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Listeriolysin O: a phagosome-specific cytolysin revisited

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Abstract

Listeriolysin O (LLO) is an essential determinant of *Listeria monocytogenes* pathogenesis that mediates the escape of *L. monocytogenes* from host cell vacuoles, thereby allowing replication in the cytosol without causing appreciable cell death. As a member of the cholesterol-dependent cytolysin (CDC) family of pore-forming toxins, LLO is unique in that it is secreted by a facultative intracellular pathogen, whereas all other CDCs are produced by pathogens that are largely extracellular. Replacement of LLO with other CDCs results in strains that are extremely cytotoxic and 10,000-fold less virulent in mice. LLO has structural and regulatory features that allow it to function intracellularly without causing cell death, most of which map to a unique N-terminal region of LLO referred to as the PEST-like sequence. Yet, while LLO has unique properties required for its intracellular site of action, extracellular LLO, like other CDCs, affects cells in a myriad of ways. Because all CDCs form pores in cholesterol-containing membranes that lead to rapid Ca^{2+} influx and K^{+} efflux, they consequently trigger a wide range of host cell responses, including MAPK activation, histone modification, and caspase-1 activation. There is no debate that extracellular LLO, like all other CDCs, can stimulate multiple cellular activities, but the primary question we wish to address in this perspective is whether these activities contribute to *L. monocytogenes* pathogenesis.

Keywords

Cholesterol; toxin; bacteria; intracellular pathogen; *Listeria*; Macrophage; inflammasome

1 Introduction to Cholesterol-Dependent Cytolysins

Cholesterol-dependent cytolysins (CDCs) represent the largest family of pore-forming toxins (PFTs) and the subset of PFTs that form the largest pores (Bischofberger, Iacovache,

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& van der Goot, 2012). To date, more than 50 CDCs have been identified in *Firmicutes, Actinobacteria*, and most recently in *Proteobacteria* (Hotze et al., 2013; Tweten, Hotze, & Wade, 2015). With the exception of those produced by *Proteobacteria*, CDCs are produced by primary and opportunistic Gram-positive pathogens, and many have important roles in pathogenesis. Among CDCs that have demonstrated contributions to pathogenesis are perfringolysin (PFO) of *Clostridium perfringens,* pneumolysin (PLY) of *Streptococcus pneumoniae,* streptolysin O (SLO) of *Streptococcus pyogenes,* anthrolysin (ALO) of *Bacillus anthracis*, and listeriolysin O (LLO) of *Listeria monocytogenes.* LLO is distinct in that it is the only CDC produced by an intracellular pathogen and has specialized features that make it suitable for its intracellular localization.

The hallmarks of CDCs are their requirement of membrane cholesterol for pore-forming activity, and their extremely large pores—which can be 30 – 40 nm in diameter (Gilbert, 2010; Mulvihill, Van Pee, Mari, Muller, & Yildiz, 2015). CDCs also contain a singular conserved cysteine that makes them highly sensitive to oxidation; CDCs were once classified as ‘thiol-activated (oxygen-sensitive) cytolysins’ because they required reducing agents for maximal activity (Morgan, Andrew, & Mitchell, 1996; Smyth & Duncan, 1978).

CDCs are secreted via a Sec-dependent pathway as monomers 50–70 kDa in mass and consist of four distinct domains. Secreted monomers bind to cell membranes and oligomerize into arc and ring prepore assemblies, which may contain up to 50 subunits. Following membrane binding, α-helical regions in domain 3 of each monomer refold into two β-hairpins that insert into the membrane and form a β-barrel pore (Christie, Johnstone, Tweten, Parker, & Morton, 2018; Leung et al., 2014; Mulvihill et al., 2015). Domain 4 contains the signature undecapeptide sequence (ECTGLAWEWWR) that is the most highly conserved region in the primary CDC sequence and is required for coupling of cholesterol binding to domain 3 rearrangement (Dowd & Tweten, 2012). The cholesterol recognition/ binding motif, which consists of a threonine-leucine pair, is also located in domain 4 (Farrand, LaChapelle, Hotze, Johnson, & Tweten, 2010). Both incomplete ring oligomers (arcs or slits) and complete rings perforate cell membranes, though pores formed by arcs are considerably smaller and may only function as ion channels, while rings allow the translocation of fully folded proteins (Palmer et al., 1998). There is also evidence that CDCs translocate proteins *in vivo*, thereby acting as secretion systems (Madden, Ruiz, & Caparon, 2001).

Although cholesterol is required for CDC activity and is generally considered the CDC receptor, a number of CDCs use human CD59 as a receptor and consequently have increased specificity for human cell membranes. However, these CDCs still require cholesterol for pore formation. These include intermedilysin (ILY) of *Streptococcus intermedius*, vaginolysin (VLY) of *Gardnerella vaginalis*, and lectinolysin (LLY) of *Streptococcus mitis.* The use of CD59 as a receptor may be attributed to a proline residue in place of a tryptophan in the undecapeptide (Lawrence et al., 2016). Additionally, CDCs also have conserved lectin-binding properties (Shewell et al., 2014). Using glycan array analysis, it was shown that PLY and SLO had affinities for different glycan structures and that binding these glycans altered the hemolytic activity of these toxins. Like cholesterol, the functional domain responsible for glycan binding is domain 4. While glycan binding has not yet been

reported for LLO, many of the modeled carbohydrate binding sites within domain 4 are conserved between LLO and CDCs from extracellular pathogens. Future experiments should investigate the roles of glycosylation with respect to cellular tropism and pathogen lifestyle.

Although the structure and mechanism of pore formation of CDCs are largely conserved, several CDCs have variations in their structure that contribute to changes in function. SLO has 60 amino acids at its N-terminus that mediate specific translocation of NAD+ glycohydrolase (SPN) into keratinocytes (Madden et al., 2001). Translocation of SPN induces cell death, following depletion of cellular NAD+, and significantly increases the virulence of *S. pyogenes* (Chandrasekaran & Caparon, 2016; Zhu et al., 2017). PLY lacks a signal peptide and may be released by cell lysis or by another export mechanism (Lemon & Weiser, 2015; Price, Greene, & Camilli, 2012). PLY also localizes to the cell wall, and its cell wall localization is dependent on SecY2A2, an accessory Sec system (Bandara et al., 2017). LLO has a 26-amino acid addition (known as the PEST-like sequence) near its N-terminus that reduces the intracellular toxicity of LLO and is necessary for *L. monocytogenes* to survive intracellularly following escape from phagocytic vacuoles (Schnupf & Portnoy, 2007). The role of the LLO PEST-like sequence in pathogenesis will be discussed in depth below.

2 Cellular Responses to CDC-Mediated Pore Formation

CDCs can induce a wide range of effects in cells, including activation of membrane damage responses and alteration of immune cell function. Among other things, CDCs can activate MAPKs, caspase-1, and TLR4, modulate SUMOylation, induce mitochondrial fragmentation, cause T cell apoptosis, and enhance bacterial internalization (Cajnko, Mikelj, Turk, Podobnik, & Anderluh, 2014; Cassidy & O'Riordan, 2013; Seveau, 2014). These responses are usually common to membrane insult by a range of PFTs and are often the direct result of Ca^{2+} influx and/or K^+ efflux. As a result of these numerous and diverse effects, LLO has been called the 'Swiss-army knife of *Listeria'* (Hamon, Ribet, Stavru, & Cossart, 2012; Osborne & Brumell, 2017). However, the role of these cellular responses in pathogenesis is not clear.

For more details on cellular responses to pore formation, refer to the following references: Cajnko et al., 2014, Cassidy & O'Riordan, 2013, Seveau, 2014, and Gonzalez, Bischofberger, Pernot, van der Goot, & Frêche, 2008.

2.1 Mitogen-activated Protein Kinase (MAPK) activation

MAPKs are involved in the initiation of signaling cascades that activate cellular responses to many stimuli. Cell membrane damage by PFTs causes the rapid efflux of intracellular K^+, and activation of the MAP kinases p38, extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK), mitogen- and stress-activated kinase 1 and 2 (MSK1/2), and cAMP response-element binding protein (CREB). Activation of p38 and ERK are required for recovery of intracellular K^+ levels following treatment of cells with sublytic concentrations of LLO and aerolysin, a non-CDC PFT that forms 2 nm pores (Cabezas et al., 2017; Gonzalez et al., 2011). In *Caenorhabditis elegans,* p38 and JNK MAPK pathways, and importantly one downstream target, activator protein 1 (AP-1), provide protection against

PFT toxicity (Kao et al., 2011). Thus, the restoration of ion homeostasis is one effect of MAPK activation in response to membrane perforation by PFTs.

2.2 Histone Modification

Histone modification has been observed in response to multiple pathogens and their CDCs. *L. monocytogenes* infection causes phosphorylation or dephosphorylation of Ser10 in histone H3 and acetylation or deacetylation of histone H4, depending on the experimental conditions (Hamon et al., 2007; Schmeck et al., 2005). In human umbilical vein endothelial cells, *L. monocytogenes* infection caused phosphorylation of Ser10 in histone H3, leading to increased expression of numerous cytokines in a p38 MAPK-dependent manner (Schmeck et al., 2005). Conversely, in human cervical epithelial cells (HeLa cells), *L. monocytogenes* infection or LLO alone caused dephosphorylation of Ser10 in histone H3, deacetylation of histone H4, and transcriptional repression of *cxcl2*, a cytokine involved in inflammation and neutrophil chemotaxis (Hamon et al., 2007). Treatment of HeLa cells with aerolysin, PFO, and PLY also results in dephosphorylation of Ser10 in histone H3, and dephosphorylation is dependent on K^+ efflux (Hamon & Cossart, 2011). Part of the *Pseudomonas aeruginosa* Type III secretion system, the PopB-PopD translocon, can form 4 nm pores on cell membranes that also result in K^+ efflux-dependent dephosphorylation of Ser10 in histone H3 in HeLa cells (Dortet, Lombardi, Cretin, Dessen, & Filloux, 2018). Thus, K^+ efflux resulting from membrane pore formation may have the ability to alter transcriptional profiles in ways that affect inflammation, although a role *in vivo* has not been demonstrated.

2.3 Alteration of Mitochondrial Dynamics

Treatment of HeLa cells with a sublytic concentration of recombinant LLO caused mitochondrial fragmentation, defined by breakage of the mitochondrial network into visually punctate structures. Mitochondria fragmentation occurred quickly after LLO treatment, and was transient; *L. monocytogenes*-infected cells completely recovered their normal mitochondrial network phenotype overnight, and did not become apoptotic. Mitochondrial fragmentation was dependent on Ca^{2+} influx, as cells incubated in Ca^{2+}-free media did not undergo mitochondrial fragmentation upon LLO addition (Stavru, Bouillaud, Sartori, Ricquier, & Cossart, 2011). In addition to causing histone modifications, infection of HeLa cells with *P. aeruginosa* also caused mitochondrial fragmentation, and was dependent on the expression of PopB and PopD (Dortet et al., 2018).

2.4 SUMOylation

SUMOylation is a eukaryotic post-translational modification, similar to ubiquitylation, in which small ubiquitin-like modifier (SUMO) is covalently attached to proteins. Most commonly, SUMOylation of transcriptional regulators leads to transcriptional repression (Gill, 2005). Treatment of HeLa cells with LLO, PFO, PLY, and suilysin (SLY)—a CDC produced by *Streptococcus suis*—resulted in degradation of Ubc9, an E2 SUMO enzyme, and a reduction in SUMO-conjugated proteins (Li, Lam, Lai, & Au, 2017; Ribet et al., 2010). Interestingly, the patterns of SUMOylated proteins were different for each CDC. Blockage of K^+ efflux prevented LLO-induced degradation of Ubc9, but enhanced Ubc9 degradation induced by PFO, PLY, and SLY, suggesting CDCs have different mechanisms for inducing Ubc9 degradation (Li et al., 2017). Overexpression of SUMO 1 and SUMO 2 in

HeLa cells limited infection of *L. monocytogenes* 2-fold (Ribet et al., 2010). Based on the observation that mice deficient for promyelocytic leukemia protein (PML), a known target of SUMO, had a defect in controlling *L. monocytogenes,* a recent study investigated the relationship between LLO, PML and SUMO during infection (Lunardi et al., 2011; Ribet et al., 2017). The authors reported that treatment with LLO, PFO, and PLY caused deSUMOylation of PML in Chinese hamster ovary (CHO) cells, and that gene expression of many cytokines was reduced in $pml^{-/-}$ mouse embryonic fibroblasts (MEFs) compared to $pml^{+/+}$ MEFs after *L. monocytogenes* infection, though the altered gene expression was not dependent on LLO (Ribet et al., 2017). A better understanding of how SUMOylation affects pathogenesis can be acquired by performing studies in mice with conditional deletions of *Ubc9* (Demarque et al., 2011; Fritah et al., 2014; Wang et al., 2017).

2.5 Caspase-1 activation

One of the hallmarks of the innate immune system is that activation of host pattern recognition receptors by conserved microbial products, known as pathogen-associated molecular patterns (PAMPs), and aberrant structure or localization of host molecules that result during disease, often referred to as damage-associated molecular patterns (DAMPs), leads to the initiation of immune responses (Brubaker, Bonham, Zanoni, & Kagan, 2015; Jounai, Kobiyama, Takeshita, & Ishii, 2012; Land, 2015; Schaefer, 2014; Vénéreau, Ceriotti, & Bianchi, 2015). An example of a DAMP is extracellular ATP, which binds and activates P2X7, an ATP-gated ion channel. Binding of ATP to P2X7 results in K^+ efflux and K^+ efflux-dependent NLRP3 inflammasome activation (Ayna et al., 2012; Pétrilli et al., 2007; Walev, Reske, Palmer, Valeva, & Bhakdi, 1995). Caspase-1 cleaves pro-interleukin 1 beta (IL-1β) into active IL-1β downstream of NLRP3 activation, which can occur spontaneously in low concentrations of K^+ (He, Zeng, Yang, Motro, & Núñez, 2016; Pétrilli et al., 2007). Many PFTs activate the NLRP3 inflammasome, including the pore-forming component of adenylate cyclase toxin (CyaA) produced by *Bordetella pertussis*, aerolysin, SLO, LLO and tetanolysin, a CDC produced by *Clostridium tetani* (Ayna et al., 2012; Chu et al., 2009; Dunne et al., 2010; Gurcel, Abrami, Girardin, Tschopp, & van der Goot, 2006; Harder et al., 2009; Idzko, Ferrari, & Eltzschig, 2014; Walev et al., 1995). Roles for the inflammasome in the pathogenesis of *B. pertussis, S. pneumoniae,* and *L. monocytogenes* have been reported, although in the case of *L. monocytogenes*, excess inflammasome activation reduces virulence (Dunne et al., 2010; Brian T Edelson & Unanue, 2002; Hassane et al., 2017; Karmakar et al., 2015; W. Li et al., 2016; J.-D. Sauer et al., 2011; Tsuji et al., 2004; Witzenrath et al., 2011).

3 Contributions of CDCs to Pathogenesis of Extracellular Pathogens

Many CDCs have clear roles in pathogenesis that are often related to the recruitment of immune cells or disruption of tissue barriers. Though all CDCs function by forming pores on host membranes, there are some differences in how they contribute to pathogenesis. Differences in how CDCs function *in vivo* are likely due to differences in infection sites and the simultaneous effects of other bacterial factors.

3.1 PFO

Upon introduction of *C. perfringens* into soft tissue, often by a traumatic injury, *C. perfringens* can cause clostridial myonecrosis, a necrotic infection of muscle that is largely devoid of infiltrating leukocytes (Soltani, Hotze, Johnson, & Tweten, 2007; Titball, 2005). Although *C. perfringens* produces many toxins, PFO (also called θ–toxin) is critical for severe pathology. PFO acts synergistically with α-toxin, a phospholipase produced by *C. perfringens*, in the development of clostridial myonecrosis (Ellemor et al., 1999). α-toxin causes most of the damage to the muscle tissue, while PFO contributes to pathology by inducing leukocyte aggregation in the vasculature—thereby preventing infiltration of leukocytes into the site of infection (Awad, Ellemor, Boyd, Emmins, & Rood, 2001). Deletion of PFO, but not α-toxin, in a mouse muscle model resulted in an almost complete reduction in severe leukocyte accumulation, which was likely caused by the upregulation of leukocyte and endothelial cell adhesion factors (Bryant & Stevens, 1996; Ellemor et al., 1999; Verherstraeten et al., 2015). Treatment of mice with a PFO-neutralizing antibody prior to infection with a lethal dose of intramuscular *C. perfringens* significantly reduced mortality (Bryant et al., 1993). Additionally, PFO is required for persistence of *C. perfringens* in a low-dose mouse femoral muscle infection model (O'Brien & Melville, 2004). Disruption of leukocyte migration and subsequent inflammation induced by PFO contributes to the disease progression of clostridial myonecrosis.

3.2 PLY

S. pneumoniae is the causative agent for a number of diseases, including pneumonia, otitis media, meningitis, and sepsis (Mitchell & Dalziel, 2014). PLY has a significant role in the pathogenesis of pneumococcal pneumonia and sepsis (Benton, Everson, & Briles, 1995; Berry, Yother, Briles, Hansman, & Paton, 1989). In a mouse *S. pneumoniae* upper respiratory tract infection model, *S. pneumoniae* strains with PLY were shed in nasal secretions at higher levels than PLY-deficient strains, and PLY was required for transmission (Zafar, Wang, Hamaguchi, & Weiser, 2017). In an intranasal mouse infection model of pneumonia and septicemia, mice infected with PLY-negative bacteria did not develop severe disease, whereas mice infected with *S. pneumoniae* expressing PLY were moribund within 48 hours. Mice infected with the PLY-negative mutant had 4-logs less bacteria in both the lungs and blood (Kadioglu et al., 2000). In addition, mice treated with a PLY-neutralizing antibody prior to infection with a lethal dose of *S. pneumoniae* administered intranasally or intraperitoneally had significantly increased survival (Del Mar García-Suárez et al., 2004; Musher, Phan, & Baughn, 2001). PLY reduced ciliary beating and caused reduced or disorganized cilia on the epithelial cells of human adenoid organ cultures. This result correlated with increased numbers of *S. pneumoniae* adhering to the organ cultures, mostly on damaged cells near disrupted tight junctions (Rayner et al., 1995). Therefore, PLY may contribute to dissemination of *S. pneumoniae* during infection of the lungs by disrupting mucociliary elevator-mediated bacterial clearance and allowing the bacteria to invade deeper tissues through disrupted tight junctions.

PLY-induced IL-1β secretion provides partial protection to the host during *S. pneumoniae* infections. IL-1β secretion in response to PLY is caspase-1, NLRP3, and ASC-dependent in many cell types (Hassane et al., 2017; Karmakar et al., 2015; Mariathasan et al., 2006).

IL-1β$^{-/-}$, caspase-1/11$^{-/-}$, ASC$^{-/-}$ and NLRP3$^{-/-}$ mice had 1-log increased bacterial burdens compared to WT mice in a *S. pneumoniae* keratitis model (Karmakar et al., 2015). In a mouse lung infection model, *S. pneumoniae* infection caused increased lung permeability, which was exacerbated in mice lacking NLRP3 (Witzenrath et al., 2011). IL-1β contributed to the activation of γδT cells, and their production of IL-17A. IL-17A-deficient, TCRδ-deficient, and neutrophil-depleted mice had significantly reduced survival compared to WT mice (Hassane et al., 2017). Thus, inflammasome activation by PLY leads to the generation of the Th17 response, which provides some protection against *S. pneumoniae* infection.

3.3 ALO

Bacillus anthracis is capable of causing severe disease in humans after inhalation, ingestion, or cutaneous introduction of bacterial spores. In all cases, the disease begins as a localized infection that can quickly lead to sepsis (Owen, Yang, & Mohamadzadeh, 2015). ALO plays a significant role in pathogenesis (Shannon, Ross, Koehler, & Rest, 2003). Administration of 100 μg of ALO-neutralizing antibody to mice infected with a lethal intravenous dose of *B. anthracis* significantly increased survival (Nakouzi, Rivera, Rest, & Casadevall, 2008). ALO is required for disruption of tight junctions and the gut epithelial barrier, and for the apical to basolateral translocation of *B. anthracis* across C2BBE monolayers, suggesting that the function of ALO is to facilitate the early establishment and penetration of *B. anthracis* into the gut epithelium (Bishop, Lodolce, Kolodziej, Boone, & Tang, 2010). Though significant, the role of ALO in systemic infection is not well characterized.

3.4 Varying Roles for CDCs in Pathogenesis

The role of CDCs in the pathogenesis of extracellular pathogens often involves damaging, but not necessarily killing, cells in and around the site of infection. CDCs cause cell remodeling, such as ciliary rearrangement, which may promote bacterial adhesion; they can disrupt tight junctions, facilitating bacterial translocation through epithelial and endothelial barriers and thus facilitating dissemination; and they can alter the expression of adherence factors, which can lead to recruitment of phagocytes and inflammation, or prevention of immune cell infiltration. Though many of these functions do not result in cell death, host cell lysis can also be a survival strategy for extracellular pathogens. Indeed, both *C. perfringens* and *B. anthracis* use their CDC to lyse host cells subsequent to phagocytosis, thereby releasing the bacteria back into the extracellular space and promoting bacterial growth (Heffernan, Thomason, Herring-Palmer, & Hanna, 2007; O'Brien & Melville, 2004). Cell lysis may also provide extracellular pathogens with nutrients. These CDC-mediated effects are well suited to the needs of extracellular pathogens.

4 The Role of LLO in Disease

L. monocytogenes is a facultative intracellular pathogen that, in humans, primarily causes self-resolving gastrointestinal infections. In immunocompromised individuals, *L. monocytogenes* can cause fatal systemic infections and, in pregnant women, placental infections that lead to pregnancy loss and systemic disease that results in death to the neonate (Lecuit, 2007). LLO is required for virulence in most if not all *L. monocytogenes*

animal disease models, including acute systemic infection in mice, neonatal mice, pregnant mice and pregnant guinea pigs (Bakardjiev, Stacy, & Portnoy, 2005; Gaillard, Berche, & Sansonetti, 1986; Kathariou, Metz, Hof, & Goebel, 1987; Le Monnier et al., 2007; W. Li et al., 2016; McKay & Lu, 1991; Portnoy, Jacks, & Hinrichs, 1988). The requirement for LLO in virulence can be recapitulated in tissue culture where it is required for *L. monocytogenes* to escape from phagosomes. Mutants lacking LLO are unable to escape from the phagosome and consequently unable to grow intracellularly (Tilney, L. G., Portnoy, 1989). In a mouse systemic infection model, LLO-negative mutants are 5-logs less virulent. The requirement for LLO in escape from the phagosome *in vivo* has been observed in real-time in infected zebrafish (Levraud et al., 2009). Strikingly, replacement of LLO with other CDCs results in strains that can escape from a phagosome but then kill the infected host cell, thereby eliminating the intracellular replicative niche (Decatur & Portnoy, 2000; S. Jones & Portnoy, 1994; Portnoy, Tweten, Kehoe, & Bielecki, 1992; Wei et al., 2005). It is important to note that there are populations of *L. monocytogenes* that replicate extracellularly in the gut and gallbladder, and LLO is not required for the establishment of infection at these sites. However, while wildtype *L. monocytogenes* can disseminate from the gut to establish infection in systemic organs, LLO-deficient bacteria cannot efficiently disseminate from the gut to systemic sites (Hardy et al., 2004; G. S. Jones et al., 2015; Roll & Czuprynski, 1990).

4.1 LLO Activity is pH-Dependent

The optimal pH for LLO activity is 5.5, while extracellular CDCs such as PFO and SLO have similar activities at pH 5.5 and pH 7, suggesting that LLO has adapted to the specific setting of the acidified phagosome (Geoffroy, Gaillard, Alouf, & Berche, 1987; Portnoy et al., 1992). An early study into the molecular basis of this low optimal pH found that amino acid L461 was the main determinant, and that this leucine is not conserved in CDCs from extracellular pathogens (Glomski, Gedde, Tsang, Swanson, & Portnoy, 2002). Nonsynonymous mutations of L461 affect LLO activity and cytotoxicity. Mutants with a threonine substitution, the residue common in extracellular pathogen CDCs, were 100-fold less virulent in mice due to their increased cytotoxicity. The pH insensitivity of L461T may be caused by an increase in the rate of oligomerization. Later it was reported that LLO is denatured at neutral pH at temperatures greater than 30 C, and that this was caused by charged amino acids within the transmembrane helices of domain 3 that act as a pH sensor (Schuerch, Wilson-Kubalek, & Tweten, 2005). Thus, while LLO is maximally active in acidified phagosomes, in the host cell cytosol its activity is partially reduced and it has the potential to denature. This mechanism is not solely responsible for limiting the activity of LLO to the phagosome, but it does contribute to reducing LLO-mediated cytotoxicity and preserving the replicative niche.

4.2 The LLO PEST-like Sequence

The most distinctive and single largest contributing feature of LLO for the *L. monocytogenes*-specific lifestyle is a PEST-like sequence at the amino terminus of the protein (Decatur & Portnoy, 2000; Lety et al., 2001). PEST-like sequences were originally described in eukaryotic proteins with short half-lives and were thought to mediate those short half-lives, but it is now appreciated that they often include another domain known as a polyproline type II (PPII) helix that mediates protein-protein interactions (Köster et al.,

2014; Rechsteiner & Rogers, 1996; Rogers, Wells, & Rechsteiner, 1986). Structural and *in vitro* analyses have indicated that residues in the PPII helix region play a role in oligomerization through intermolecular contacts (Köster et al., 2014). Deletion of 26 amino acids of LLO that include the PEST-like sequence has a minor effect on hemolytic activity; however, the bacteria are extremely cytotoxic in tissue culture and 10,000-fold less virulent in mice (Decatur & Portnoy, 2000).

Intracellular LLO exists in multiple forms, including 58kDa and 55kDa molecular weight species. The lighter species is absent during infection with the PEST-deletion mutant or mutants deficient in actin-based motility, suggesting the PEST-like sequence contributes to subcellular compartmentalization or processing of LLO (Schnupf, Portnoy, & Decatur, 2006). Additionally, independently of the PEST-like sequence, LLO is ubiquitylated and accumulates as a ladder of higher molecular weight species in the presence of proteasome inhibitors. LLO has an N-terminal lysine that serves as a destabilizing signal for the N-end rule pathway, which involves ubiquitylation and proteasomal degradation. Indeed, the short intracellular half-life of LLO was extended by replacing the N-terminal lysine with stabilizing amino acids. However, the half-life extension only marginally affected cellular toxicity or virulence unless combined with mutations in the PEST-like sequence (Schnupf, Zhou, Varshavsky, & Portnoy, 2007). Future studies should aim to identify the precise site or sites of ubiquitylation and their roles in pathogenesis and cell biology.

Consistent with the hypothesis that the LLO PEST-like sequence is important for intermolecular interactions, the PEST-like sequence contains three residues (S44, S48, and T51) that are predicted targets for MAPKs, and one or all of these residues are important for LLO phosphorylation inside of infected host cells (Schnupf, Portnoy, et al., 2006). Studies on phosphorylation of the PEST-like sequence have been confounded by the observation that point mutations in the region result in increased protein production and cytotoxicity, and attenuated virulence (Schnupf, Portnoy, et al., 2006). For example, mutations that change the S44 codon to alanine, thereby preventing phosphorylation, have increased translation of LLO. However, mutations that change the S44 codon to other serine codons also have increased translation— suggesting that the PEST-like sequence acts at the mRNA level to affect translation. Further evidence of translational regulation is supported by the observation that mutations in the 5’ UTR alter protein expression (Schnupf, Hofmann, et al., 2006; Shen & Higgins, 2005). The unexplained effect of mutations in the PEST-like sequence on translation complicates the study of post-translational modifications in the PEST-like sequence.

In addition to the above modifications, LLO is covalently modified by exogenously- and endogenously-produced S-glutathione at its cysteine residue (Portman, Huang, Reniere, Iavarone, & Portnoy, 2017). Modification of this residue may modulate the activity of all extracellular CDCs or restrict their activity to phagosomes containing oxidoreductases. For example, this cysteine has been implicated as a target for the phagosomal thiol-reductase known as GILT (Singh, Jamieson, & Cresswell, 2008). GILT$^{-/-}$ mice and macrophages were more resistant to *L. monocytogenes* due to a defect in phagosomal escape, presumably because LLO activity was reduced by modification with glutathione or another low molecular weight thiol. Thus, the presence of a host oxidoreductase, such as GILT, can

confer cellular specificity to CDC-producing pathogens by activating CDCs in the phagosome and promoting escape. However, mutant *L. monocytogenes* in which the LLO cysteine is substituted with an alanine have a very small virulence defect (Portman et al., 2017).

Although a lot of work is still required to understand the role of LLO modifications inside host cells, recent work has provided a detailed mechanism describing how its N-terminus uses host cell machinery to promote LLO degradation (Chen et al., 2018). Within cells, LLO localized to puncta within the cytosol while LLO lacking the PEST-like sequence was found on the host plasma membrane. This was due to interaction of the PEST-like sequence with the host Ap2a2 subunit of the clathrin-dependent endocytosis machinery, supporting a model in which LLO prevents cytotoxicity by accelerating the removal of membrane-associated LLO by endocytosis and targeting to autophagosomes. Interestingly, replacement of the LLO PEST-like sequence with the PEST-like sequence of human calcium receptor protein (HCaR), a G protein-coupled receptor that also interacts with Ap2a2, restored much of the virulence defect seen in a PEST deletion mutant. Though there are still some unanswered questions about the individual functions of this region of LLO, it is clear that the PEST-like sequence reduces the cytotoxicity of LLO.

4.3 Contribution of LLO to *L. monocytogenes* Pathogenesis

As discussed above, LLO has many other putative functions that are shared with CDCs produced by extracellular pathogens. Antibody to CDCs can often dramatically affect pathogenesis, as has been shown for PFO, PLY, ALO, and SLY (Bryant et al., 1993; Del Mar García-Suárez et al., 2004; Musher et al., 2001; Nakouzi et al., 2008; Takeuchi et al., 2014). In the case of *L. monocytogenes*, pretreatment of mice with 1 mg of LLO neutralizing antibody, 10-times the amount of antibody required to effectively neutralize ALO and PLY *in vivo*, resulted in reduced bacterial burden (B T Edelson, Cossart, & Unanue, 1999). However, it was later shown that this amount of antibody blocked the activity of LLO inside of cells and prevented vacuolar escape (Asano et al., 2016; B. T. Edelson & Unanue, 2001). These results suggest that LLO is required for pathogenesis of *L. monocytogenes* because it enables vacuolar escape, and that extracellular LLO has little if any effect on pathogenesis.

5 Future Considerations

Tissue culture models of infection provide a convenient way to study the effects that pathogens exert upon cells, and can shed insight into the host and bacterial factors required for any observed phenotypes. Bacterial mutants or antibody can be used to demonstrate the requirement for specific bacterial gene products in a given phenotype, and host mutants or specific inhibitors can be used to demonstrate host requirements. These are powerful strategies that have been used often to show the role of CDCs in the induction of host responses. For example, the conclusion that CDCs of *S. pneumoniae,* and *L. monocytogenes* induce inflammasome-dependent IL-1β secretion results from two distinct findings (1) deletion of the CDCs diminished IL-1β secretion and (2) deletion of inflammasome components also diminished IL-1β secretion from cells. However, once the host and bacterial requirements for an *in vitro* phenotype have been established, how do we

accurately determine if and how the phenotype translates into an effect on pathogenesis *in vivo*?

If a host is genetically tractable and the host factor in question is nonessential, it is possible to use a similar combinatorial approach, which can appropriately be called 'genetics-squared' (Persson & Vance, 2007). For some of the proposed LLO functions, genetic models can be used to verify the role of the host factors in pathogenesis. For example, *Ubc9*$^{+/-}$ mice have been used to demonstrate the importance of SUMOylation in control of *Shigella flexneri*, which is also a facultative intracellular pathogen, and could be used similarly for *L. monocytogenes* (Fritah et al., 2014). Host gene deletions were used to understand the role of IL-1β in the pathogenesis of *S. pneumoniae* and *L. monocytogenes.* Caspase-1/11$^{-/-}$ mice infected with *S. pneumoniae* had increased bacterial burdens compared to WT mice, effectively demonstrating the role of caspase-1 in control of *S. pneumoniae* (Karmakar et al., 2015). However, PLY-deficient strains were not used in the *in vivo* experiments and thus we are left with questions: would the PLY-deficient strain grow better than wildtype *S. pneumoniae* in WT mice as a result of not activating the inflammasome, and if so, would that benefit still occur in caspase-1-deficient mice? These approaches are not straightforward because of the multiple effects of individual virulence factors but nevertheless should be performed whenever possible. In *L. monocytogenes,* LLO showed the same capacity for activating caspase-1 as other PFTs *in vitro*, while infection of caspase-1/11$^{-/-}$ mice yielded opposing results *in vivo* and, in our hands, had no effect on infection or immunity in mice (Sauer et al., 2011). Thus, similar tissue culture model results do not always translate directly to similar effects on pathogenesis. Furthermore, evaluating the role of LLO on caspase-1 activation—and most phenotypes for that matter—*in vivo* is difficult to assess because LLO-negative bacteria cannot grow intracellularly.

How, then, can the role of LLO *in vivo* be validated separately from its essential role in vacuolar escape? One strategy used to validate the significance of extracellular CDCs to pathogenesis is the use of neutralizing antibodies. Treatment of mice with PFO- PLY-, SLY- and ALO-neutralizing antibodies prior to infection with their respective pathogens resulted in a reduction in disease, thereby providing evidence for their role in disease. Many of the proposed functions of LLO, including MAPK activation, histone dephosphorylation, mitochondrial fragmentation, Ubc9 degradation, and caspase-1 activation occur upon addition of purified LLO to cells. It has been proposed that extracellular LLO that is secreted before bacterial invasion could cause the same effects *in vivo.* We propose the following experimental process to confirm or disprove that extracellular LLO causes these effects *in vivo* and that they have an effect on pathogenesis. First, these phenotypes must be identified following infection of mice; second, administration of an LLO-neutralizing antibody must reduce or abrogate the phenotypes; and third, the administration of LLO-neutralizing antibody must affect pathogenesis.

Lastly, how can roles for cytosolic LLO be elucidated when deletion of the gene prevents phagosomal escape thereby preventing secretion of cytosolic LLO? Various approaches including inducible promoters have been used to show that LLO was necessary for cell-to-cell spread (Dancz, Haraga, Portnoy, & Higgins, 2002). An alternative approach would be to incorporate an inducible degradation tag such as the auxin-inducible degron, where LLO

could be targeted for degradation in the cytosol (Holland, Fachinetti, Han, & Cleveland, 2012; Nishimura, Fukagawa, Takisawa, Kakimoto, & Kanemaki, 2009). However, this would be very difficult to adapt to animal experiments. Another approach has recently been developed in our lab in which the gene encoding LLO was bracketed by *loxP* sites in a strain that induces the expression of Cre upon reaching the host cytosol. In this strain, LLO mediates escape from a phagosome, but is rapidly deleted upon reaching the cytosol. This system revealed that intracellular LLO has the potential to be cytotoxic, but that cytotoxicity is reduced by subversion of host endocytosis machinery to remove LLO from the host plasma membrane (Chen et al., 2018). Others have proposed that LLO participates in cell-to-cell spread by causing localized damage in membrane protrusions, resulting in markers of apoptosis that allow those protrusions to be recognized and subject to efferocytosis by adjacent macrophages (Czuczman et al., 2014). The foundation of this concept, and other concepts, could be strengthened by using the strain of *L. monocytogenes* that escapes the vacuole and deletes LLO in the cytosol. Although the strain is not ideal for all experiments, because it is defective in cell-to-cell spread, it provides a valuable starting point for the evaluation of intracellular LLO phenotypes that was not previously available.

The question remains, is LLO a phagosome-specific cytolysin or a multifunctional virulence factor? LLO has an abundance of features throughout its structure that allow it to mediate the escape of *L. monocytogenes* from a vacuole without causing excess cytotoxicity in the cytosol. Furthermore, it is absolutely required for disease because of its role in vacuolar escape. Thus, we believe that most evidence points to LLO being a phagosome-specific cytolysin. However, LLO may act extracellularly under some circumstances, perhaps in the intestine or during extracellular growth in the gall bladder (Hardy et al., 2004). The notion that LLO can activate many of the same pathways as extracellular CDCs is intriguing, and the tools exist to validate whether or not LLO activates these pathways in the host in ways that affect the outcome of disease.

		Primary function *in vivo*	**K+ dependent MAPK activation**	**Histone Modification**	**Mitochondrial fragmentation**	**deSUMOylation/ Ubc9 degradation**	**Caspase-1 activation**	**Macrophage TLR4 activation**	**Effects on cell adhesion**	**Protein translocation**	**Vacuolar Escape**
CDC	**Organism**										
Listeriolysin O (LLO)	*Listeria monocytogenes*	Escape from the vacuole	(Gonzalez et al., 2011; Tang, Rosenshine, Cossart, & Finlay, 1996)	(Hamon et al., 2007)	(Stavru et al., 2011)	(Ribet et al., 2010)	(Meixenberger et al., 2010; Sauer et al., 2010)	(Park, Ng, Maeda, Rest, & Karin, 2004)	(Drevets, 1997; Kayal et al., 1999; Krüll et al., 1997)	(Sibelius et al., 1996)	(Tilney, L. G., Portnoy, 1989)
Perfringolysin (PFO)	*Clostridium perfringens*	Disruption of neutrophil migration (Ellemor et al., 1999)		(Hamon et al., 2007)		(Ribet et al., 2010)		(Park et al., 2004)	(Bryant & Stevens, 1996; Rafii, Park, Bryant, Johnson, & Wagner, 2008)		(O'Brien & Melville, 2004)
Pneumolysin (PLY)	*Streptococcus pneumoniae*	Epithelial barrier disruption (Rayner et al., 1995) Transmission (Zafar et al., 2017)	(Aguilar et al., 2009)	(Hamon et al., 2007)		(Li et al., 2017; Ribet et al., 2010)	(Fang et al., 2011; Hassane et al., 2017; Karmakar et al., 2015; Witzenrath et al., 2011)	(Malley et al., 2003; Srivastava et al., 2005)	(Nel et al., 2017; Thornton & McDaniel, 2005; Zhang et al., 2016)		
Streptolysin O (SLO)	*Streptococcus pyogenes*	Cell killing by SPN translocation	(Stassen et al., 2003)			(Li et al., 2017)	(Harder et al., 2009; Keyel et al., 2013)	(Park et al., 2004)	(Bryant et al., 2005;	(Madden et al., 2001)	

		Primary function *in vivo*	K+ dependent MAPK activation	Histone Modification	Mitochondrial fragmentation	deSUMOylation/ Ubc9 degradation	Caspase-1 activation	Macrophage TLR4 activation	Effects on cell adhesion	Protein translocation	Vacuolar Escape
		(Zhu et al., 2017)							Zhang et al., 2016)		
Suilysin (SLY)	*Streptococcus suis*		(Bi et al., 2015)			(Li et al., 2017)		(Bi et al., 2015)	(Zhang et al., 2016)		
Anthrolysin O (ALO)	*Bacillus anthracis*		(Ratner et al., 2006)					(Park et al., 2004)			(Tonello & Zornetta, 2012)
Vaginolysin (VLY)	*Gardnerella vaginalis*		(Gelber, Aguilar, Lewis, & Ratner, 2008)								
Other Pore-forming molecules											
T3SS				(Dortet et al., 2018)	(Dortet et al., 2018)		(McCoy, Koizumi, Higa, & Suzuki, 2010)			(Wagner et al., 2018)	
Aerolysin	*Aeromonas hydrophila*		(Gonzalez et al., 2011)	(Hamon & Cossart, 2011)			(Gurcel et al., 2006; McCoy et al., 2010)				

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