112,127,128].

## (b) Membrane binding and pore formation

### (i) Cholesterol recognition

Mutagenesis analysis on perfringolysin O (PFO), streptolysin O and pneumolysin (PLY) revealed that two conserved residues in loop 1 of D4, corresponding to Thr515 and Leu516 on LLO, were essential for cholesterol binding and haemolytic activity [129]. Although the insolubility of cholesterol has limited our structural understanding of its recognition by CDCs, the 3-β-hydroxyl group appears important since epicholesterol, an isomer that differs only in the orientation of the 3-β-hydroxyl group, was not bound by PFO [130]. Subsequent to cholesterol recognition, PFO becomes anchored to the membrane by insertion of the undecapeptide, and loops L2 and L3 [115].

### (ii) Listeriolysin O oligomerization

Following membrane attachment, CDC monomers oligomerize into a pre-pore complex that extends 113 Å above the membrane [131,132]. The oligomeric interface is located in D3, with β1 of one monomer binding to β4 of the neighbouring monomer. PFO has poor spontaneous aggregation because β4 is normally shielded by β5 [115]. Membrane binding caused a conformational shift that reoriented β5, exposing the oligomeric interface [115]. LLO shows strong charge complementarity between D1 and D3 of neighbouring monomers. Inverting residue charges along these regions abolished LLO activity and prevented oligomeric ring formation; instead granular protein complexes or discontinuous protein fibres were formed [112].

AFM has recently enabled visualization of LLO oligomers. LLO formed arcs, slits and rings in a cholesterol- and time-dependent manner that progressively fused to form larger rings [133]. Other groups failed to observe ring structures using high-speed AFM but instead visualized rapid arc formation that stalled after incorporation of 20 monomers. Multiple arcs then annealed to form larger, ring-like, oligomers [134].

### (iii) The pre-pore to pore transition

Formation of ring-like LLO pre-pores precedes membrane insertion. There are conflicting reports on the degree of LLO oligomerization required for transition from the pre-pore to pore. One group observed membrane insertion of arcs and slits followed by further oligomerization [133]. Others observed membrane insertion only with higher-order oligomers with no further oligomerization following pore formation [134].

The transition from the pre-pore to pore state involves a vertical collapse of 40 Å with large conformational changes as the central α-helices convert to transmembrane β-hairpins [25,135]. Cryo-EM maps of the PLY pore show a variable 320–430 Å pore diameter [25]. CDC oligomers appear to have variable pore sizes, with 35–47 subunits forming the pore. These size differentials support the observation that multiple arcs appear to anneal to form a ring-like structure rather than a single oligomeric ring [134].

## (c) Listeriolysin O lineactivity

Recent AFM observed that in addition to its membrane pore formation activity, LLO had subsequent lineactivity: LLO could cause further, large-scale membrane damage from the membrane edge [134]. Such an activity could be unique to CDCs as lineactant activity was not observed with the aerolysin-like pore-forming toxin lysenin. Lineactant activity requires a membrane edge and may act to enlarge existing LLO pores. This is consistent with the previously discussed finding of graded pore formation where small fluorescent dextrans moved across membranes rapidly following LLO treatment and, over time, larger dextrans subsequently transversed the membrane.

# 4. Conclusion

It is clear from the wealth of studies on LLO that its activity cannot be simplified as an on–off switch. Based on the research discussed above, it is becoming clear that LLO has at least two modes of action: membrane perforation/lineactivity and the formation of large pores. This versatility allows LLO to promote its many functions in different environments during infection of its host. The ability of LLO (and other CDC) monomers to undergo conformational changes upon membrane binding appears to be the key feature that links these activities. In other words, the 'moving parts' of LLO allow it to do many things in different places. The bacterial and host factors that control LLO activities are now recognized as critical determinants in both the initiation of LLO-associated phenotypes and the survival of host cells during the infection. Membrane repair pathways in particular are likely to be important determinants of the outcome of infection by *Lm* and other bacteria expressing CDC toxins. LLO is clearly not behaving as an unrestricted 'bazooka', even under the most severe (and artificial) infection conditions seen *in vitro*. Rather, LLO is now recognized as a precise tool used by *Lm* to modulate host cellular pathways in a manner that promotes infection. Indeed, Cossart and colleagues have referred to LLO as a 'Swiss army knife', a fitting analogy based on our new appreciation of LLO as a multi-functional tool used by *Lm* to promote infection. We anticipate that LLO has a great deal more to teach us about bacterial pathogenesis and host innate immune responses to infection.

Competing interests. We declare we have no competing interests.

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