A Role for Taok2 in Listeria monocytogenes Vacuolar Escape

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The bacterial pathogen Listeria monocytogenes invades host cells, ruptures the internalization vacuole, and reaches the cytosol for replication. A high-content small interfering RNA (siRNA) microscopy screen allowed us to identify epithelial cell factors involved in L. monocytogenes vacuolar rupture, including the serine/threonine kinase Taok2. Kinase activity inhibition using a specific drug validated a role for Taok2 in favoring L. monocytogenes cytoplasmic access. Furthermore, we showed that Taok2 recruitment to L. monocytogenes vacuoles requires the presence of pore-forming toxin listeriolysin O. Overall, our study identified the first set of host factors modulating L. monocytogenes vacuolar rupture and cytoplasmic access in epithelial cells.

Keywords. Listeria monocytogenes; Taok2; STE1-like kinase; vacuolar escape; siRNA screen.

The gram-positive pathogen Listeria monocytogenes is a major model microorganism to understand bacterial subversion of host functions in the context of disease. L. monocytogenes is a facultative intracellular pathogen able to invade mammalian cells. Upon interaction of bacterial surface molecules (InlA, InlB) with host receptors (E-cadherin, Met), signaling cascades are triggered to promote actin rearrangements and bacterial engulfment. Rupture of the internalization vacuole allows L. monocytogenes to reach the cytosol, where it replicates [1].

Three L. monocytogenes exotoxins control vacuolar escape: a phosphatidylinositol-specific phospholipase C (PlcA), a broad-range phospholipase C (PlcB), and the cholesterol-dependent cytotoxin listeriolysin O (LLO) [1]. In phagocytic cells, host factors have also been recognized as modulators of L. monocytogenes vacuolar rupture, including γ-interferon–inducible lysosomal thiol reductase (GILT), cystic fibrosis transmembrane conductance regulator (CFTR), and calpain [2]. Whether host factors in nonphagocytic cells control L. monocytogenes vacuolar escape has not been explored so far.

In the present study, we used a high-content microscopy approach to identify epithelial host cell components specifically required for efficient rupture of the L. monocytogenes-containing vacuole.

METHODS

Bacterial and Mammalian Growth Conditions, Plasmids, Drugs, and Infections

Bacterial strains were: L. monocytogenes EGDe PrfA* (BUG 3057), EGDe PrfA*Δlac (BUG 3358), EGDe PrfA*ΔplcAΔplcB (BUG3620), EGDe PrfA*ΔhlyΔplcAΔplcB (BUG 3648), L. innocuaΔlac/ΔIfS (BUG 3614) [3], Shigella flexneri wild-type strain M90T expressing the adhesin AfaI [4], Francisella tularensis subsp. novicida strain U112 (or F. novicida wild type), and an isogenic strain with the Francisella pathogenicity island (APFI) deleted [5]. The plasmid encoding YFP-CBD, a yellow fluorescent protein (YFP) chimera protein of the cell wall binding domain (CBD) from the Listeria phage endolysin Ply118, has been described [6]. The plasmids encoding actin-mOrange and galectin-3-enhanced green fluorescent protein (EGFP) were used as markers for ruffle formation and vacuole lysis by S. flexneri [4]. Human HeLa cells (American Type Culture Collection CCL-2) were transfected with 1.5 μg of the plasmids encoding YFP-CBD, actin-mOrange, or galectin-3-EGFP using Lipofectamine LTX for 24 hours. Bacterial strains and HeLa cells were grown as described [3]. The specific Taok2 inhibitor SW172006 (Chembridge) [7] was added to HeLa cells at 50 μM for 24 hours before infection.

L. monocytogenes infections were performed as described [8, 9] to: (1) assess the efficiency of L. monocytogenes EGDe PrfA* invasion into HeLa cells treated with dimethyl sulfoxide (DMSO) or SW172006 at 1 hour post infection (hpi); (2) identify cytosolic bacteria labeled with CBD following vacuolar escape after Taok2 knockdown using small interfering RNA (siRNA) or after Taok2 kinase activity inhibition using SW172006 at 1 hpi (100 cells were counted in 3 representative fields to estimate the number of CBD-labeled bacteria); (3) perform immunofluorescence studies to analyze...
the distribution of Taok2 in HeLa cells at 30 minutes post infection (mpi); and (4) quantify the number of intracellular bacteria that escape from vacuoles and are associated with actin at 5 hpi after Taok2 siRNA knockdown. The multiplicity of infection (MOI) used was 25 (EGDe PrfA*), 50 (EGDe PrfA* ΔlplcΔbpbc), or 125 (EGDe PrfA* ΔhlyΔlplcΔbpbc). *S. flexneri* infections were performed as described [3]. HeLa cells transfected with scrambled or Taok2 siRNAs were infected (37°C, MOI 50), and live imaging of *S. flexneri* vacuolar rupture was monitored and quantified as the time interval between ruffle formation and appearance of a galectin-3 signal around the bacteria. Images were recorded in a Nikon Ti-E microscope (×20 objective) with a time lapse of 2 minutes for 2 hours. *F. novicida* infections were performed using a MOI of 1000. Bacterial entry synchronization was performed by a 5-minute centrifugation at 1000 rpm and plates incubated at 37°C. After 1 hour plates were incubated at 37°C in fresh medium supplemented with gentamycin for 24 hours. Bacterial multiplication was monitored by plating cell lysates on chocolate agar plates. Each experiment was conducted at least twice in triplicates.

**siRNA Library Screen**

Four distinct oligos were used for each target gene. The screen was performed as described [4]. Each 96-well screen plate contained: 8 positive control wells transfected with scrambled siRNA and challenged with *L. monocytogenes* EGDe PrfA*ΔlplcΔbpbc* [3]; 8 negative control wells transfected with scrambled siRNA and challenged with invasive *L. innocua* ΔlplcΔbpbc* (which invades host cells but remains trapped in vacuoles); and 4 replicates for each siRNA target infected with *L. monocytogenes* EGDe PrfA*ΔlplcΔbpbc*. Reverse siRNA transfection of HeLa cells was performed using Lipofectamine RNAi Max at a 10-nM siRNA concentration for 72 hours. The CCF4 assay for *L. monocytogenes* vacuole rupture quantification and differential staining of extracellular versus total *L. monocytogenes* for cell entry quantification at 1 hpi was performed as described [3, 7]. Images were acquired on a confocal microscope Opera QEOS (PerkinElmer) as described [3, 7]. The strictly standardized mean difference (SSMD) statistical test was used for quality control analysis and for hit scoring [3, 7].

**Image Acquisition**

Bacterial cytoplasmic access was determined by actin comet tail formation using phalloidin [9]. Briefly, extracellular *L. monocytogenes* were labeled with a primary goat anti-*L. monocytogenes* serum and with a secondary chicken anti-goat Alexa 647. Cells were permeabilized using 0.1% Triton X-100 and total *L. monocytogenes* were labeled with the same primary antibody and a secondary chicken anti-goat Alexa 488. Actin was labeled with phalloidin Alexa 546. DNA was stained with Hoechst 33342.

**Statistical Analysis**

Statistical analysis was performed with GraphPad Prism v6. Differences between control and experimental groups were evaluated by 2-tailed unpaired *t* tests or 1-way ANOVA followed by a Dunnett multiple comparison test. *P < .05* was considered as significant.

**RESULTS**

**Identification of Host Factors Controlling *L. monocytogenes* Vacuolar Escape in Epithelial Cells**

To identify epithelial host cell factors specifically involved in *L. monocytogenes* vacuolar escape, we exploited a previous genome-wide siRNA screen that distinguished host genes modulating the cytoplasmic accumulation of the *L. monocytogenes* secreted molecule InlB (an event requiring *L. monocytogenes* cell invasion, vacuolar escape, and cytoplasmic proliferation) [10]. We siRNA-inactivated the 165 most significant hits identified in this previous screen and performed a novel high-content microscopy screen in which we combined: (1) a CCF4 fluorescence resonance energy transfer (FRET)-based assay to measure *L. monocytogenes* vacuolar rupture and cytoplasmic access [3, 7], and (2) a differential immunofluorescence staining to distinguish extracellular versus total *L. monocytogenes* [3, 7].

Fifty siRNA targeted genes were identified as extremely strong hits affecting *L. monocytogenes* vacuolar escape (Figure 1A–C and Supplementary Table 1). Some of these genes displayed either inhibition of bacterial entry into host cells (PODXL, SLC12A4, USE1, and TTYC19) or induced strong cytotoxicity (PPP3R2) (Supplementary Table 1). However, we also identified genes that had a bona fide effect on *L. monocytogenes* cytoplasmic access and did not affect bacterial entry or cell integrity (EED, KATS, MTR, SYK, TKAO2, DPEP1, and PIGL) (Figure 1A–C and Supplementary Table 1).
Taok2 Controls *L. monocytogenes* Vacuolar Escape

We selected Taok2 for further validation based on its strong phenotype and on reports showing its localization to vesicular compartments and its implication in surface receptor endocytosis [11, 12]. Taok2 depletion efficiency was verified by quantitative reverse transcription polymerase chain reaction...
Figure 2. Taok2 siRNA knockdown or inhibition of its kinase activity by the chemical compound SW172006 impairs efficient rupture of *Listeria monocytogenes*-containing vacuoles. A, Taok2 knockdown by siRNA (left) or inhibition of its kinase activity (right) results in reduced cytoplasmic access. HeLa cells were transfected with the YFP-CBD of the *L. monocytogenes* phage endolysin Ply118, which identifies cytosolic bacteria shortly after escape. The red line indicates the mean. Results were from 100 cells counted in 3 representative fields to estimate the number of CBD-labeled bacteria. Statistical analysis: 2-tailed unpaired *t* tests. **P < .01, ****P < .0001. B and C, Taok2 accumulation
We next analyzed the distribution of Taok2 in L. monocytogenes-infected HeLa cells. Using anti-Taok2 antibodies, we observed rare events of Taok2 association with the L. monocytogenes EGDe PrfA* strain (not shown), suggesting that Taok2 recruitment to L. monocytogenes vacuoles is transient. We hypothesized that by delaying L. monocytogenes vacuolar escape, we might increase the probability of detecting Taok2 recruitment. Indeed, when using an EGDe strain lacking both phospholipases C and displays delayed vacuolar escape [14], we were able to observe a clearer association of Taok2 with the L. monocytogenes vacuole and which plays a more direct role on facilitating vacuolar rupture. Our results indicate that Taok2 association with L. monocytogenes vacuoles is dependent on LLO production suggest either that specific membrane damages produced by LLO are the signal triggering Taok2 vacuolar recruitment or, alternatively, that Taok2 recruitment is driven by other L. monocytogenes components being exposed to the cytosolic environment upon effective vacuolar rupture mediated by LLO. The molecular cascade of events downstream of Taok2 that leads to facilitated L. monocytogenes vacuolar rupture remains to be identified.

From Taok2, we also found other host genes with a strong effect on L. monocytogenes vacuolar rupture and no effect on entry. These results propose a multifaceted mechanism
where several host pathways are hijacked by *L. monocytogenes* to control vacuolar rupture.

**Supplementary Data**

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

**Notes**

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