Listeria monocytogenes exploits cystic fibrosis transmembrane conductance regulator (CFTR) to escape the phagosome

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Virulence of the intracellular pathogen *Listeria monocytogenes* (*Listeria*) requires escape from the phagosome into the host cytosol, where the bacteria replicate. Phagosomal escape is a multistep process characterized by perforation, which is dependent on the pore-forming toxin listeriolysin O (LLO), followed by rupture. The contribution of host factors to *Listeria* phagosomal escape is incompletely defined. Here we show that the cystic fibrosis transmembrane conductance regulator (CFTR) facilitates *Listeria* cytosolic entry. CFTR inhibition or mutation suppressed *Listeria* vacuolar escape in culture, and inhibition of CFTR in wild-type mice before oral inoculation of *Listeria* markedly decreased systemic infection. We provide evidence that high chloride concentrations may facilitate *Listeria* vacuolar escape by enhancing LLO oligomerization and lytic activity. We propose that CFTR transiently increases phagosomal chloride concentration after infection, potentiating LLO pore formation and vacuole lysis. Our studies suggest that *Listeria* exploits mechanisms of cellular ion homeostasis to escape the phagosome and emphasize host ion-channel function as a key parameter of bacterial virulence.

ion flux | listeriosis | membrane integrity

The bacterial pathogen, *Listeria monocytogenes*, enters mammalian hosts by oral infection, traversing the intestinal barrier to cause systemic disease. Upon entering a host cell, *Listeria* must escape from the pathogen-containing vacuole, or phagosome, into the cytosol to replicate (1). Phagosomal escape is largely mediated by the cholesterol-dependent cytolysin listeriolysin O (LLO), which is essential for *Listeria* virulence. Rupture of the *Listeria*-containing phagosome is a dynamic multistep process. After internalization, an LLO-dependent perforation of the phagosome distinct from rupture occurs, resulting in transient changes in vacuolar pH and calcium (2). Although LLO is the only bacterial factor required for vacuole perforation and escape, the contribution of host factors is less well defined.

Host regulation of the biochemical environment of the phagosome can facilitate bacterial virulence. Phagosome acidification by the vacuolar ATPase is sensed by intracellular pathogens and can increase activity or expression of bacterial virulence determinants, e.g., low pH triggers the PhoQ sensor kinase of *Salmonella enterica*, leading to changes in bacterial physiology and membrane permeability (3, 4). LLO is regulated at multiple levels by the bacterium, but host regulatory mechanisms also modulate LLO function during infection (5). Maximizing LLO activity in the vacuole but not in the cytosol is a critical aspect of the intracellular lifestyle of *Listeria* because LLO mutations with increased expression or pore-forming activity destroy the host cell and decrease virulence (6). LLO pore formation proceeds by oligomerization of cholesterol-bound monomers into a prepore complex, followed by insertion into the lipid bilayer (7). LLO oligomerization increases at low pH, suggesting optimal activity in acidifying phagosomes (8). A recent study also showed regulation of bacterial escape by γ -IFN-induced lysosomal thiol re-

ductase, which reduces the single cysteine of LLO to permit pore formation (9). Thus, *Listeria* relies on host regulation of the phagosome for efficient escape into the cytosol. The phagosomal environment is dynamically modulated by many host proteins, including ion channels and transporters (10). Because ion flux occurs while *Listeria* is in the phagosome, we hypothesized that host ion transport could affect *Listeria* escape by altering activity of host or bacterial factors (2).

Results

Previous studies demonstrated optimal hemolytic activity from supernatants when *Listeria* were grown in 428 mM KCl, and increased oligomerization of recombinant LLO (rLLO) occurs when purified in high-salt buffer, suggesting that high chloride concentrations could alter virulence properties of *Listeria* (8, 11). To determine whether chloride transport aids *Listeria* escape from the phagosome, we used host chloride channel inhibitors during infection. We treated the murine peritoneal macrophage cell line RAW264.7 (RAW) with the anion channel inhibitor diphenylamine-2-carboxylic acid (DPC) at the indicated times and infected with *Listeria*. Infected cells were fixed at 2 h post-infection (pi) and subjected to immunofluorescence staining to measure percentage actin colocalization with intracellular bacteria as an indicator of phagosomal escape (Fig. 1A). DPC treatment significantly decreased *Listeria* escape into the cytosol, even when added at 60 min pi. One DPC-sensitive chloride channel is the cystic fibrosis transmembrane conductance regulator (CFTR) (12). We asked whether CFTR contributed to *Listeria* phagosomal escape by infecting RAW cells in the presence of CFTR inhibitors CFTR(inh)-172 or GlyH-101 (Fig. 1A and Fig. S1A) (13, 14). *Listeria* escape into the cytosol was decreased in cells treated with CFTR inhibitor when added up to 30 min pi, compared with untreated cells. To confirm CFTR expression in RAW macrophages, cell lysates were analyzed for the presence of CFTR protein, which could be detected by immunoprecipitation, in agreement with previous work implicating low endogenous CFTR function in these cells (15) (Fig. 1B). CFTR was also required for efficient *Listeria* escape in the human intestinal epithelial cell line Caco-2 (Fig. S1B). Inhibition of CFTR by CFTR(inh)-172 did not significantly alter bacterial uptake, as indicated by similar cfu at 0.5 h pi (Fig. 1C). CFTR inhibition resulted in decreased numbers of intracellular bacteria in a dose-dependent

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508(ΔF), which results in decreased trafficking of CFTR and associated proteins to the plasma membrane (21). To further define the contribution of CFTR to *Listeria* infection, we isolated primary peritoneal macrophages (pM ϕ) from wild-type mice or littermates homozygous for the CFTR ΔF allele (Fig. 2A). *Listeria* escape was suppressed in the CFTR ΔF pM ϕ compared with wild type, suggesting that CFTR must reach the plasma membrane, where it may be incorporated in phagosomes, to mediate *Listeria* vacuolar escape. We also infected pM ϕ from wild-type or CFTR ΔF mice and analyzed changes in CFU over time (Fig. 2B). Bacterial numbers at 1 h pi were not significantly affected by CFTR mutation, suggesting unimpaired phagocytosis, but intracellular replication in this short-term infection was delayed in CFTR ΔF pM ϕ , consistent with an early defect in escape from the phagosome, as observed in RAW macrophages. The growth phenotype in infected primary CFTR ΔF pM ϕ was more pronounced than in RAW cells with CFTR(inh)-172 likely because of increased bactericidal capacity associated with primary peritoneal macrophages. From these data, we conclude that CFTR dysfunction impedes *Listeria* vacuolar escape in primary peritoneal macrophages.

CFTR expression is most abundant in epithelial cells of the lungs and gastrointestinal tract of mammals (17, 21). As a food-borne pathogen, the natural route of infection of *Listeria* is through the gastrointestinal tract, where *Listeria* could exploit CFTR to infect epithelial cells, a natural bottleneck, followed by systemic dissemination (22). CFTR-deficient mice suffer from intestinal dysfunction and hyperinflammatory responses, com-

plicating interpretation of in vivo *Listeria* infection (23–25). As an alternative, we treated wild-type mice with GlyH-101, which exhibits superior pharmacokinetics in mice compared with CFTR(inh)-172 (26), and had similar effects on *Listeria* escape in cell culture (Fig. S14). At 30 min after i.p. injection with one dose of GlyH-101 or DMSO control, mice were orally infected with 2×10^9 *Listeria*, and systemic dissemination was measured at 18 h pi (Fig. 2C). We observed ≈ 100 -fold less *Listeria* in the liver and spleen of GlyH-101-treated mice compared with controls. The magnitude of the effect of the CFTR inhibitor on *Listeria* infection in mice compared with results in cell culture may reflect the importance of early translocation across the epithelium, which is not modeled in cell culture. In contrast, i.v. *Listeria* infection, which bypasses this bottleneck, revealed no difference in bacterial burden at 18 h pi between mice treated with one dose of DMSO or GlyH-101; thus, a limited window of CFTR inhibition is not sufficient to alter the course of systemic infection (Fig. 2D). These results suggest that functional CFTR is critical for *Listeria* to establish infection via the oral route in a murine model of listeriosis.

Host ion channel function could contribute to phagosomal escape of *Listeria* by altering function of bacterial or host factors. Results from previous studies led us to hypothesize that high NaCl or KCl concentrations could stabilize LLO oligomerization, increasing hemolytic activity (8, 11, 27). We therefore compared oligomerization and hemolytic activity of wild-type rLLO $\pm$ 428 mM KCl. Oligomers of the cholesterol-dependent cytolysin family of toxins are partially resistant to denaturation by heat and detergent, so we heated rLLO-containing lysates $\pm$ 428 mM KCl in

Fig. 2. CFTR potentiates *Listeria* vacuolar escape and infection. (A) Wild type or ΔF primary pM ϕ were untreated or treated for 1 h with CFTR(inh)-172 or DPC and infected with *Listeria*. Cells were fixed at 3 h pi, stained with rhodamine-phalloidin and anti-*Listeria* antibody, and analyzed by epifluorescence microscopy. Percentages represent number of bacteria per 100 colocalized with actin ($n = 3$; ** $P < 0.001$ compared with untreated wild-type pM ϕ). Data shown are representative of at least three independent experiments. (B) pM ϕ were infected as in A, and, at the time points indicated, infected cells were lysed to enumerate CFU. (C) C57BL/6 mice were i.p. injected with Gly-H101 (6 mg/kg) or DMSO and, 30 min later, orally inoculated with 2×10^9 *Listeria* in 0.1 mL of PBS. At 18 h pi, spleens and livers were harvested to enumerate CFU. These data represent pooling of two independent experiments. The limit of detection (dotted line) was 2 CFU per organ (* $P < 0.05$). (D) C57BL/6 mice were i.v. injected with Gly-H101 (6 mg/kg) or DMSO concomitantly with 2×10^6 *Listeria* in 0.05 mL of PBS and analyzed as in C. The limit of detection (dashed line) was 100 CFU per organ.

SDS sample buffer, followed by SDS-agarose gel electrophoresis to permit visualization of high molecular weight protein complexes (Fig. 3A) (28). We observed an increase in higher molecular weight complexes of wild-type rLLO treated with 428 mM KCl, suggesting enhanced oligomerization. To determine the effect of chloride on LLO activity, we briefly treated rLLO with increasing KCl and measured hemolysis of sheep red blood cells (SRBC) at pH 5.5 (Fig. 3B). High salt enhanced hemolytic activity of rLLO but did not alter hemolytic activity of control lysate or Triton X-100 (Fig. 3C). Treatment of rLLO with 428 mM KCl or NaCl increased hemolytic activity, but 428 mM KOAc did not, indicating specificity for the chloride anion (Fig. 3D). If high KCl increased hemolytic activity by promoting LLO oligomerization, we reasoned that KCl might not enhance activity of a gain-of-function LLO mutant that oligomerizes faster than wild-type LLO because of a leucine substitution at threonine 461 (rL461T) (8). High KCl enhanced hemolytic activity of wild-type rLLO but did not increase activity of rL461T (Fig. 3E). These in vitro data suggest that a high chloride environment could potentiate oligomerization and activity of LLO.

Based on our in vitro data, we hypothesized that CFTR-mediated chloride flux enhanced LLO oligomerization within the vacuole, resulting in efficient phagosome rupture. This hypothesis predicts that CFTR would be preferentially required for escape of the wild-type strain over *Listeria* expressing LLO L461T because this mutant protein oligomerizes faster than wild-type LLO (8). We first tested whether faster oligomerization would result in more efficient escape by infecting RAW cells with *Listeria* expressing wild-type LLO or L461T (Fig. 4A). *Listeria* expressing LLO L461T escaped more quickly than wild-type bacteria, although maximal escape was similar. We then treated RAW cells with CFTR(inh)-172 or DPC and measured *Listeria* escape (Fig. 4B). Escape of wild-type *Listeria* was markedly inhibited by CFTR

(inh)-172 and DPC, whereas the strain expressing LLO L461T was only modestly affected by CFTR inhibition. Escape of the L461T mutant was still strongly inhibited by DPC, suggesting that CFTR-independent anion channels may play a distinct role in phagosome rupture. Moreover, the L461T mutant could escape more efficiently from CFTRΔF pMφ than wild-type *Listeria* (Fig. S3). The L461T expressing strain escaped somewhat less from CFTRΔF pMφ than from wild-type pMφ + CFTR inhibitor, suggesting that loss of surface CFTR in the mutant pMφ impacted escape even by the L461T-expressing strain, perhaps because of other proteins that require CFTR for localization or function. These data provide evidence for a model in which CFTR-mediated chloride flux contributes to efficient *Listeria* phagosomal escape by favoring increased LLO oligomerization and hemolytic activity in a compartmentalized manner.

Discussion

Our studies reveal an unanticipated role for CFTR in promoting escape of *Listeria* from the phagosome. CFTR was previously reported to act as a binding determinant for *Pseudomonas aeruginosa* and *Salmonella typhi* (20, 29). In the case of *Listeria* infection, upon CFTR inhibition or mutation, we observed no reproducible differences in bacterial uptake in cultured cells that might suggest a role in mediating recognition or phagocytosis. Our favored model is that CFTR-mediated chloride movement into the phagosome enhances *Listeria* escape. CFTR-dependent chloride flux into phagosomes has been demonstrated in neutrophils and contributes to the substantial production of hypochlorous acid in that compartment (30, 31). Enteroinvasive bacteria can induce chloride secretion by intestinal epithelial cells; similarly, upon *Listeria* infection, host cells could transiently upregulate chloride flux by both CFTR-dependent and -independent mechanisms (32,

Fig. 3. LLO oligomerization and hemolytic activity is enhanced in vitro by high KCl concentration. (A) SDS/agarose gel electrophoresis analysis of lysates followed by immunoblotting using an anti-LLO antibody. (B and C) Hemolytic activity of rLLO lysates at pH 5.5 without (closed triangles) or with (open symbols) increasing KCl. BL21(DE3) lysate (triangles) and Triton X-100 (diamonds) represent negative and positive controls, respectively. (D) Hemolytic activity of rLLO lysates at pH 5.5 in HAB (closed triangles) or HAB with 428 mM KCl (open squares), NaCl (open diamonds), or KOAc (open circles). (E) Hemolytic activity of BL21(DE3) lysates expressing rLLO (triangles) or rL461T (squares) with (open symbols) or without (closed symbols) 428 mM KCl on SRBC at pH 5.5. Results shown in all panels represent at least three independent experiments.

Fig. 4. CFTR function is not required for escape of *Listeria* expressing a gain-of-function LLO allele. (A) RAW macrophages were infected with *Listeria* expressing wild-type LLO or LLO L461T. Cells were fixed at the times indicated pi, stained with rhodamine-phalloidin and anti-*Listeria* antibody, and analyzed by fluorescence microscopy. Percentages represent number of bacteria per 100 colocalized with actin ($n = 3$; $**P < 0.001$ for comparison between strains). (B) RAW cells were untreated or treated for 1 h with CFTR(inh)-172 or DPC, infected with wild-type or the L461T mutant *Listeria*, fixed at 2 h pi, and analyzed as in A ($n = 3$; $P < 0.05$ and $**P < 0.001$, comparing treated with untreated cells or as indicated by the bracket).

33). Transient acute increases in chloride in the lumen of the phagosome would then enhance LLO oligomerization and pore formation in the vacuolar membrane. Although we observed optimal LLO hemolytic activity at 428 mM KCl in vitro, the concentration of chloride in phagosomes is likely to be dynamic, and whether it reaches 428 mM is unclear. A study of elemental concentrations in *Mycobacterium avium*-containing phagosomes within BALB/c macrophages indicated that phagosomal Cl levels were markedly higher than the cytosol or free bacteria (34). It is therefore possible that, in the phagosome, chloride ions may transiently reach high concentrations in a spatially and temporally regulated manner. It is also worth noting that CFTR has many cellular functions, including glutathione flux, which may additionally alter the dynamics of intracellular infection (35). Further, CFTR inhibition or mutation can affect cholesterol homeostasis, with long-term CFTR inhibition decreasing cellular cholesterol, whereas cells with the CFTR Δ F508 mutation exhibit increased cholesterol (36). Because we observe similar escape trends in cells with CFTR inhibition or mutation rather than opposite phenotypes, the effects of CFTR on cholesterol homeostasis are unlikely to be the key parameter affecting *Listeria* escape. CFTR may play an underappreciated role in modulating ionic strength and membrane integrity of the phagosome during host-microbe interactions. Indeed, the physiological function of CFTR in macrophage phagosomes remains poorly understood and is a matter of current debate (37). Studies characterizing the phagosome proteome have revealed the presence of multiple ion channels and transporters (10, 38). Other than the vacuolar ATPase, the contribution of these proteins to phagosome function and microbial infection is largely unknown. Further investigation of the role of ion transport in bacterial infection at the molecular and cellular level is likely to yield insights into key interactions of bacterial pathogens with their host that alter the outcome of infection.

Methods

Antibodies and Reagents. RAW cells were treated with 0.25 mM DPC, 10–25 μ M CFTR(inh)-172, or 50 μ M Gly-H101 unless otherwise indicated. Antibodies and chemical reagents are fully described in *SI Methods*.

Cell Culture, Bacterial Strains, and Infections. RAW cells were grown as previously described (39). The wild-type *Listeria* strain used was 10403S; the LLO-deficient strain (DP-L2161) and LLO L461T strain (DP-L4017) are derivatives of 10403S. rLLO was expressed as described (8). For infections in cell culture, bacteria were grown in brain-heart infusion (BHI) broth at 30 °C overnight.

Host cells were infected at a multiplicity of infection (MOI) of 1 for 30 min, then washed three times with PBS, followed by addition of medium with 10 μ g/mL gentamicin.

Isolation and Culture of Peritoneal Macrophages. *Cftr*(Δ F/ Δ F) mice have a 3-bp (CTT) deletion resulting in loss of a Phe residue corresponding to position 508 of human CFTR (40). *Cftr*(Δ F/WT) breeders were genotyped as described (40). Offspring from *Cftr*(Δ F/WT) breeders were fed a liquid elemental diet containing hydrolyzed milk protein (Peptamen; Nestle Clinical Nutrition) beginning at 10 d (41). To isolate peritoneal macrophages, *Cftr*(Δ F/ Δ F) mice or littermates were euthanized, and peritoneal lavage collected for culture in DMEM + 20% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1% 2-mercaptoethanol, and 30% L929 conditioned medium.

SDS Gel Electrophoresis, Immunoprecipitation, and Immunoblotting. SDS/agarose gel electrophoresis was performed as previously described (28). Samples were prepared by incubating 5 μ L of reduced rLLO or rL461T *Escherichia coli* lysate in 428 mM KCl or PBS for 5 min at 37 °C. SRBC (0.5%) were added and incubated for 5 min at 37 °C before adding 5 μ L of 5 $\times$ SDS sample buffer, boiling, and running electrophoresis on a 1% SDS-agarose gel, followed by immunoblotting. Immunoprecipitation of CFTR from 2×10^6 RAW cells using 1.2 μ g of M3A7 and 1.2 μ g of 24-1 anti-CFTR antibodies was performed as previously described (42). Samples were run on 6% SDS-PAGE gels and immunoblotted with anti-CFTR antibody clone 24-1.

Microscopy. An Olympus BX60 microscope with epifluorescence was used to quantitate *Listeria* colocalized with F-actin as described (43). To determine pH of *Listeria*-containing phagosomes, RAW cells were incubated with CFTR(inh)-172 or DMSO for 1 h and infected with SNARF-1-labeled DP-L2161 with 0.75 mg/mL 10-kDa Oregon Green/dextran. After 5 min, cells were rinsed and imaged in Ringer's buffer with 10 μ M CFTR(inh)-172 or DMSO. Images were collected as described (2). The pH of *Listeria*-containing vacuoles was determined by dividing average fluorescence (AF) of the endosome excited at 500 nm by AF of the endosome excited at 435 nm. This ratio was used to calculate endosomal pH based on a standard curve of 492 nm/435 nm ratio plotted against pH.

Animal Infections. For oral infections, food was removed 4 h before infection of female C57BL/6J mice (12–16 wk; Jackson Laboratory). At 30 min before infection, mice were fed 50 μ L of 10% NaHCO₃ orally and injected i.p. with 6 mg/kg Gly-H101 or DMSO in PBS. At 30 min after inhibitor treatment, mice were orally gavaged with 2×10^9 midlog *Listeria* in 0.1 mL of PBS. For i.v. infections, mice were injected with 2×10^4 midlog *Listeria* and either 6 mg/kg Gly-H101 or DMSO in a total volume of 50 μ L. For both routes of infection, mice were euthanized at 18 h pi, and organs were homogenized in 0.2% Nonidet P-40 in PBS and plated on LB-agar to enumerate cfu.

Hemolytic Assay. Hemolytic assays were performed as described (43). Lysates were used for the hemolytic assay rather than purified protein, as previously published purification procedures included high salt elution. Lysates were diluted 1:10 in hemolytic assay buffer (HAB; 35 mM sodium phosphate, 125 mM NaCl, and 0.1 mg/mL BSA, pH 5.5) and reduced for 1 h with 5 mM DTT. Lysates were serially diluted in HAB with indicated concentrations of KCl, NaCl, or KOAc in 96-well plates before adding 1% SRBC (43).

Statistical Analysis. Data were analyzed by using Student's unpaired *t* test. For in vivo experiments, outlier box and quartile box plots were used to identify outliers with the definition of up-outlier > upper quartile + 1.5

(interquartile range) and down-outlier < lower quartile – 1.5(interquartile range). For all experiments, error bars indicate SD.

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