Listeria monocytogenes virulence factors, including listeriolysin O, are secreted in biologically active extracellular vesicles

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This article contains Movie S1, Tables S1–S4, and Figs. S1–S4. Multimicroscopy data were deposited in the MassIVE repository under accession number MSV000081402, MSV000081403, and MSV000081404.  

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3 The data were from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Sue Golding Graduate Division of Medical Science, Albert Einstein College of Medicine, Yeshiva University, Bronx, NY.  

4 Supported by National Institutes of Health Training Grant GMS GM08752.  

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The abbreviations used are: LLO, listeriolysin O; PC, phosphatidylcholine; PLC, phospholipase C; ActA, Actin assembly–inducing protein; EV, extracellular vesicles; OMV, outer membrane vesicle; MPLEX, metabolite, protein, and lipid extraction; TEM, transmission electron microscopy; rLLO, recombinant LLO; RBC, red blood cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; hpi, h post-infection; HK, heat killed; BHI, brain heart infusion; ER, endoplasmic reticulum; DMEM, Dulbecco’s modified Eagle’s medium; BisTris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)-propane-1,3-diol; EM, electron microscopy; m.o.i., multiplicity of infection.
In the cytoplasm, *L. monocytogenes* replicates rapidly and produces the surface actin assembly-inducing (ActA) protein (17–19). ActA induces actin formation creating a comet tail, ultimately pushing the bacterium toward the host cell surface to invade neighboring cells. In this manner, *L. monocytogenes* replicates and spreads within the host avoiding the extracellular space and evading the immune system.

The use of extracellular vesicles (EVs) to secrete compounds to the extracellular space is established in mammals and is described in a variety of microorganisms, suggesting these structures are produced by all domains of life (1, 3, 4, 6, 12, 20–24). EVs are small, lipid-bilayered spheres ranging in diameters from ~20 to 500 nm. In Gram-negative bacteria, the outer membrane is lost, resulting in the formation of outer membrane vesicles (OMVs). OMVs have been associated with, but not limited to, adhesion, immunosuppression, cytotoxicity, virulence, and stress response (20–22, 25, 26) and are postulated to be “virulence bags.” The study of EVs in cell-walled organisms such as Gram-positive bacteria, mycobacteria, and fungi was neglected due to the erroneous inference that the combination of a thick cell wall and lack of outer membrane would preclude release of such structures. However, the discovery that fungi produced EVs despite having cell walls (24, 25) stimulated the search for EV in cell-walled organisms. EVs were found in *Bacillus anthracis* and, consistent with the idea of virulence bags, were implicated in the delivery of anthrax toxin to host cells (12). Packaging of toxins in EVs is widespread: both pneumococcal pneumolysin and staphyloccocal α-toxin are in EVs (4, 27, 28). In *Staphylococcus aureus* and *Mycobacterium ulcerans*, intact toxin-associated EVs are more cytotoxic than disrupted EVs or purified toxin alone, indicating that the EV structure is required for efficient delivery of the virulence “package” (3, 29, 30).

There have been two prior published reports of EVs in *L. monocytogenes* (17, 31) associating LLO with vesicles but neither unequivocally establishes toxin transport in vesicles. In addition, vesicles from *L. monocytogenes* have been reported to carry a variety of components including RNAs (32). In this study, we apply a variety of techniques to establish that *L. monocytogenes* packages virulence factors including LLO in EVs that are cytotoxic to mammalian cells due to packaging of LLO, characterized the secreted EVs using simultaneous metabolite, protein, and lipid extraction (MPLEx) multimetrics approach (33, 34). Finally, using EM and high-resolution fluorescence imaging, we observed EVs secreted into the cytosol of mammalian cells by intracellular bacteria. Our findings, together with prior reports (17, 31, 32), provide a compelling body of evidence that *L. monocytogenes*, like other Gram-positive bacteria (6, 25) use vesicular transport to deliver a concentrated and varied toxin cargo to host cells.

**Results**

*L. monocytogenes* produce EVs which carry virulence factors and are hemolytic

Production of EVs has been reported in several species of Gram-positive bacteria (25). To visualize EVs released from *L. monocytogenes*, we isolated cells or EVs from the extracellular media and imaged these by transmission electron microscopy (TEM) (Fig. 1, A–C). Lipid bilayer vesicles, consistent with EVs, andvesicle-like structures were visualized protruding from bacterial cells (Fig. 1, see Table 1 for strain description). We performed a density gradient on EVs and show that LLO immunodetection from extracted EVs is different from the pattern of recombinant LLO (rLLO), consistent with its localization within EVs. The fractions containing LLO showed structures consistent with EVs by negative staining TEM (Fig. 1, D and E, Fig. S2). We tracked the presence of LLO and PI-PLC during our EV purification protocol to obtain biochemical evidence for the association of virulence factors, such as LLO and PI-PLC, with EVs (Fig. 1, F and G). Our data shows that LLO and PI-PLC sediment into a pellet by ultracentrifugation at 100,000 × g, consistent with secretion in EVs. Additionally, we explored the temporal relationship between LLO-EV association and bacterial growth by purifying EVs from the WT strain (WT) at various time intervals during broth culture growth and observed LLO in the EV fraction at all phases of bacterial culture growth (Fig. S1A).

Protease protection assays were performed to ascertain the proportion of hemolytic activity in *L. monocytogenes* supernatants and investigate the alternate possibilities that LLO protein aggregates were precipitating with EVs in some artificial fashion. These assays show LLO from culture supernatants (Fig. 1H) and in EVs (Fig. S1, B and D) are protected from protease digestion. The finding that all hemolytic activity of culture supernatants was preserved in the presence of trypsin, whereas that of recombinant LLO added to the same media was destroyed implies that naturally produced LLO is protected from the protease, consistent with the location inside EVs. To ascertain the presence of functional LLO within EVs, we tested all stages of the purification process for lysis of erythrocytes (red blood cells, RBC) (Fig. S1C). The fractions: Sup, 100 kDa conc, Sup2 (as defined in diagram in Fig. 1C), and EVs could lyse RBC. We note that after the EV purification steps some LLO appears in filtrates implying that these procedures disrupt some vesicles. However, we show EVs from WT lysis more RBC than EVs from Δhly (Fig. S1D). We conclude that LLO and PI-PLC are secreted within EVs. The loss of activity from the Sup to EV fraction suggests EVs rupture in the extracellular milieu or during experimental handling of samples and release the cargo in soluble form.

To measure LLO-derived toxicity of EVs in nucleated mammalian cells, J774.16 cells were incubated with EVs purified from cultures of WT and avirulent strains Δhly (LLO−) and ΔplcAB (PI-PLC−, PC-PLC−), and viability of macrophages was determined based on the MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) assay. EVs purified from the Δhly mutant strain exhibited only residual cytotoxicity, whereas EVs purified from the ΔplcAB strain were more cytotoxic than EVs from WT bacteria (Fig. 2A). To determine which gene deletion was responsible for increased hemolysis we tested EVs purified from strains with single mutations ΔplcA and ΔplcB strains. EVs purified from the PI-PLC mutant (ΔplcA) were more cytotoxic to macrophages than EVs from other strains. To determine whether increased cytotoxicity of
Lm produces extracellular vesicles with virulence factors
EV from ΔpIC could be reversed by addition of PI-PLC we performed a rescue assay by combining EVs from the WT strain and ΔpIC mutant strain, whereas keeping constant the total amount of EVs, and conceivably LLO, added to each sample (Fig. 2B). We found that WT EVs could partially reverse increased cytotoxicity of the ΔpIC strain. A similar experiment performed with addition of Δhly EVs to ΔpIC EVs confirmed this reversal in cytotoxicity. We investigated whether LLO production was increased in ΔpIC EVs when compared with the WT cells but could not detect an increase in LLO secretion (Fig. S3), as shown before for ΔpIC (35). To rule out that ΔpIC deletion affected gross composition of EVs we compared EVs by SDS-PAGE gel analysis and dynamic light scattering to find distinct protein electrophoretic patterns but with a similar size of EVs (Fig. 2, C and D). The diameter and morphology of EVs from L. monocytogenes is similar to EVs produced by S. aureus and Streptococcus pneumoniae (1, 3, 4). Hence, the stronger hemolytic activity observed for the strain lacking pIC was not due to increased production of LLO or EV structural differences.

**MPLEx characterization of L. monocytogenes cells and EVs**

EVs typically carry proteins, lipids, RNA, and metabolites. EVs from L. monocytogenes have been characterized for their protein content (17), but no information is available about other components. We performed MPLEx analysis of bacterial cells and EVs to characterize their composition (Fig. 3, Tables S1–S4). We found striking differences in protein, lipid, and metabolite compositions between cells and EVs. The EVs were enriched in proteins from peptidoglycan synthesis and carbohydrates synthesis (Fig. 3, A and D), suggesting a role in the synthesis of the cell wall. EVs are depleted in proteins associated with translation machinery, and enzymes from fatty acid or amino acid metabolism, but enriched in ABC transporters. We also identified the classical virulence factors of L. monocytogenes such as LLO, InlA, InlB, plcB, and ActA associated with vesicles, although the origin of these vesicles, if bacterial or host cell-derived, could not be determined (Fig. 4F). Gold particles were abundantly detected in the nucleoplasm of host nuclei, mainly on the heterochromatic area (Fig. 4C). However, data from immunofluorescence (see below) shows unspecific staining of the mAb to LLO to mammalian cell nuclei, and therefore we advise cautious interpretation of immunodetection when performing studies of LLO location within host cells. A noticeable feature of Listeria-infected cells is LLO-mediated induction of autophagy (38). We noted the accumulation of autophagosomes and autolysosomes in infected cells, and detected vesicles containing LLO adjacent to autophagic structures (Fig. 4D). Additionally, LLO was observed as vesicular structures and free aggregates in the host cytosol (Fig. 4, E and F), without any discernable membrane surrounding or binding the toxin. Another set of host organelles targeted by LLO included tubules of the ER (Fig. 4G) and the mitochondria, in which LLO was detected on the outer and inner membranes (Fig. 4H). The transport of LLO to host mitochondria may be vesicular as several LLO-containing vesicles were observed surrounding these organelles. The ER or mito-

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**Table 1**

| Strain        | Genotype | Phenotype  | Source or Ref. |
|---------------|----------|------------|----------------|
| 10403s        | Wildtype | Wildtype (WT) | 71             |
| DP-L2161      | 10403s Δhly | LLO         | 72             |
| DP-L1936      | 10403s ΔpICAB | PI-PLC–, PC-PLC– | 15            |
| DP-L1552      | 10403s ΔpICA | PI-PLC–      | 35             |
| DP-L1935      | 10403s ΔpICB | PC-PLC–      | 15             |
| GFF           | 10403s GFP | Wildtype-GFP | 73             |

Ev from ΔpIC could be reversed by addition of PI-PLC we performed a rescue assay by combining EVs from the WT strain and ΔpIC mutant strain, whereas keeping constant the total amount of EVs, and conceivably LLO, added to each sample (Fig. 2B). We found that WT EVs could partially reverse increased cytotoxicity of the ΔpIC strain. A similar experiment performed with addition of Δhly EVs to ΔpIC EVs confirmed this reversal in cytotoxicity. We investigated whether LLO production was increased in ΔpIC EVs when compared with the WT cells but could not detect an increase in LLO secretion (Fig. S3), as shown before for ΔpIC (35). To rule out that ΔpIC deletion affected gross composition of EVs we compared EVs by SDS-PAGE gel analysis and dynamic light scattering to find distinct protein electrophoretic patterns but with a similar size of EVs (Fig. 2, C and D). The diameter and morphology of EVs from L. monocytogenes is similar to EVs produced by S. aureus and Streptococcus pneumoniae (1, 3, 4). Hence, the stronger hemolytic activity observed for the strain lacking pIC was not due to increased production of LLO or EV structural differences.

**EVs are produced in infected human epithelial cells**

We performed immunogold EM (immuno-EM) in Listeria-infected MCF-7 cells for information on LLO distribution at the ultrastructural level, and in an attempt to visualize association of EVs and LLO in the context of mammalian infection. The specificity of the immuno-EM staining using anti-LLO antibodies was determined by assessing LLO-gold particle density. Immuno-EM labeling was negligible for the Δhly strain compared with WT strain-infected cells (Fig. S4). At 3 h post-infection (hpi) in MCF7 cells, the vast majority of Listeria were detected as free organisms in the host cytoplasm, with only 5% enclosed in a phagosomal compartment (Fig. 4A). Some LLO-gold particles were observed in close proximity to free Listeria and associated with vesicles, although the origin of these vesicles, if bacterial or host cell-derived, could not be determined (Fig. 4B). Gold particles were abundantly detected in the nucleoplasm of host nuclei, mainly on the heterochromatic area (Fig. 4C). However, data from immunofluorescence (see below) shows unspecific staining of the mAb to LLO to mammalian cell nuclei, and therefore we advise cautious interpretation of immunodetection when performing studies of LLO location within host cells. A noticeable feature of Listeria-infected cells is LLO-mediated induction of autophagy (38). We noted the accumulation of autophagosomes and autolysosomes in infected cells, and detected vesicles containing LLO adjacent to autophagic structures (Fig. 4D). Additionally, LLO was observed as vesicular structures and free aggregates in the host cytosol (Fig. 4, E and F), without any discernable membrane surrounding or binding the toxin. Another set of host organelles targeted by LLO included tubules of the ER (Fig. 4G) and the mitochondria, in which LLO was detected on the outer and inner membranes (Fig. 4H). The transport of LLO to host mitochondria may be vesicular as several LLO-containing vesicles were observed surrounding these organelles. The ER or mito-

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**Figure 1. Analysis of EVs from L. monocytogenes.** EVs were isolated from 18-h cultures in BHI broth. A, TEM micrograph of EVs purified from Δhly strain (LLO–) culture supernatants. B, TEM micrographs of putative EV protrusions releasing from L. monocytogenes cells. C, diagram illustrating isolation of EVs. D, OptiPrep gradient fractionation of EVs was followed by immunoblot to detect LLO. The experiment was repeated three times and one representative experiment is shown. E, OptiPrep gradient fractionation of LLO was negative staining TEM. F and G, immunoblot of LLO and PI-PLC for each step of the EV isolation protocol. Controls were supernatants from Δhly (LLO–) and ΔpIC (PI-PLC–) strains. Aliquots were taken at each step and protein extracted by TCA precipitation. Each lane was loaded with 7 μl of each protein extract equivalent to 70 ml of culture. Numbers represent fractions from C, lanes left empty. Experiments were repeated three times and one representative experiment is shown. Scale bars, 100 nm. H, EVs from Δhly (LLO–) and ΔpIC (PI-PLC–) strains were digested with trypsin and LLO function was measured via RBC lysis assay. To ascertain the role of soluble LLO we added rLLO to Δhly EVs followed by trypsin treatment when appropriate. Experiments were performed four times and mean ± S.E. of all experiments are shown. Data from Δhly supernatant shown in both graphs for comparison purposes. Statistical analysis two-way analysis of variance. Top, statistical analysis for WT versus WT + trypsin. Bottom: statistical analysis for WT versus Δhly. ***, p < 0.001; n.s., nonsignificant.
Mitochondrial staining was not observed when cells were infected with \( \Delta hly \) bacteria. In summary, quantitative distribution of gold particles in mammalian cells containing replicating bacteria reveals that LLO is present at the surface of various host organelles, mainly ER- and mitochondria-associated, and within membrane vesicles whose origin could not be determined by immuno-EM.

**Figure 2. Association between LLO and PI-PLC in EVs.**

A, EVs were incubated with J774.16 cells whose viability was determined by MTT assay. EVs from the \( \Delta plcA \) (PI-PLC\(^{-}\)) strain were more cytotoxic than WT EVs or \( \Delta plcB \) (PC-PLC\(^{-}\)). B, EVs from WT and \( \Delta hly \) (LLO\(^{-}\)) prevent toxicity by \( \Delta plcA \) (PI-PLC\(^{-}\)). Half the volume of EVs from each individual strain was added to maintain equal total volumes of EVs. *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \); ****, \( p < 0.0001 \) with two-way analysis of variance of \( \Delta plcA \) with the WT or \( \Delta plcB \). Shown is mean ± S.D. C, protein content of EVs from \( L. monocytogenes \) as revealed by silver-stained SDS-PAGE. D, mean EV diameters from dynamic light scattering. Shown is mean ± S.E. Experiments were performed in triplicate wells and at least three times.
To concurrently detect bacterial EVs and EV-association during mammalian infection we developed a high-resolution imaging approach to detect *Listeria*-derived lipids. To label *L. monocytogenes* lipids, we grew bacteria in the presence of 1/1000 M BODIPY 558/568-C12 (BODIPY-C12), a fluorescent saturated fatty acid analogue (39) for 18 h. Bacteria were grown at 30 °C to inhibit the in vitro production of virulence genes, including LLO (40). After labeling, part of the bacteria were heat killed (HK) followed by growth at 37 °C for 6 h to extract EVs or addition to cultures of MCF-7 breast cancer epithelial cells. We note that the pre-growth at 30 °C may delay progression of infection when compared with samples imaged by immuno-EM. BODIPY-C12 effectively labeled bacterial cells (Fig. 5), presumably inserted into the phosphatidylylycerol-dodecanoic acid lipid species (see lipid analysis in Table S2). BODIPY-C12 signal was consistent with localization within EVs and its BODIPY-C12 density was distinct from density of structures isolated from HK bacteria, presumably debris. High-resolution imaging of MCF-7 cells infected with live *L. monocytogenes* (Fig. 5C, yellow circles). These puncta were proximal and distant to bacterial cells suggesting release and mobility of bacterial lipids, presumably EVs, in the host cytoplasmic compartment. Time-lapse imaging was used to capture the dynamics of puncta formation. We detected that BODIPY-C12–labeled regions formed bulges (Fig. 6, blue arrow), which underwent rapid (<5 s) separation from the bacterium. These data (summarized in Fig. 7) support the notion of compartmentalization and release of virulence factors within EVs as part of the pathogenic strategy of *L. monocytogenes*.

**Discussion**

The production of EVs has now been described in all domains of life. OMVs from Gram-negative bacteria have been studied for over half a century, whereas only recently were EVs found in Gram-positive bacteria as diverse as *S. aureus* (1), *B. anthracis* (12), *Bifidobacterium* spp. and *Lactobacillus* spp. (41), *Bacillus subtilis* (23), *Pneumococcus pneumoniae* (4), *Streptomyces lividans* (42), *Clostridium perfringens* (20), and *L. monocytogenes* (17, 31, 32). One advantage for toxin packag-
Lm produces extracellular vesicles with virulence factors

**Figure 4. Ultrastructural localization of LLO in L. monocytogenes-infected cells.** Wildtype (WT) *L. monocytogenes* was used to infect MCF7 cells at a m.o.i. of 20 and at 3 hpi cells were fixed and processed for LLO immuno-EM. A, panel a: large view of infected cells showing free *L. monocytogenes* (L) in the host cell cytoplasm (hc). hPM, host plasma membrane. Panels b and c illustrate free bacteria and intracellular bacteria within phagosomes (P), respectively. B, a cluster of vesicles containing LLO (arrows) in close proximity to *L. monocytogenes* (L). C, host nucleus (n) containing LLO-gold particles. D, detection of many host autophagic profiles (au) in infected cells (panel a), with some LLO-stained vesicles in their vicinity (panel b). E, LLO on the limiting membranes of many host-dispersed cytosolic vesicles (arrows). F, free aggregates of LLO in the host cytosol. G and H, host ER tubules (G, ER) and mitochondria (m) positively labeled for LLO distributed both on the outer (panel a) and inner membranes (panel b). Note the presence of LLO-positive vesicles close to mitochondria (arrow). All scale bars are 1 μm. I, stereological analysis of gold labeling demonstrating the specific localizations of LLO in cells infected with *L. monocytogenes*. Density (gold particles per mm²) of labeled structures was determined from 55 to 62 cryosections. Percentage of individual intracellular compartment density was determined from the sum of gold density normalized for the variation in distribution of LLO.

In EVs is that it allows their delivery as a concentrated warhead that is not diluted as a function of distance from the bacterial surface and the contents are protected from proteolytic attack and neutralization factors. The bacterial EV field has historically been plagued by controversy whether these structures were real cellular products or the result of lipid association following lysis of bacteria (43). For Gram-positive bacteria these questions are further complicated by concerns as to how EVs traverse the bacterial cell wall (23). Raising additional suspicion for their existence was the absence of EV-null mutants, although recent work have identified genes modulating EV synthesis in Gram-positive bacteria (44) and mycobacteria (43), as well as gene deletions that alter morphology of EVs (17). In this study we establish that EVs are associated with LLO via biochemical purification approaches, functional detection, immuno-EM and fluorescence microscopy. We show that the hemolytic activity...
Lm produces extracellular vesicles with virulence factors

A

+ Bodipy-C12

16 h at 30°

Heat-kill Lm 2h at 72°

Live Lm

30 min infection

IF: 2-4 h

B

Density Gradient Fraction (top to bottom)

1 2 3 4 5 6 7 8 9 10

Input

Bodipy-C12 + Live Lm

Bodipy-C12 + HK Lm

DMSO + Live Lm

C

DNA Lm

HK Lm

Live Lm

D

Bodipy-C12 Puncta per MCF-7 cell

Live Lm n=97

HK Lm n=176

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of *L. monocytogenes* supernatants is maintained despite trypsin digestion consistent with protection of hemolysins in EVs. We provide evidence against their formation as by-products of cell autolysis by showing major differences in lipid and protein composition from parent bacteria. Critically, we provide the first microscopic evidence of real-time EV release from bacterial cells. These findings together with prior reports (17, 31) are a strong and compelling narrative for the notion that *L. monocytogenes* releases EVs packed with virulence factors. Incorporating EVs and their cargo into current views of *L. monocytogenes* pathogenesis will provide new insights into the mechanisms for host cell subversion during infection.

Prior studies that recovered LLO from culture supernatant using protein purification techniques would have included the vesicular fraction and therefore those results are compatible with our findings (45). Those procedures are likely to have disrupted EVs and their cargo into current views of *L. monocytogenes* pathogenesis will provide new insights into the mechanisms for host cell subversion during infection.

Figure 5. Specific labeling of bacterial lipids using the fluorescent free fatty acid analogue BODIPY-C12. A, schematic describing labeling and infection strategy. GFP-expressing WT *L. monocytogenes* (Lm) were grown overnight at 30 °C in the presence of 1 μM BODIPY-C12. A, MCF-7 cells were infected with live bacteria at m.o.i. of 10 for 30 min, and imaged at 2 hpi. Time-lapse imaging of infected, live MCF-7 cells was performed at 1 image/5 s. Scale bar, 10 μm. B, puncta dynamics in a region of interest (9.19 × 9.19 μm). Puncta formation is show by blue arrows, tracking is show in blue overlay (see Movie S1).

Figure 6. Release of EV structures from bacteria during mammalian infection using the fluorescent free fatty acid analogue BODIPY-C12. GFP-expressing WT *L. monocytogenes* were grown overnight at 30 °C in the presence of 1 μM BODIPY-C12. A, MCF-7 cells were infected with live bacteria at m.o.i. of 10 for 30 min, and imaged at 2 hpi. Time-lapse imaging of infected, live MCF-7 cells was performed at 1 image/5 s. Scale bar, 10 μm. B, puncta dynamics in a region of interest (9.19 × 9.19 μm). Puncta formation is show by blue arrows, tracking is show in blue overlay (see Movie S1).
these findings. First, it is possible that Δ*plcA* loss subtly affects EVs into more efficient vehicles of LLO delivery, because we could not detect gross EV structural abnormalities relative to WT strains. Second, the pattern of secreted proteins is not identical, implying different compositions of EVs for different strains that could affect toxicity in some yet to be determined manner. Third, it is possible that PI-PLC interacts with LLO to reduce its toxicity in some manner (47). For example, it is possible that the mode of cellular death/lysis is affected by PI-PLC, but the mechanism by which this phenomenon occurs is unclear at this time or perhaps there is some molecular interaction between these two virulence factors. The cell death pathway triggered in mammalian cells by EV treatment, the comparison of EV-induced cell death versus soluble LLO-cell death, and the observed interaction by PI-PLC is of paramount importance and should be addressed in future studies. Interestingly, Vdovikova et al. (31) described autophagy alterations in mammalian cells exposed to EVs.

LLO secreted by *L. monocytogenes* in the extracellular compartment or addition of recombinant LLO to mammalian cells
EVs were found to fuse with host cells (act as intracellular danger signals and trigger autophagy (38), such that tight control of toxin synthesis and activity is needed to avoid killing the host cell (49). It remains to be determined how LLO delivery in EVs will be reconciled with current paradigms of its function in \textit{L. monocytogenes} intracellular pathogenesis (50). The fact that LLO is vesicle associated is a new consideration in evaluating the equilibrium between LLO oligomers and monomers and how they affect the toxin physical and virulence properties. It is tempting to hypothesize that packaging of LLO in EVs facilitates assembly of oligomers, simply due to the high concentration achieved by packaging monomers in the small volume of an EV. It is conceivable that the functions of intracellular EVs, and their LLO cargo, in the host simply recapitulate those of exogenous LLO, such that its actions are indistinguishable. Alternatively, the finding of delivery of LLO in EVs implies a new set of interactions for further study that could significantly alter our understanding of \textit{L. monocytogenes} pathogenesis.

Another intriguing aspect is how exogenously added EVs exert their toxicity. Possibilities include fusing with host plasma membrane and release of contents into the host cytosol or ingestion by target cell followed by trafficking in the endocytic/phagocytic compartments before toxin release (31). In other bacterial pathogens (\textit{S. aureus} and \textit{Legionella pneumophila}), EVs were found to fuse with host cells (51) but it has been suggested that EVs from \textit{L. monocytogenes} are internalized in endosomes (31). An alternative possibility is that EVs are disrupted by host components and their associated proteins releasing vesicular contents in the immediate vicinity of the target cell. In this regard, we note that bacterial and fungal EVs are disrupted by some host proteins including albumin (52). In the specific case of \textit{L. monocytogenes}, EVs may simply be internalized by endocytosis (31), or, given the invasive arsenal packaged in EVs it is equally probable that EVs induce their ingestion by host cells, either via internalin (InlA and InlB) interactions with lipid microdomains at the cell surface (53), or by LLO pore-triggered entry mechanisms analogous to what has been observed for \textit{L. monocytogenes} cells invading human hepatocytes (9).

Overall, our data show that EVs have distinct compositions from bacterial cells, a finding providing strong evidence that EVs are specialized structures. In mammalian cells, exosomes have a lipid composition that is distinct from the plasma membrane of the parent cell that is enriched in sphingomyelin and cholesterol (29). Saturated lipids in \textit{L. monocytogenes} cells are necessary in low temperature conditions (27, 28). We found EVs are enriched in unsaturated branching, sphingolipids, and phosphatidylethanolamine, and that EV composition was distinct from other bacterial EVs: EVs from Group A \textit{Streptococcus (44)} displayed an enrichment in phosphatidylcholine and monoacylglycerol. Lipid composition of EVs may vary with the bacterial species and possibly is finely adapted for each survival niche. We note that our experimental approach is not suitable to detect steroid lipids, such as bacterial hopanoids or mammalian cholesterol, but the importance of steroids in lipid bilayer stability warrants a detailed study of the steroid lipid class in EVs. The same pattern emerged for detection of metabolites: EVs are strikingly different from bacterial cells. It is also likely that EV composition will dramatically change in response to the surrounding environment. EVs are enriched in proteins from peptidoglycan synthesis as well as carbohydrates, suggesting a function in the synthesis of the cell wall. It is conceivable that some of these enzymes may be involved in biofilm formation (33). However, we could not detect in our EV protein dataset any of the enzymes reported to be involved in biofilms of \textit{L. monocytogenes}. EVs were enriched in the classical virulence factors of \textit{L. monocytogenes} (36), greatly supporting the paradigm of EVs as virulence bags. The concentrated delivery would allow interactions between all the EV-associated virulence factors into the targeted host, a concerted attack.

We observed in mammalian cells infected with \textit{L. monocytogenes} that LLO associated with vesicular structures whose size was compatible with that of EVs. The observed size difference (150–250 nm in average diameter in infection versus 90 nm average diameter by dynamic light scattering) could be due to different growth conditions, host versus laboratory-rich broth. However, the origin of these EVs could not be ascertained and they could be either bacterial-derived or the result of host membrane sequester by LLO. LLO molecules could hijack mammalian lipid structures via interaction with cholesterol, the only known mammalian cell receptor for LLO (54), which would explain the LLO-vesicular association observed. Regardless of the origin of EVs, the proximity of these EVs to host organelles suggests a vesicular delivery of LLO, preceding insertion into organelle membranes.

The majority of previous studies focused on exogenous addition of EVs and its effects on host cells (31), but unequivocal demonstration of EV secretion within infected host cells had not been shown. Two reports describe synthesis of EVs by \textit{Mycobacterium tuberculosis} (55) and \textit{L. pneumophila} (56), during intracellular residence in macrophages. The infected macrophage cells secreted disparate populations of EVs with one population carrying solely host proteins, whereas the second population carried mostly bacterial products. We developed a protocol to label and observe secretion of EVs into the host compartments. We demonstrated, for the first time, bacterial lipids distal to bacterial cells within the host cytosol indicative of EV release into the host cell, presumably carrying a cargo of virulence factors. Strategies akin to ours will greatly benefit from identification of a lipid species that is specific to bacterial EVs, facilitating a finer distinction between bacterial-host-derived lipids. This proof-of-concept experiment paves the way for discoveries on the interactions of EVs with the host, for example, the molecular mechanism for EV uptake and how EVs release cargo.

In summary, \textit{L. monocytogenes} provides yet another example of a Gram-positive bacterium packaging its toxins into EVs (17, 24, 41). LLO, “the Swiss army knife” (8), is continuously produced to regulate multiple steps of the life cycle of \textit{L. monocytogenes} and we show vesicular LLO is a major component of LLO secreted. The fact that many Gram-positive and cell-walled microbes produce EVs raises fascinating questions in cell biology regarding the mechanisms for transit across cell

\textbf{Lm produces extracellular vesicles with virulence factors}
walls and the packaging of the EV cargo. Our results establish that *L. monocytogenes* release LLO and other virulence packaged in EVs, characterize composition of EVs, and establish production of EVs during mammalian infection. Overall these findings provide fertile ground for future investigations in this rapidly developing field.

**Experimental procedures**

**Bacterial strains and mammalian cell lines**

*L. monocytogenes* strains were maintained at −80 °C (Table 1). All cultures were grown in brain heart infusion (BHI) broth (Difco) with 180 rpm shaking at 37 °C. J774.16 macrophage-like cells were maintained in 10% fetal bovine serum, 10% NCTC-109, 1% nonessential amino acids, in Dulbecco’s modified Eagle’s medium (DMEM catalogue number 10-013-CV). Human MCF-7 breast cancer cells (ATCC HTB22) were maintained in DMEM, supplemented with 10% fetal bovine serum, L-glutamine, nonessential amino acids, and penicillin/streptomycin/amphotericin B (all from Thermo Scientific).

**EVs purification**

Cultures were incubated at 180 rpm shaking at 37 °C for 18 h of growth unless specified otherwise. EVs were purified from planktonic BHI cultures (see diagram in Fig. S1). Cultures were centrifuged at 16,000 × g for 15–20 min to remove cells and subsequently filtered through a 0.2-μm filter to remove remaining cells or debris. Cell-free supernatant (“Supernatant”) was concentrated using a 100-kDa cutoff membrane on an Amicon ultrafiltration device (Millipore). In some experiments the fraction that was able to flow-through the membrane was collected (“flow-through”). Concentrated supernatant was washed with PBS (with Ca2+ and Mg2+), brought down to 10–50 ml while the flow-through was collected as a separate fraction (“PBS Wash 1”). About 10–50 ml remaining on the top of the membrane were then ultracentrifuged at 100,000 × g for 1 h in a Beckman Optima-XL to pellet EVs, supernatant was removed (“Supernatant 2”), washed once with PBS, and re-centrifuged. At the end of this centrifugation, supernatant (“PBS Wash 2”) was collected and pellets (“EVs”) were resuspended in PBS.

**OptiPrep density gradients and protection assays**

EVs were subject to gradient centrifugation with 45% to 15–10% of Optiprep (Sigma) in 10 mM HEPES with 0.85% NaCl, as previously described (57, 58). For protection assays the “100-kDa cone” was subject to 0.1% SDS, pH adjusted to 7.4, or treated with 0.005% trypsin (Corning) at 180 rpm shaking for 1 h at 37 °C, followed by regular purification of EVs. For this experiment Sup2 was concentrated using a 10-kDa Centricron. Protease inhibitors (cOmplete, Roche) were added to all samples after ultracentrifugation steps or after completion of TCA extraction. For a protection assay followed by RBC lysis, trypsin was buffer exchanged to PBS, pH 5.5, to avoid inactivation of LLO (and rLLO) activity due to high pH. Sup or EVs were incubated in equal volumes with buffer-exchanged trypsin for 1 h, 37 °C, and then RBC lysis was assessed.

**Transmission electron microscopy**

Cell and EV samples for TEM were processed as previously described (23, 45). Samples were viewed on a JEOL 100CXII or JEOL 1200EX at 80 kV. For measurements of EV diameter by TEM, a 1.27 correction factor was applied to compensate for the fact that EM diameters are derived from spheres (59). Negative staining was performed after OptiPrep density gradients by adsorbing 10 μl of each fraction to glow-discharged 400-mesh ultrathin carbon-coated grids (EMS CF400-CU-UL) for 2 min, followed by 3 quick rinses of TBS and stained with 1% uranyl acetate and 0.05% tylse. Grids were immediately observed with a Philips CM120 at 80 kV and images were captured with an AMT XR80 high-resolution (16-bit) 8 Mpixel camera.

**SDS-PAGE gels and Western blots**

To generate bacterial whole cell lysates, ~100 ml of culture was spun to obtain bacterial cells, bacteria were disrupted by sonication (4 rounds of 20 s sonication and 30 s rest on ice) (Sonic Disembrator model 100; Fisher Scientific). EVs were isolated from 1-liter cultures and supernatants were obtained from 1 liter of culture, concentrated using 100-kDa ultrafiltration and then further concentrated on a 30-kDa ultrafiltration device (Amicon, Millipore). The equivalent of 1 ml of cells, and 1 liter of EVs were supplemented with 4 × Sample Buffer and 20 μl used for each sample. All buffers used with intact cells and EVs were at mammalian physiological pH (7.0–7.4). Culture BHI broth after ON culture had pH 6.3. Samples were kept on ice at all times, except ultrafiltration was performed at room temperature. For experiments in Fig. S1, EVs were isolated as described previously with aliquots corresponding to 1/2 of the culture taken at each step (see diagram on Fig. 1) and subject to TCA precipitation. Samples were then incubated with 15% TCA with 1/100 of its volume of 2% deoxycholate or with 10% TCA overnight at 4 °C. The resulting pellet was washed 2 times with acetone, resuspended in protein sample buffer (50 mM Tris, 2% SDS, 100 mM DTT, 10% glycerol) in a volume proportional to the equivalent starting culture material (100 μl/1000 ml of starting culture). Equal volumes of samples were run on a 10% BisTris (Nupage, Life Technologies) denaturing gel, according to the manufacturer’s instructions. Positive control was a recombinant ~53 kDa LLO fragment (Abcam, ab83345) and negative control was supernatant from the deletion strain. PI-PLC rabbit antiserum was a kind gift of Dr. Howard Goldfine (60). For the dot-blots, samples were resuspended in PBS, pH 5.5. Samples were transferred into a polyvinylidene difluoride membrane and immunoblotted using anti-LLO polyclonal rabbit IgG (Abcam, ab43018, lotGR56599-8,17, or ab200538, lot GR211194-7,12) in blocking buffer (5% nonfat dry milk in TBS, 1% Tween) overnight at 4 °C. The secondary antibody, goat anti-rabbit IgG-horseradish peroxidase (Southern Biotech) was incubated for 1 h at room temperature. Blots were developed using SuperSignal West Pico Chemoluminescent Substrate (Thermo Scientific). For dot-blot experiments, samples (supernatants, EVs, or OptiPrep fractions) were applied onto the polyvinylidene difluoride membrane using a vacuum dot-blot apparatus and immunoblot was performed as

*Lm produces extracellular vesicles with virulence factors*
**Lm produces extracellular vesicles with virulence factors**

described above. For visualizing proteins, gels were incubated at room temperature in silver stain (Pierce) according to manufacturer’s instructions.

**Erythrocyte (RBC) lysis assay**

Erythrocyte (RBC) lysis was measured as described previously. Briefly sheep RBC (Innovative Research) were resuspended in 1% BSA at 6 × 10⁸ RBC/ml. Each of the purification step fractions was diluted serially in 1:3 dilutions. Assay was performed in total volume of 300 μl and a rLLO concentration of 350 ng/ml. EVs (obtained as described above) were resuspended in PBS or 10 mM dextrose, filter sterilized, and diluted in 1% BSA. Samples were incubated with RBC for 45 min to 1 h at 37 °C, briefly spun to sediment intact RBC, and absorbance of the supernatant was measured at 594 nm.

**Cytotoxicity MTT assay in J774.16 cells**

MTT colorimetric assay was utilized to determine EV cytotoxicity to J774 murine macrophages. EVs were purified from 1 or 2 liters of broth culture and filter (0.22 µm) sterilized. A 96-well plate was seeded with 5 × 10⁴ macrophages per well and incubated overnight at 37 °C. EVs were resuspended in PBS or 10 mm dextrose, filter sterilized, and added to cells in triplicate with a serial dilution of 1:2 in DMEM. The plate was incubated for 4 h at 37 °C. At the end of 4 h, sterile MTT solution (5 mg/ml in PBS) was added to wells and incubated for an additional 2 h at 37 °C. A purple color results from viable macrophages that can reduce MTT to formazan.

**Erythrocyte (RBC) lysis**

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**Dynamic light scattering**

Hydrodynamic diameters of EVs were measured with 90Plus/BI-MASS Multi-Angle Particle Sizing analyzer (Brookhaven Instruments Corp.), as described previously (23, 52).

**Multimomics experimental design and statistical rationale**

For the multimomics experiment *L. monocytogenes* cells were grown and harvested in biological triplicates, which is enough for studying samples derived from established cell cultures (61). Furthermore, we observed that with the recent improvements of the mass spectrometers the technical variability is much smaller than natural biological variability, avoiding the necessity of collecting data for technical replicates. To ensure the performance of the mass spectrometer, quality control samples were run before and after each sample batch, with the quality monitored as previously described (62). All samples were randomized for sample preparation and again for data collection. Enrichment or repletion of molecules in EVs was determined by t test considering two-tailed distribution and equal variance.

**MPLEx**

*L. monocytogenes* cells were lysed by vigorous shaking with 0.1-mm zirconia/silica beads as in 50 mM NH₄HCO₃ buffer, pH 7.8. Then cell lysates and EVs were submitted to MPLEx as previously described (34). Briefly, 5 volumes of −20 °C chloroform/methanol (2:1) were added and the samples incubated for 5 min on ice, before vortexing for 1 min and centrifuging at 12,000 rpm at 4 °C for 10 min. The top and bottom phases, containing metabolites and lipids, respectively, were collected into autosampler vials and dried in a vacuum centrifuge (Labconco, Kansas City, MO). The protein pellet was washed by adding 1 ml of methanol, −20 °C, and centrifuging at 12,000 rpm at 4 °C for 10 min. Then the supernatant was discarded and the precipitated protein was dried in a vacuum centrifuge.

**Proteomic analysis**

Proteins were digested with trypsin as described elsewhere (34) and the resulting peptides were analyzed by LC tandem MS (LC-MS/MS) in NanoAcquity UPLC (Waters) connected to a Q-Exactive mass spectrometer (Thermo Fisher Scientific). Peptides were loaded into trap column (5 cm × 360 μm OD × 150-μm ID-fused silica capillary tubing, Polymicro, Phoenix, AZ; packed with 3.6-μm Aeries C18 particles, Phenomenex, Torrance, CA) and the gradient was performed in a capillary column (70 cm × 360 μm OD × 75 μm ID packed with 3-μm Jupiter C18 stationary phase, Phenomenex): 1–8% solvent B in 2 min, 8–12% B in 18 min, 12–30% B in 55 min, 30–45% B in 22 min, 45–95% B in 3 min, hold for 5 min in 95% B, and returning to 1% B in 10 min. Solvent A was water with 0.1% formic acid and solvent B was acetonitrile containing 0.1% formic acid. Spectra were collected in 400–2000 m/z range with a resolution of 35,000 at m/z 400. MS/MS were performed once on the top 12 most intense ions with ±2 charges using high-collision energy (30% normalized collision energy and resolution of 17,000 at m/z 400) and dynamic exclusion was set to 30 s. Spectra were searched against the *L. monocytogenes* 10304s protein sequences from Uniprot Knowledgebase (2815 sequences downloaded on January 17, 2017) using MaxQuant (v. 1.5.5.1) (63), considering only fully tryptic peptides with two missed cleavages allowed, methionine oxidation as a variable modification, and cysteine carbamidomethylation as fixed modification. Peptide mass tolerance was set at 20 ppm for the first database search and at 4.5 ppm after calibration for the main peptide search. Fragment mass tolerance was set at 20 ppm for both searching rounds. The peptide score threshold was set as the default parameter from MaxQuant and the FDR was limited to 1% in both peptide-spectrum match and protein levels. Quantification was performed using the LFQ function of MaxQuant using a minimum ratio count of 2, minimum number of neighbors of 3, and average number of neighbors of 6. Redundant peptides were assembled into protein groups and quantification of proteins was performed based on unique peptides plus razors. Extracted LFQ intensities were submitted to linear regression and central tendency normalization with InfernoRDN (formerly DAnTE) (64). Function-enrichment analysis was performed using the KEGG annotation (65) and consider-
ing only pathways with more than 3 entries, fold-enrichment ≥1.5, and p value ≤0.05 (Fisher’s exact test).

**Metabolomic analysis**

Polar metabolites from both the EV’s and cell lysates were derivatized and analyzed as described previously (34) using a GC 7890A GC-MS system (Agilent Technologies). Blanks and fatty acid methyl ester samples were included in the analyses for background reference and room temperature calibration purposes, respectively. GC-MS data were processed in Metabolite Detector as previously stated (66). For identification purposes metabolites were identified by matching experimental spectra to a PNNL augmented version of the FiehnLib library (61), containing spectra and validated retention indices of more than 900 metabolites. As for the unidentified metabolites, these were screened against the NIST14 GC-MS Spectral Library by comparing their spectra alone (denoted with “NIST”). The curated dataset of identified metabolites, unidentified features, and their abundances for each sample were then subjected to multivariate data analysis by making use of MetaboAnalyst (62). For identification purposes metabolites were identified by matching experimental spectra to a PNNL augmented version of the FiehnLib library (61), containing spectra and validated retention indices of more than 900 metabolites. As for the unidentified metabolites, these were screened against the NIST14 GC-MS Spectral Library by comparing their spectra alone (denoted with “NIST”). The curated dataset of identified metabolites, unidentified features, and their abundances for each sample were then subjected to multivariate data analysis by making use of MetaboAnalyst (62).

**Lipidomic analysis**

Lipids were analyzed by LC-MS/MS both positive and negative ionization modes in a LTQ ObTrap Velos mass spectrometer (Thermo Fisher Scientific) as described in detail previously by (67). Lipid species were identified using the LIQUID tool (67) followed by manual inspection. Confidently identified lipid species were quantified using MZmine 2 (68) and the peak intensities were normalized by linear regression and central tendency with InfernoRDN.

**Immunoelectron microscopy of L. monocytogenes-infected MCF-7 cells**

Cell lines were plated at 1 × 10^5/ml the day before infection in 2 ml for 6-well plates. *L. monocytogenes* cells from an overnight starvation culture in BHI 37 °C were inoculated in fresh media at 1:100 dilution and grown for 1–2 h in the same conditions. Monolayers of MCF-7 cells were infected with *L. monocytogenes*, WT or LLO-deficient, at the m.o.i. of 1:20 for 3 h before fixation, first in 4% paraformaldehyde (Electron Microscopy Sciences, PA) in 0.25 M HEPES, pH 7.4, for 1 h at room temperature, then in 8% paraformaldehyde in the same buffer overnight at 4 °C. Samples were infiltrated, frozen, and sectioned as previously described (69). The sections were immunolabeled with mouse anti-rabbit LLO antibody (Abcam, ab200538) at 1:20 dilution in PBS, 1% fish skin gelatin, then with IgG antibodies, followed directly by 12-nm protein A-gold particles before examination with a Philips CM120 electron microscope (Eindhoven, the Netherlands) under 80 kV.

**BODIPY-C12 labeling and microscopy of L. monocytogenes-infected MCF-7 cells**

To label *L. monocytogenes* membrane lipids, bacteria were incubated in Vegitone broth (Sigma) with 1 µM BODIPY 558/568-C12 (Thermo Fisher Scientific) for 18 h at 30 °C, to repress LLO expression, and excess dye was washed off. Cells were heat killed by incubating at 70 °C for 10 min (infection and microscopy) or 2 h (EV extraction). For EV extraction cells were then further grown at 37 °C for 6 h. MCF-7 cells were plated and infected in 8-well microscopy µ-slides (ibidi, Germany) in antibiotic-free media and infected (m.o.i. indicated in the figure legends). Gentamicin was added 30 min after the start of infection to repress extracellular growth of bacteria. For time-lapse imaging WT GFP-expressing *L. monocytogenes* was used and imaged 2–4 h post-infection. For immunofluorescence staining, cells were fixed for 15 min with 4% paraformaldehyde (in PBS), pH 7.4, at the indicated time points. Cells were then permeabilized with 0.3% Triton X-100 in PBS overnight at 4 °C. DNA was labeled using Hoechst 33342 (1 µg/ml; Thermo Fisher Scientific). Three-dimensional stacks of infected cells were obtained using a DeltaVision. For time course experiments, images were acquired every 5 s for 2 min, at 37 °C, in an Elite microscope system (GE Healthcare) equipped with a ×60 oil immersion objective. Images were deconvolved (SoftWoRx) and analyzed using ImageJ. Colocalization was performed using the Colormap plug-in (70).

**Statistics**

Statistical tests were performed using GraphPad Prism.

**Author contributions**—C. C., L. C. B., M. C. B., J. E. K., H. M. H., R. P.-R., E. S. N., N. R. B., A. H.-B., I. C., and A. C. conceptualization; C. C., L. C. B., M. C. B., J. E. K., H. M. H., E. S. N., N. R. B., A. H.-B., I. C., and A. C. formal analysis; C. C., G. L., E. S. N., A. H.-B., and A. C. supervision; C. C., E. S. N., and N. R. B. validation; C. C., L. C. B., M. M., R. V., D. F. S., M. C. B., J. E. K., H. M. H., J. R., R. P.-R., E. S. N., N. R. B., A. H.-B., and I. C. investigation; C. C., L. C. B., R. V., D. F. S., M. C. B., J. E. K., H. M. H., R. P.-R., E. S. N., N. R. B., A. H.-B., and I. C. methodology; C. C., L. C. B., E. S. N., and A. C. writing-original draft; C. C., E. S. N., and A. C. project administration; C. C., L. C. B., E. S. N., N. R. B., A. H.-B., I. C., and A. C. writing-review and editing; L. C. B. data curation; M. C. B., J. E. K., H. M. H., E. S. N., N. R. B., A. H.-B., and I. C. visualization; G. L. resources; A. C. funding acquisition.

**Acknowledgments**—We thank Geoffrey Perumal, Benjamin Clark, and Leslie Gunther at the Analytical Imaging Facility, Albert Einstein College of Medicine, for assistance with the transmission EM. Negative staining was performed at the Johns Hopkins Microscopy Facility, and we particularly acknowledge Barbara McClintock. We also thank the technical competence of Kimberley Zichichi from the Electron Microscopy Facility at Yale University for the immuno-EM. We thank Howard Goldfine for the kind gift of PI-PLC antiserum. The MPLEx experiment was performed in the Environmental Molecular Science Laboratory, a United States Department of Energy (DOE) national scientific user facility at the Pacific Northwest National Laboratory (PNNL) in Richland, WA. Battelle operates PNNL for the DOE under contract DE-AC05-76RL01830.

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