Live Imaging Reveals *Listeria* Hijacking of E-Cadherin Recycling as It Crosses the Intestinal Barrier

**Highlights**

- *Listeria* translocates across organoid goblet cells in an InlA-Ecad-dependent manner
- Real-time imaging reveals *Listeria* transcytosis
- Ecad endocytosis is involved in *Listeria* entry and microtubules in translocation
- *Listeria* hijacks Rab11-dependent Ecad recycling for transcytosis

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**In Brief**

Kim et al. develop an intraluminal microinjection-based intestinal organoid model of *Listeria* infection, which allows real-time imaging. *Listeria* specifically translocates through goblet cells, on which each receptor Ecad is luminally exposed. *Listeria* transcytoses within its membrane internalization vacuole, hijacking Rab11-mediated Ecad recycling.

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Live Imaging Reveals Listeria Hijacking of E-Cadherin Recycling as It Crosses the Intestinal Barrier

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SUMMARY

Listeria monocytogenes is a foodborne bacterial pathogen that causes human listeriosis, a severe systemic infection.1 Its translocation across the intestinal epithelium is mediated by the interaction of internalin (InlA), a Listeria surface protein, with its host-species-specific receptor E-cadherin (Ecad).2–5 It occurs through goblet cells, on which Ecad is luminally accessible,6 via an unknown mechanism. In the absence of cell lines recapitulating this phenotype in vitro, we developed an ex vivo experimental system, based on the intraluminal microinjection of Listeria in untreated, pharmacologically treated, and genetically modified intestinal organoids. Using both live light-sheet microscopy and confocal imaging, we show that Listeria translocates through goblet cells within a membrane vacuole in an InlA- and microtubule-dependent manner. As Ecad undergoes constant apical-basal recycling,7,8 we hypothesized that Lm may transit through goblet cells by hijacking Ecad recycling pathway. Indeed, Listeria is stuck at goblet cell apex when Ecad endocytosis is blocked and remains trapped intracellularly at the basolateral pole of goblet cells when Rab11-dependent Ecad recycling is compromised. Together, these results show that Listeria, upon docking onto its luminally accessible receptor Ecad, hijacks its recycling pathway to be transferred by transcytosis across goblet cells. Live imaging of host-pathogen interactions in organoids is a promising approach to dissect their underlying cell and molecular biology.

RESULTS AND DISCUSSION

In order to decipher the cell biology mechanisms of Listeria translocation across the intestinal epithelium, we developed a genetically amenable experimental system permissive to InlA-Ecad-dependent trans-epithelial crossing. Deciphering the detailed cell biology mechanisms of Listeria translocation across the intestinal epithelium in vivo would require interfering with cellular pathways that may disrupt its barrier function. Additionally, it would be extremely challenging to capture Listeria translocation in real time across intestinal villus epithelium, given its rarity and intestinal peristalsis. Furthermore, no adherent cell, including human colonic cell lines T84, HT29, and Caco-2, displays an apical-basal polarization and apical accessibility of Ecad, which are both critical for Listeria InlA-Ecad-dependent trans-epithelial crossing to occur (unpublished data). We therefore set up an ex vivo experimental system based on the microinjection of Listeria in the lumen of intestinal organoids. Intestinal organoids derive clonally from intestinal stem cells, which give rise to a fully differentiated, polarized intestinal epithelium that forms a so-called “minigut” centered by a lumen and contains differentiated intestinal cell subtypes.9 Intestinal organoids are genetically amenable,10–12 can be subjected to pharmacological interventions,14 and can also be imaged both fixed and alive.9,15

Intestinal Organoids Contain Goblet Cells

We generated intestinal organoids from the small intestine of knockin E16P (E16P KI) mice, in which the endogenous mouse Ecad is punctually modified to express a proline at position 16 of the mature protein in place of a glutamic acid. This modification enables this “humanized” mouse Ecad to interact with InlA and mediate Listeria internalization (see STAR Methods).1,4,16 As expected,17 E16P organoids grown in Matrigel exhibit a fully mature apical-basal polarity and display cell subtype heterogeneity. Organoids intestinal stem cells differentiate into enterocytes (villin+), enteroendocrine cells (chromogranin A+), goblet cells (wheat germ agglutinin [WGA]+/lysozyme–), and Paneth cells (WGA–/lysozyme+; Figure S1A). Additionally, and as previously reported,17 intestinal organoids do not contain M cells (GP2+; Figure S1A). As Listeria crosses the intestinal barrier via goblet cells in vivo, it is important to unambiguously identify this cell subtype in our experimental model. Goblet cells are
A. Organoids passage → Microinjection → 1 to 16 hours → Fix & stain → Live Imaging

B. Exterior

C. Number of infected cells

D. Time after microinjection (h:mins:sec)

E. Non-permeabilized

F. Permeabilized

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mucus-secreting cells with a characteristic goblet-shaped cell morphology, in which nucleus location does not align with neighboring enterocytes. Additionally, goblet cells can be identified by immunolabeling of the Muc2 mucin, a major mucus component, following Carnoy hydrophobic fixation. However, Muc2 labeling cannot be applied to our experimental system: Carnoy fixation damages plastic wells in which Matrigel-embedded organoids are located and, more importantly, renders Matrigel opaque. Therefore, we used WGA, which labels mucus by binding to sialic acid and N-acetyl-glucosaminyl carbohydrate residues on mature, modified mucins. WGA-positive cells were co-labeled for cytokeratin-18, which is specifically expressed in goblet cells in the gut (Figure S1B). Of note, paraformaldehyde (PFA) fixation can dissolve mucus, resulting in bona fide WGA-negative goblet cells. Therefore, we used the following criteria to identify goblet cells: (1) WGA labeling, (2) goblet-shaped cellular morphology, and (3) misaligned nucleus relative to neighboring cells. When WGA-negative cells strictly met the last two conditions, we also considered them as goblet cells (e.g., in Figure S1D, right).

**InIA Is Necessary and Sufficient for *Listeria* Translocation across Intestinal Organoid Goblet Cells**

We microinjected 5 × 10⁵ colony-forming units (CFUs) of wild-type (WT) *Lm* into the lumen of mature organoids and investigated bacteria interactions with the intestinal epithelium (Figure 1A). Confocal imaging of intestinal organoids fixed 1 h after microinjection allowed the detection of bacteria both inside and underneath the basolateral pole of goblet cells (Figure 1B; Video S1). Quantification studies 16 h post-infection revealed that bacteria were all located exterior to microinjected organoids (Figure 1C). Of note, the microinjection procedure and/or presence of bacteria in the organoid lumen did not modify the proportion of WGA+ cells (10.87% ± 3.07%) compared to non-injected organoids (9.56% ± 2.62%; Figure S1C). Consistent with our previous in vivo studies in humanized mice permissive to InIA-Ecad interaction, this phenotype was strictly InIA dependent. In contrast, it was independent of listeriolysin O (LLO) and ActA, which mediate *Lm* escape from its internalization vacuole and actin-based motility, respectively (Figure 1C). *L*(*inIA*) is derived from *L*. *innocua*, a non-pathogenic *Listeria* species devoid of *L. monocytogenes* virulence factors, which has been genetically modified to express InIA, and enters into cells in an *Ecad*-dependent manner. Upon microinjection in intestinal organoids lumen, *L*(*inIA*) was also located extracellularly at basolateral pole of goblet cells, as wild-type *Lm* (Figures 1C, S1D, and S1E; Video S1). This indicates that *Listeria* crossing of the intestinal epithelium does not require escape from the vacuole and actin-based motility. In some cases, bacteria underneath goblet cells were surrounded with WGA-labeled material, implying that bacteria can translocate with mucus (Figure S1E). Of note, bacteria underneath goblet cells cannot replicate or move within Matrigel, as it contains gentamicin and is bactericidal. Together, these results show that *Listeria* microinjection in the lumen of intestinal organoids allows to faithfully recapitulate *Listeria* translocation across the intestinal epithelium in vivo. These results also confirm that InIA is necessary and sufficient to mediate *Listeria* translocation across goblet cells, although LLO and ActA are dispensable, and justify the use of *L*(*inIA*) for further experiments, in order to minimize the potential cytotoxicity of LLO to microinjected organoids.

**Real-Time Imaging of *Listeria* Transcytosis across Intestinal Organoid Epithelium**

Despite converging evidence indicating that *Listeria* transcytoses through goblet cells in vivo, live imaging has not been performed to prove it actually occurs, i.e., translocation of a bacterium surrounded by its internalization vacuole. We therefore set up experimental conditions to image in real-time translocation of Li-GFP(InIA) across intestinal organoids, in which cell membranes are constitutively red fluorescent (mtd-Tmt; E16P KI). *Listeria* translocation across the intestinal epithelium is a rare event in vivo, as only 3% to 4% of intestinal villi are infected in 45-min-long intestinal ligated loop assays (unpublished data). Moreover, *Lm* translocation across the small intestinal...

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**Figure 1. *Ex Vivo* Intestinal Organoid Model of *Listeria* Infection**

(A) Experimental scheme and example of *Listeria* microinjection (details in the STAR Methods).
(B) Example images of *Listeria* translocation (optical section). *Lm* within a goblet cell is shown, fixed 1 h post-microinjection (left panel; goblet cell marked with an arrow). *Lm* basolaterally extracellular beneath a goblet cell is shown (right panel; goblet cells are marked with arrows). See also Video S1. Translocated bacteria were distinguished from the total bacteria by consecutive immunostaining, first against *Lm* without permeabilization followed by tissue permeabilization and labeling (STAR Methods). Scale bars, 10 μm.
(C) Quantification of *Listeria* translocation. Top: number of cells associated with bacteria either intracellularly or basolaterally extracellularly per organoid is shown. Bottom: number of translocated bacteria per organoid is shown. Counts were performed in 12 organoids for each condition. Two-way ANOVA test; NS, not significant; ***p < 0.0001. See also Figures S1D and S1E and Video S1.
(D) Light-sheet live imaging at indicated time point (top, maximum intensity projection [MIP]; bottom, optical section). Within 12 min, bacteria-containing vesicle reaches the basolateral side of the cells and then exits from the cell in the following time points. Intracellular *Listeria* is surrounded with membrane tomato (arrow). See also Video S3.
(E) Left: scheme depicting detection of luminally accessible Ecad from side and top view. Organoids were fixed, embedded, sectioned to open the lumen, and immunolabeled from the luminal side without permeabilization. Ecad in the sectioned plane is exposed and thus accessible without permeabilization and stained throughout the cutting plane (marked with green on the drawing). Right: 3D view of opened organoids from the side (top left) and top (bottom left) is shown. Enlargement of boxed area on the left 3D-reconstructed organoids containing goblet cells expressing luminally accessible Ecad is shown (center and right). Scale bars, 10 μm. See also Video S3.
(F) Left: scheme depicting detection Ecad from side and top view. Organoids were fixed, embedded, sectioned to open the lumen, permeabilized, and immunolabeled from the luminal side. Total Ecad is stained (marked with green on the drawing). Right: 3D view of opened organoids from the side (top left) and top (bottom left) is shown. Enlargement of boxed area on the left 3D-reconstructed organoids containing goblet cell is shown (center and right). Scale bars, 10 μm. See also Video S3.
Figure 2. Endocytosis and Microtubule Dynamics Are Required for Bacterial Translocation
(A) Top: experimental scheme of microinjection. Bottom: confocal imaging of permeabilized, whole-mount stained organoids treated with indicated inhibitors is shown. In colchicine-treated organoids, epithelium displayed more metaphase-blocked cells (+, enlarged), indicating that colchicine treatment worked.
(B) Quantification of number of cells associated with bacteria. NS, not significant.

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epithelium occurs within 30 min in vivo. The rarity of translocation events requires that entire organoids are scanned to be captured, and this may exceed the time needed for bacterial translocation when using classical confocal imaging. Additionally, laser power has to be minimal to preserve the fluorescent signal of individual bacteria and cell membrane over prolonged imaging. Furthermore, imaging has to be performed at the adequate spatial resolution to track micron-sized bacteria within intestinal organoids, the diameter of which ranges from hundreds to thousands of microns. To accommodate all these experimental requirements, light-sheet microscopy was used, which can image intestinal organoids at least thirty times faster than a regular confocal microscope with minimal phototoxicity. 5 × 10^3 CFUs of Li-GFP(InlA) were microinjected intraluminally into mtd-Tmt; E16P KI organoids. Real-time imaging revealed that a bacterium reached basolateral pole surrounded by a cell membrane in less than 12 min. During the following 36 min, the bacterium remained at the basolateral pole of the cell, surrounded by mtd-Tmt signal, and then exited from the cell basolaterally (Figure 1D; Video S2). This allowed to image for the first time Listeria transcytosis across the intestinal epithelium directly and unambiguously.

**Ecad Is Luminally Accessible on Goblet Cells in Intestinal Organoids**

InlA-dependent translocation of Listeria specifically through goblet cell (Figures 1B, 1C, S1D, and S1E) suggests that translocation is mediated by InlA interaction with luminally accessible Ecad on goblet cells, as it has been observed in vivo. To assess luminal accessibility of Ecad in organoids, we applied an Ecad antibody directed against its ectodomain (ECCD-2) to the accessible luminal side of fixed and sectioned organoids (Figure 1E). To stain only accessible Ecad, we performed surface immuno-labeling without cell permeabilization. Junctional proteins in the sectioned plane are exposed, accessible without permeabilization, and are therefore also labeled. However, junctional proteins located below the cutting plane cannot be labeled in the absence of cell permeabilization, unless they are inherently luminaly accessible (Figures 1E and 1F). As expected, given the specific location of InlA-Ecad Listeria translocation (Figure 1C), luminally accessible Ecad was detected only on the apical side of mucus-expelling goblet cells in non-permeabilized organoids (Figure 1E; Video S3). In contrast, in permeabilized organoids, Ecad was detected at adherens junctions (AJs), down to the basolateral membrane in all cells, regardless of the subtype (Figure 1F; Video S3). Together, these results indicate that the cells on which Ecad is luminally accessible in intestinal organoids are mucus-expelling goblet cells and that bacteria located beneath the basal pole of goblet cells after intraluminal microinjection have undergone InlA-Ecad-mediated transcytosis. This establishes the specificity of this ex vivo experimental system to study the cell biology of Listeria InlA-Ecad translocation across the intestinal epithelium.

**Endocytosis and Microtubule Dynamics Are Required for Listeria Translocation through Goblet Cells**

Previous in vivo investigations have shown that InlA-Ecad-dependent Listeria crossing of the intestinal barrier depends on microtubule and the exocyotic machinery. We therefore hypothesized that Listeria, upon its docking on luminally accessible Ecad on goblet cells, hijacks Ecad recycling pathway to cross the intestinal epithelial barrier, from its dynamin-mediated endocytosis and endosomal trafficking along microtubules to its Rab11-dependent release at the cell basolateral pole. Ecad recycling has not been studied in goblet cells, but it is also expected to involve its endocytosis at the apical pole and basolateral recycling in a microtubule-dependent manner. We therefore tested this hypothesis by dissecting the role of Ecad recycling pathway on Listeria transcytosis.

In polarized differentiated cells, Ecad, which forms AJs, is endocytosed in a clathrin-dependent manner. To inhibit endosomes are excised from the plasma membrane by the guanosine triphosphatase (GTPase) dynamin. Ecad is trafficked in a microtubule-dependent manner and recycled to the basolateral membrane. To first inhibit Ecad endocytosis, we used dynasore, a dynamin inhibitor that prevents the fission of clathrin- and caveolin-dependent endocytic vacuoles. To inhibit microtubule-based Ecad trafficking, we used colchicine, which blocks microtubule polymerization. In organoids treated 2 h with dynasore, cytosolic endocytic Ecad punctae were drastically reduced (Figure S2C), as previously reported in cultured cells. In presence of colchicine, more cells with metaphase-blocked mitoses were observed (Figure 2A), as expected, indicating that both drugs are active in our system. Nevertheless, the overall morphology and polarity of the organoids were comparable to non-treated controls (Figure 2A), and we observed no difference in total bacterial association to cells (either apically, intracellularly, or basolaterally; Figure 2B). Yet there was a significant decrease of extracellular translocated bacteria in dynasore- and colchicine-treated organoids, relative to control organoids (Figure 2C). Consistent with a similar total bacterial association to cells in all conditions, accessible Ecad was detected similarly on goblet cells in dynasore- and colchicine-treated conditions (Figures 2D and S2; Video S4), as in untreated organoids (Figure 1E). This suggests that the interaction between Listeria and its receptor Ecad is not impaired in dynasore- and colchicine-treated organoids, whereas Listeria transcellular transport is blocked within goblet cells. To examine where bacteria were trapped in cells, we investigated their location in three distinct compartments: apically associated to the cell membrane, intracellular, and extracellular at their basal pole. In dynasore-treated organoids, bacteria were mostly apically associated (Figures 2C...
and 2E, dynasore, and S3; Video S4). These bacteria were likely trapped in elongated invaginations of the plasma membrane, as observed both in Drosophila and mammalian epithelial cells when dynamin is inhibited.\textsuperscript{35–37} In colchicine-treated organoids, where microtubule dynamics is inhibited, intracellular bacteria were located in the median part of the cells (Figures 2C, 2E, colchicine, and S3; Video S4). In all experimental conditions, intracellular bacteria were surrounded with Tomato red-labeled membrane, indicating that bacteria were within a vacuole (Figure 2E). Together, these results show that, upon InLA-mediated Listeria docking to Ecad, Ecad endocytosis is required to complete bacterial internalization into goblet cells, and microtubules dynamics is required for bacterial trans-epithelial transit in a vacuole.

**Rab11 Is Required to Complete Listeria Intestinal Translocation through Goblet Cells**

We next investigated whether recycling of Ecad endosomes is required for Listeria InLA-dependent translocation across the intestinal epithelium. Ecad is recycled and trafficked via Rab11-positive recycling endosomes, which recruit the exocytosis machinery.\textsuperscript{8,26,27,36,38} Rab11 is also involved in basolateral sorting of newly synthesized Ecad in polarized mammalian cells \textit{in vitro}.\textsuperscript{38} The kinetics of Rab11-based trafficking is around 30 min,\textsuperscript{40,41} which fits with our live imaging of Listeria crossing the epithelium (Figure 1D; Video S2). Therefore, to perturb Ecad recycling, we targeted Rab11. Because Rab11-null mutations are embryonically lethal in mice\textsuperscript{42,43} and constitutive knockout of Rab11 may be detrimental for the development of intestinal organoids, we generated lentivirus-transduced organoids in which a dominant-negative (DN) and myc-tagged version of Rab11 can be induced by doxycycline. As Rab7-dependent Ecad degradation pathway\textsuperscript{44} is not expected to be involved in bacterial translocation, we also generated lentivirus-transduced organoids in which a dominant-negative Rab7 can be induced to use them as negative controls. To visualize successful transduction and transcription induction, we used a bi-cistronic reporter system where an internal ribosome entry site (IRES) is placed downstream of each DN mutant and upstream of a mCerulean coding sequence (Figures 3A and 3B). Organoids were grown and maintained in presence of antibiotics to select for transduced cells. When organoids were properly formed and mature, doxycycline was added 12–16 h before microinjection to induce the transcription of dominant-negative Rab11 and Rab7 variants Rab11DN and Rab7DN, respectively (Figure 4A). Organoids expressing Rab11DN exhibited cytosolic Ecad puncta that accumulated at basolateral side of epithelial cells compared to control organoids, consistent with a blockade of Ecad release when Rab11 is non-functional (Figure S2C). Induced expression of Rab7DN caused general enrichment of cytosolic Ecad (Figure S2C), and large Ecad aggregates formed throughout the apical-basal axis (Figure S2C), suggesting that Ecad degradation is affected when Rab7 is non-functional.\textsuperscript{44} However, inducing Rab11 and Rab7 dominant-negative proteins for a short period of time (less than 24 h) did not detectably impair epithelium morphology (Figure 3C) and Ecad luminal accessibility compared to WT organoids (Figures 4B and S2; Video S5).

Microinjection experiments revealed that induction of Rab7DN did not affect bacterial translocation through goblet cells. Listeria was found extracellular at the basolateral pole of goblet cells of Rab7DN-induced organoids, to the same degree as in transduced but non-induced control organoids (NI). This shows that bacteria complete translocation normally within goblet cells in presence of Rab7DN (Figures 4C–4E, Rab7DN, and S3; Video SS). In contrast, in mycRab11DN-induced transduced organoids, a significant decrease of translocated bacteria beneath goblet cells was observed, together with a corresponding significant increase of intracellular bacteria. In all experimental conditions, intracellular bacteria were mostly located at the basolateral pole of goblet cells (Figures 4C–4E, Rab11DN, and S3; Video S5). This was also the case for wild-type Listeria microinjected in mycRab11DN-induced organoids, even 16 h post-microinjection (Figures S2D–S2F). Note that prolonged exposure to doxycycline might have prevented Listeria vacuolar escape and cell-to-cell spread at this late time point. Taken together, these data show that Rab11, in contrast to Rab7, is required for the release of bacteria from the basal pole of goblet cells, highlighting that Listeria hijacks Rab11-dependent Ecad recycling for trans-epithelial translocation via goblet cells.

**Organoid as a Model for Real-Time Investigations of Host-Pathogen Interactions**

Here, we have developed an \textit{ex vivo} minigut system in which Listeria crosses the intestinal barrier by transcytosis through goblet cells as it does in \textit{vivo}.\textsuperscript{1} We have harnessed the power of this experimental system to directly image, in real time, the trans-epithelial translocation of a microbial pathogen and to dissect the underlying cell biology and molecular mechanisms. Listeria crossing of the intestinal barrier relies on the specific interaction of InLA with luminaly accessible Ecad at the apical pole of goblet cells. We have shown that (1) bacterial Ecad-dependent internalization in goblet cells requires dynamin-mediated endocytosis, (2) bacteria transit through these cells in a microtubule-dependent manner as \textit{in vivo},\textsuperscript{1} and (3) Rab11 is required for the release of Listeria at the basolateral pole of goblet cells, thereby showing that Listeria hijacks E-cadherin recycling pathway to cross the intestinal barrier (Figure S3). Although organoids have been used to investigate infection with human norovirus and SARS-CoV-2,\textsuperscript{45,46} and the interactions of enteropathogens, such as \textit{Salmonella enterica}\textsuperscript{47} and \textit{Cryptosporidium parvum} with the intestinal epithelium,\textsuperscript{48} here, we have microinjected for the first time microbes in the lumen of...
Listeria Translocation Requires Functional Rab11, but Not Rab7

(A) Experimental scheme of microinjection. The organoids were grown in presence of antibiotics (penicillin-streptomycin, geneticin, and puromycin), and doxycycline was added 12–16 h before the microinjection. Induction of mCer was detected by eyes with Zeiss Filter set 38 HE (excitation BP 470/40; beamsplitter (legend continued on next page)

Figure 4. Listeria Translocation Requires Functional Rab11, but Not Rab7

(A) Experimental scheme of microinjection. The organoids were grown in presence of antibiotics (penicillin-streptomycin, geneticin, and puromycin), and doxycycline was added 12–16 h before the microinjection. Induction of mCer was detected by eyes with Zeiss Filter set 38 HE (excitation BP 470/40; beamsplitter
intestinal organoids, imaged in real time microbial crossing of the intestinal barrier, and deciphered the underlying molecular mechanisms of microbial translocation. This novel ex vivo system of infection opens a wide range of opportunities to study pathogen interactions with host barriers in a direct manner, in contrast to what has been done so far. Additionally, this study also pioneers the use of genetically modified inducible organoids to address the cellular and molecular mechanisms of host-pathogen interactions in a tissue context. Rab11 has been shown to be necessary for Ecad recycling in mammalian cells and in vivo in *Drosophila melanogaster.* Moreover, Rab11 is required for basolateral trafficking of newly synthesized Ecad in polarized Madin-Darby Canine Kidney (MDCK) cells. However, the cellular trafficking of Ecad from its apical to basolateral pole from either side of adherens junctions had not been thoroughly investigated in a tissue context to our knowledge. It has been previously shown in non-polarized epithelial intestinal Caco-2 and trophoblastic JEG3 cell lines that, upon binding of InlA to Ecad, the plasma membrane is remodeling in an actin-dependent manner and leads to caveolin- and clathrin-dependent bacterial endocytosis. In cultured epithelial cells, *Listeria* internalization occurs mainly at the edge of cell islets, where cells are non-polarized and Ecad is broadly accessible. Once internalized into these non-polarized cells, *Lm* escapes from the vacuole in an LLO-dependent manner and propels itself through ActA in the cytosol. Here, we have shown that, in polarized differentiated goblet cells in a tissue context, the initial step of internalization through accessible Ecad is also dynamin dependent. However, after completing bacterial internalization, *Lm* is rapidly transferred and released to the basolateral pole of goblet cells, owing to the rapid Rab11-dependent recycling of Ecad. This may protect invading bacteria from epithelial innate immune responses and favor their dissemination in host tissues. Live imaging of host-pathogen interactions in organoids is a promising approach to dissect their underlying cell and molecular biology.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.11.041.

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**AUTHOR CONTRIBUTIONS**

M. Lecuit conceived the project, designed the study, directed the research, participated in the analysis of the data, and edited the manuscript. M.K. designed and performed experiments, analyzed data, and wrote the manuscript. C.F. designed and performed some experiments and analyzed some of the data. M. Lavina provided technical help. O.D. participated in the supervision of the study, data analysis, and writing and editing of the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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FT 495; emission BP 525/50; **Figure 3B.** Induced organoids were selected and microinjected, incubated for 2 h (DN proteins still being induced), and followed by fixation and imaging.

(B) Luminally accessible Ecad of the sectioned organoids induced with indicated DN proteins. Accessible Ecad staining in XY, XZ, and YZ planes from indicated fixation and imaging.

(C) Optical sections from 3D reconstruction of microinjected organoids induced for indicated DN proteins.

(E) Quantification of number of bacteria per organoid in indicated location. Experiments were repeated 2 to 3 times. The results combine all experiments. Counts were performed in 19 non-induced, 18 Rab7DN, and 24 Rab11DN organoids. The results for WT organoids are those from Figures 2B and 2C. Kruskal-Wallis test; comparison to WT; "p < 0.05; "*"p < 0.01. See also **Figure S3.**
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# STAR Methods

## Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies and fluorescent dyes | | |
| Rat anti-E-cad (ECCD-2) | Takara | #M108 |
| Rabbit anti Rab7 | Sigma | R4779; RRID:AB_477460 |
| Rabbit antisera against *Listeria monocytogenes* | | 55 |
| Mouse IgG1 anti-myc (71D10) | Cell signaling | #2278; RRID:AB_490778 |
| WGA-conjugated with Alexa Fluor 647 | Invitrogen | W32466 |
| Hoechst 33342 | Invitrogen | H3570 |
| Goat anti-Rat Alexa Fluor 546 | Invitrogen | #A-11081; RRID:AB_141738 |
| Goat anti-Rabbit Alexa Fluor 405 | Invitrogen | #A-31556; RRID:AB_221605 |
| Goat anti-Mouse Alexa Fluor 405 | Invitrogen | #A-31553; RRID:AB_221604 |
| Bacterial and Virus Strains | | 56 |
| *Lm* strain EGD | BUG600 |
| *EGD ΔinlA* | BUG 947 |
| *EGD Δhly* | BUG 2132 |
| *EGD ΔactA* | BUG 2140 |
| *Li WT* | BUG 499 |
| *Li (ΔInlA+)* | BUG 1489 |
| *Li-GFP (ΔInlA+)* | This paper | MBHL 366 |
| rLV.EF1.Tet3G-9 | Takara | 631311 |
| Chemicals, Peptides, and Recombinant Proteins | | 56 |
| N2 Supplement | GIBCO Invitrogen | #17502048 |
| B27 Supplement | GIBCO Invitrogen | #17504044 |
| N-Acetylcysteine | Sigma Aldrich | #A9165-5G |
| Human recombinant R-spondin 1 (final 500 ng/ml) | R&D systems | #4645-RS250 |
| Mouse recombinant Noggin (final 100 ng/ml) | Peprotech | #250-38-20ug |
| Mouse recombinant EGF (final 50 ng/ml) | Invitrogen | #PMG8044 |
| Y-27632 (final 10 uM) | Sigma Aldrich | #Y0503-1MG |
| Mouse recombinant Wnt3a (final 100 ng/ml) | Millipore | #GF160 |
| Nicotinamide (final 10 mM) | Sigma Aldrich | #N6363-100 g |
| CHIR99021 (final 10 uM) | Stemgent | #248040004 |
| Doxycycline (final 2 ug/ml) | Takara | #631311 |
| Dynasore (final 80 uM) | Sigma | 324410-10MG |
| Colchicine (final 10 ug/ml) | Sigma | C3915 |
| DATP (final 10 uM) | Stemgent | #04-0041 |
| IL-13 (final 20 ng/ml) | R&D systems | #413-ML-005 |
| TransDux MAX™ | System Bioscience | LV860A-1 |
| Matrigel | Corning | 356231 |
| Cell recovery solution | BD | 354253 |
| Advanced DMEM/F12 | GIBCO Invitrogen | 12634010 |
| DMEM/F12 phenol red-free | GIBCO Invitrogen | 21041025 |
| GlutaMAX | GIBCO Invitrogen | 35050038 |
| 1M HEPES | GIBCO Invitrogen | 15830056 |
| Penicillin-streptomycin | GIBCO Invitrogen | 15140163 |
| Puromycin (final 1 ug/ml) | Sigma | 540411-25MG |
| Neomycin (Geneticin, final 1X) | GIBCO Invitrogen | 10138031 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Marc Lecuit (marc.lecuit@pasteur.fr).

Materials Availability
All plasmids generated in this study are available upon request.

Data and Code Availability
This study did not generate/analyze datasets or codes.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacteria
For Figures 1B and 1C, Lm strain EGD (BUG 600) and isogenic deletion mutants ΔinA (BUG 94725), Δhly (BUG 213257), ΔactA (BUG 214025), Li WT (BUG 499), Li-expressing InLA (Li (InLA+), BUG 148925) were used. We generated Li-expressing both InLA and GFP in tandem under the phyer promoter of pAD vector, separated by the terminator sequence (MBHL 366). Bacteria were transformed by electroporation. GFP expression was confirmed by fluorescent microscopy, function of InLA was confirmed by in vitro invasion assay using mouse fibroblast L2071 expressing human E-cadherin.

Mice
E16P KI mice were generated in the laboratory. They were crossed with mT/mG mice to generate mtd-Tmt; E16P KI mice. Animal experiments were performed according to the Institut Pasteur guidelines for laboratory animals’ husbandry and in compliance with...
European regulation 2010/63 EU. They were approved by the ethical committee CETEA/CMEA No. 89 of Institut Pasteur under the number DHA180011.

**Organoids**

Intestinal organoids were generated and cultured from the crypts recovered from small intestines of 6- to 8-week old KIE16P and mtd-Tmt; E16P KI mice using EDTA dissociation method. They were grown in ENR medium (Advanced DMEM/F12 with EGF (50 ng/ml), Noggin (100 ng/ml) and R-spondin1 (500 ng/ml)).

**Cells**

HEK293T cells (ATCC CRL-11268) were grown and passaged in DMEM containing 2% glutamine, 10% Tet-system approved FBS (Takara # 631106) and penicillin-streptomycin.

**METHOD DETAILS**

**Organoid microinjection**

Mature organoids growing in ENR medium were mechanically dissociated and passed into the gridded, low-bottom iBidi-injection plate (Clinisciences # 80156). Result of Figures 1B and 1C was obtained with ENR medium while all others were obtained using the following condition: Organoids were passed in injection plates with 2:1 ratio of ENR and 50% of L-WRN cell-conditioned media (made with Tet System Approved FBS, Takara # 631106) for 1-2 days to have a round center to facilitate microinjection. When the organoids produced spherical centers, the organoids were washed several times with pre-warmed Advanced DMEM/F12 to remove the serum and excessive growth factors, replaced with either ENR or 5% L-WRN conditioned media for minimal 1 day. A day before the microinjection, 10 μM DAPT and 20 μg/ml IL-13 were supplemented overnight to partially enrich the goblet cells and to induce mucus expulsion from the existing goblet cells. Final concentration of 80 μM dynasore or 10 μg/ml colchicine were applied 2 hours prior to microinjection. Figures 4A and 4C, final concentration of 2 μg/ml doxycycline was added to the media 12-16 hours before the microinjection. Microinjection was performed only to organoids verified to have successful induction. Results of Figures 1B and 1C were obtained by fixing 1 hour and 16 hours, respectively, after microinjection. The incubation period post microinjection of the other experiments was optimized to 2 hours followed by fixation to reduce the damage of the organoids. Drug/doxycycline treatment was maintained during this period. For live imaging, immediately after microinjection, organoids were re-sampled to the imaging chamber of light sheet microscope Zeiss Z1.

Overnight culture of bacteria was diluted (1/200) and grown until O.D 0.8, washed at least 3 times in PBS and collected as a final volume of 100 μl in DMEM/F12 phenol red-free medium. Microinjection was performed with Eppendorf InjectMan and FemtoJet system as reported using glass micropipettes injection needle (Vitromed # V-INJ-S3-35).

**Accessible Ecad detection**

Organoids were passed on the 8-well Lab-Tek plates and supplied with the media as for the microinjection: first with 2:1 ratio of ENR and 50% L-WRN cell conditioned media to grow the spherical center, followed by washing and either returned to ENR media or supplied with 5% L-WRN media for at least 1 day. A day before the fixation, organoids were treated at a final concentration of 10 μM DAPT and 20 ng/ml IL-13 overnight to induce mucus expulsion from the existing goblet cells. Organoids were fixed with 4% PFA at 4°C overnight. For the experiments in Figure 2D, final concentration of 80 μM dynasore and 10 μg/ml colchicine were applied 2 hours after fixation. Experiment of Figure 4B, final concentration of 2 μg/ml doxycycline was added 12-16 hours before fixation. When the organoids were fixed, PFA was removed, organoids were washed and 6% low-melting agarose was poured to the wells. Solidified wells were recovered, sectioned in 150-200 μm thickness with a vibratome (Micro HM 650V, Thermo Fisher Scientific). Sections containing opened organoids were blocked in 3% BSA in PBS and stained as indicated below.

**Immunofluorescence**

Accessible Ecad (Figures 1E, 2C, and 4B): Organoids were fixed with 4% PFA at 4°C overnight followed by washing and blocking with 3% BSA in PBS, overnight at room temperature. Primary and secondary antibodies were applied for 1 hour at room temperature.

Whole-mount staining: Organoids were fixed with 4% PFA for 30 min – 1 hour at room temperature followed by washing, blocking/ permeabilizing for 2 hours either in 5% goat serum with 1% Triton X-100 or in 3% BSA with 1% Triton X-100 in PBS. Primary antibodies were applied at 4°C overnight followed by washing and stained with secondary antibodies for 2 hours at room temperature or 4°C overnight.

Distinguishing intracellular versus translocated bacteria (Figure 1B): Organoids were fixed with 4% PFA at 37°C for 1 hour, washed and blocked without triton for 2 hours at room temperature. Rabbit antisera against Listeria monocytogenes (R1155) was added for 2 hours at room temperature, followed by washing and secondary antibody for 1 hour at room temperature to stain extra-organoid bacteria. Then microinjected organoids were permeabilized with 1% Triton X-100 and stained with primary antibody at 4°C overnight followed by washing and secondary antibody for 2 hours at room temperature.

The following antibodies were used: anti-Ecad (Ecd2, Takara #M108, 1:350), anti-myc (71D10, Cell Signaling # 2278, 1:500), anti-Rab7 (Sigma, #R4779, 1:200), WGA conjugated with alexa 647 (Invitrogen, whole mount 1:300, section 1:1000) and Hoechst 33342 (Invitrogen 1:5000). Secondary antibodies include goat anti-rat conjugated with alexa 546, goat anti-mouse conjugated with alexa...
405, and goat anti-rabbit conjugated with alexa 405 (all Invitrogen 1:500). To identify the goblet cells, the following criteria were used, as the Muc2 labeling method following Carnoy fixation and paraffin embedding is not compatible with our system, rendering the matrigel opaque as well as breaking the plate: WGA+, typical goblet-shaped cellular morphology including the opening of the apical area and the nucleus misaligned with neighboring cells. If the cells meet 2 or more conditions, we regarded them as goblet cells.

**Imaging**

Images were acquired either by confocal microscope (fixed image, upright Zeiss LSM 700 equipped with a water Plan-Apochromat 40x/1.0 DIC M27 objective & inverted Zeiss LSM 710 equipped with an oil Plan-Apochromat 40x/1.3 DIC M27 objective) or light sheet microscope (live image, Zeiss Z.1 equipped with a water Plan-Apochromat 40x/1.0 DIC objective). For live imaging, imaging chamber was maintained at 37°C temperature, 5% CO2 and supplemented with ENR media made with phenol red-free DMEM/F12. Three-dimension reconstruction was performed using Arivis Vision 4D. 3.0.1 software. For Figure 1D and Video S2, the image was denoised using median filter (radius 1) followed by background correction.

**Rab11DN and Rab7DN vector construction**

Rab11DN and Rab7DN were generated by mutagenesis PCR from the pCMV-intron myc Rab11WT (Addgene #46785) and pCMV-SPORT6-Rab7, respectively.

Primers used for Rab11DN are: 5' - GTGTTGGAAAGAACAACCTCCTGTCTCGATTTA-3' & 5' - GACAGGAGGTTGTTCTTTCCAACACCAGAATC-3'. Primers used for Rab7DN are: 5' - CTGGTGTTGGAAAGAACTCTCTCTCATGAACCAG – 3' & 5' - CTGGTTCATGAGTTCTTTCCAACACCAG – 3'. DN constructs were cloned into the multiple cloning site I (MCSI) and mCerulean sequence was cloned into the MCS II of Tet-On 3G Inducible Expression System (Bicistronic Version, pTRE3G-IRES, Takara # 631166)

**Lentivirus production and concentration**

Lentivirus particle containing tet-activator was purchased from Takara (#631311). Lentivirus containing inducible mycRab11DN-mCer and Rab7DN-mCer constructs were produced using calcium phosphate transfection in HEK293T cells (Takara # 631312, user protocol). 2nd generation lentiviral plasmids psPAX2 and pMD2.G were used (Addgene #12260, #12259). Lentivirus was concentrated with PEG-it™ (System Bioscience, # LV810A-1, user protocol).

**Organoids transduction**

Organoids were transduced with lentivirus using the method modified from Maru et al., 2016 and Van Lidth de Jeude et al., 2015. Briefly, 1st generation of organoids from the crypts were passed with ENR medium containing Wnt3a, CHIR99021 and Nicotinamide to enrich stem cell population. 2-3 days later when the organoids display spheroid morphology with few dead cells in the lumen, organoids were mechanically broken down and trypsinized to single cells. Cells were washed and supplemented with the Lentivirus concentrate in a final concentration of 1X ENR media containing Wnt 3a, CHIR99021, Nicotinamide, TransDuxMAX™ with enhancer. Cells and virus mixture were incubated at 37°C water bath for around 1 hour, distributed to a matrigel-coated 24 well plates, incubated in the 37°C incubator for overnight. The next day, as live cells settle on the coated matrigel, supernatant containing virus and dead cell debris was carefully removed and fresh matrigel was added to form a sandwich having the live cells in the center of the two layers of the matrigel. ENR medium containing Wnt3a, CHIR99021 and Nicotinamide (without TransDuxMAX™) was supplied for 2 more days. A final concentration of 1 μg/ml puromycin and 100 μg/ml geneticin (neomycin) were added to select for the successful transduction with pTRE3G-DN mutant-IRES;mCerulean and tet-activator, respectively, until the transduced stem cells form small visible organoids. Media was returned to regular ENR media containing three antibiotics (pen-strep, puromycin and geneticin) and transduced organoids were cultured in presence of the three antibiotics throughout.

**Quantification and Statistical Analysis**

**Statistical Analysis**

All statistical analysis has been performed using Prism 8 (Graphpad). Details for statistical tests used can be found in figure legends, including the number of replicates performed and number of organoids analyzed for each condition and p value.