

Adhesion to the host cell surface is sufficient to mediate *Listeria monocytogenes* entry into epithelial cells

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ABSTRACT The intestinal epithelium is the first physiological barrier breached by the Gram-positive facultative pathogen *Listeria monocytogenes* during an in vivo infection. *Listeria monocytogenes* binds to the epithelial host cell receptor E-cadherin, which mediates a physical link between the bacterium and filamentous actin (F-actin). However, the importance of anchoring the bacterium to F-actin through E-cadherin for bacterial invasion has not been tested directly in epithelial cells. Here we demonstrate that depleting α E-catenin, which indirectly links E-cadherin to F-actin, did not decrease *L. monocytogenes* invasion of epithelial cells in tissue culture. Instead, invasion increased due to increased bacterial adhesion to epithelial monolayers with compromised cell–cell junctions. Furthermore, expression of a mutant E-cadherin lacking the intracellular domain was sufficient for efficient *L. monocytogenes* invasion of epithelial cells. Importantly, direct biotin-mediated binding of bacteria to surface lipids in the plasma membrane of host epithelial cells was sufficient for uptake. Our results indicate that the only requirement for *L. monocytogenes* invasion of epithelial cells is adhesion to the host cell surface, and that E-cadherin–mediated coupling of the bacterium to F-actin is not required.

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INTRODUCTION

The pathogenic Gram-positive bacterium *Listeria monocytogenes* can cause severe food poisoning, which can lead to meningitis in immunocompromised individuals and newborns and spontaneous abortions in pregnant women (de Noordhout et al., 2014). *Listeria*

monocytogenes has a diverse repertoire of virulence factors that allow it to invade and survive inside phagocytic and nonphagocytic cells, such as epithelial cells lining the gut lumen (Mengaud et al., 1996), endothelial cells lining the interior of blood vessels

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Author contributions: M.R. performed experiments with A431D phosphomutant cell lines; N.C. and F.E.O. performed strain array experiments; P.R. performed structured illumination microscopy; F.E.O. performed all other experiments. M.G., J.B., and K.S. generated and validated the $\Delta\alpha$ E-catenin MDCK cell line. W.S.L. and P.L. generated the $\Delta actA/\Delta inA$ *L. monocytogenes* strain. F.E.O. and J.A.T. conceived and designed all experiments. F.E.O. and J.A.T. wrote the manuscript with input from W.J.N. All authors read and commented on the final version of the manuscript.

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Abbreviations used: ActA, actin assembly-inducing protein; BSA, bovine serum albumin; Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; DAPI, 4',6'-diamidino-2-phenylindole, dihydrochloride; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor; EGTA, ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; F-actin, filamentous actin; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; G-actin, globular actin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; inlA, internalin A; inlB, internalin B; LLO, listeriolysin O; MDCK, Madin-Darby canine kidney; mTagRFP, monomeric red fluorescent protein; NNI, nearest neighbor index; PBS, phosphate-buffered saline; PDMS, polydimethylsiloxane; PE, phosphatidylethanolamine.

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(Drevets et al., 1995; Rengarajan et al., 2016), and hepatocytes (Dramsai et al., 1995; Gaillard et al., 1996). The pathogenicity of *L. monocytogenes* depends on its colonization of the host gut, which is required for dissemination of bacteria to distant organs such as the placenta (Bakardjiev et al., 2005) and the brain (Brouwer et al., 2006). Thus characterizing the molecular requirements for *L. monocytogenes* entry into epithelial cells is important for understanding how this bacterial pathogen breaches physiological and cellular barriers to cause infection in vivo.

Listeria monocytogenes uses a variety of bacterial proteins called internalins to invade nonphagocytic epithelial cells. Different members of this protein family may interact with each other to either synergize or antagonize invasion, depending on the specific host cell type (Bergmann et al., 2002). Internalin B and internalin A contribute to invasion in multiple cell types and have been well characterized. Internalin B, encoded by the gene *inlB*, is a surface protein that activates the host cell growth factor receptor c-Met and boosts dynamin-mediated endocytosis to promote bacterial internalization (Shen et al., 2000; Veiga and Cossart, 2005; Pentecost et al., 2010). Internalin A, encoded by *inlA*, is a bacterial cell wall-anchored protein with a leucine-rich repeat domain (Gaillard et al., 1991) that

binds to the surface of intestinal epithelial cells via E-cadherin (Mengaud et al., 1996). E-cadherin is a transmembrane protein component of the adherens junction that provides cell–cell adhesion and structural integrity to epithelial tissue via homotypic binding interactions between E-cadherin molecules expressed on neighboring cells (Hartsock and Nelson, 2008). The mechanism by which internalin A/E-cadherin can act to mediate internalization of the 2- μ m-long rod-shaped bacterium has been widely presumed to require cytoskeletal rearrangements (Pizarro-Cerdá et al., 2012). Therefore the cytoplasmic tail of E-cadherin, which binds β -catenin and α E-catenin to physically link E-cadherin to F-actin under force (Buckley et al., 2014), is also thought to be required (Lecuit et al., 2000).

In previously published work, mouse fibroblasts were used to demonstrate the importance of the E-cadherin/catenin interaction for *Listeria* invasion (Lecuit et al., 2000). Nonpathogenic *Listeria innocua* expressing internalin A could not invade fibroblasts in the absence of E-cadherin. Ectopic expression of full length E-cadherin in fibroblasts resulted in increased bacterial uptake, but expression of a truncated E-cadherin missing the cytoplasmic β -catenin-binding domain, and hence linkage to F-actin through α E-catenin,

resulted in a sevenfold decrease in bacterial uptake. These data suggested that *Listeria* invasion of nonphagocytic cells might require a physical link between the E-cadherin/catenin complex and F-actin for efficient bacterial uptake (Figure 1A). While the interaction between internalin A and E-cadherin is critical for *L. monocytogenes* invasion of epithelial cells in vitro (Mengaud et al., 1996) and in vivo (Wollert et al., 2007), the requirement for the entire E-cadherin/catenin/F-actin complex for *L. monocytogenes* invasion has not been tested directly in epithelial cells.

Here we show that bacterial adhesion to the surface of the host cell is the minimal requirement for *L. monocytogenes* invasion in epithelial cells. Depleting α E-catenin or expressing truncated E-cadherin unable to interact with F-actin, including a lipid-anchored E-cadherin extracellular domain, had only mild effects on the efficiency of bacterial entry in epithelial cells. In contrast, artificial adhesion of *L. monocytogenes* to plasma membrane phospholipids was sufficient to mediate invasion. Therefore we propose that, in addition to an α E-catenin/F-actin-dependent invasion mechanism, *L. monocytogenes* can use alternative modes of entry into epithelial cells that do not require direct anchoring of the host cell surface receptor to the internal cytoskeleton.

RESULTS

An intact E-cadherin/ β -catenin/ α E-catenin/F-actin complex is dispensable for *L. monocytogenes* invasion in MDCK cells

To test whether E-cadherin/catenin-independent mechanisms could mediate *L. monocytogenes* invasion in epithelial cells,

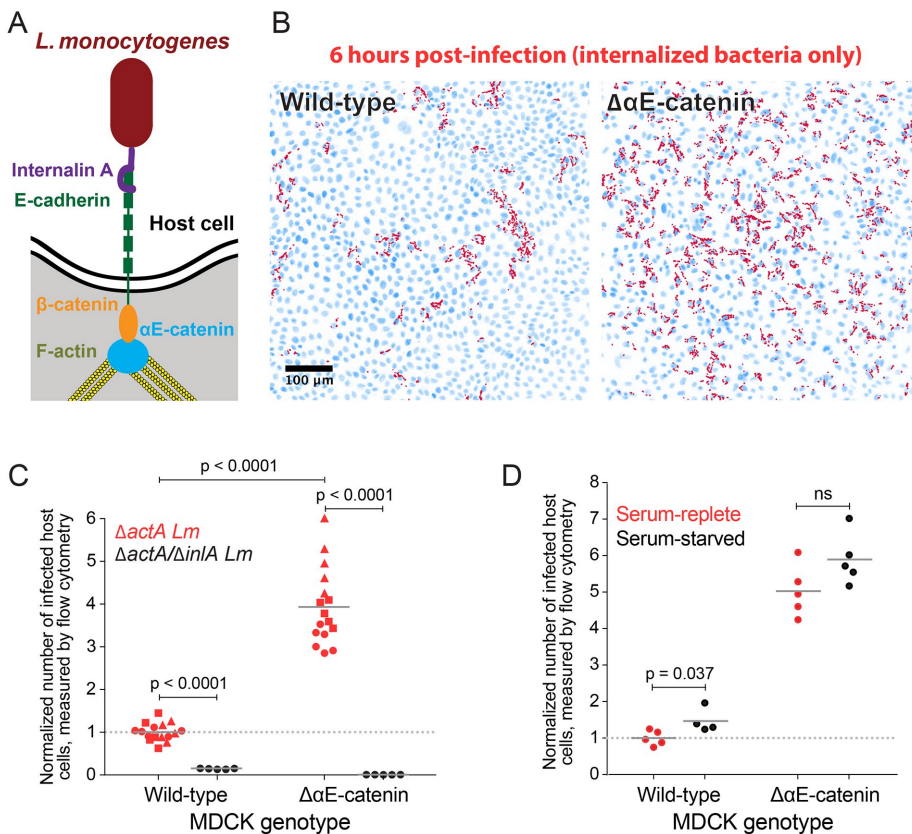


FIGURE 1: *Listeria monocytogenes* invasion in MDCK cells does not require α E-catenin. (A) “Catenin-centric” model of *L. monocytogenes* invasion of nonphagocytic cells. (B) Fluorescence micrographs showing nuclei (4',6-diamidino-2-phenylindole, dihydrochloride [DAPI], blue) and internalized bacteria (mTagRFP, red) in wild-type (left) and $\Delta\alpha$ E-catenin (right) MDCK monolayers. (C) Flow cytometry data quantifying the number of *L. monocytogenes*-containing wild-type and $\Delta\alpha$ E-catenin MDCK cells. Data were normalized to 1 for wild-type MDCK cells infected with $\Delta actA$ *L. monocytogenes* for each experiment and pooled from three independent experiments (each experiment is depicted by different symbols). (D) Flow cytometry data quantifying the effect of serum on *L. monocytogenes* invasion of wild-type and $\Delta\alpha$ E-catenin MDCK cells. For both C and D, experiments were each carried out with five replicates per condition. Each data point represents an individual replicate where 10,000 host cells were analyzed. Horizontal bars indicate the mean. *p* values were calculated with the Wilcoxon rank sum test.

we modified interactions in the E-cadherin/catenin/F-actin complex in Madin-Darby canine kidney (MDCK) epithelial cells. The current model of *L. monocytogenes* invasion predicts that $\Delta\alpha$ E-catenin MDCK cells should be protected against bacterial invasion because a physical link between E-cadherin and the actin cytoskeleton is missing. CRISPR/Cas9 gene editing was used to delete the α E-catenin gene in MDCK cells (Supplemental Figure S1A), which resulted in disruption of normal cell–cell adhesion (Supplemental Figure S1B and Supplemental Videos 1 and 2) even though levels of E-cadherin and β -catenin were similar to those in wild-type MDCK cells (Supplemental Figure S1A).

Wild-type and $\Delta\alpha$ E-catenin MDCK cells were infected with Δ actA *L. monocytogenes* with a chromosomally integrated open reading frame of the monomeric red fluorescent protein from *Entacmaea quadricolor* (mTagRFP) under the ActA promoter (Zeldovich *et al.*, 2011), which becomes transcriptionally active when the bacterium enters the host cell cytosol (Moors *et al.*, 1999). The absence of the bacterial protein ActA in this strain prevents *L. monocytogenes* from initiating actin polymerization and thus generating the force required to spread from cell to cell (Kocks *et al.*, 1992; Pistor *et al.*, 1994). *Listeria monocytogenes* invasion events are relatively rare in confluent MDCK cell monolayers, as these polarized cells form a tight junction, which establishes a circumferential seal to block access of bacteria to E-cadherin in the lateral plasma membrane (Pentecost *et al.*, 2006). Therefore, to reliably establish a baseline level of invasion in wild-type MDCK cells, we followed the standard in the field by pretreating the monolayer briefly with ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) to disrupt cell–cell junctions and expose E-cadherin (Gaillard and Finlay, 1996). Host cells were infected with Δ actA *L. monocytogenes* as described under *Materials and Methods*.

Surprisingly, more $\Delta\alpha$ E-catenin MDCK cells (Figure 1B, right panel) appeared to be infected with replicating *L. monocytogenes* than wild-type MDCK cells (Figure 1B, left panel). To quantify the level of bacterial invasion, the number of *L. monocytogenes*-containing, mTagRFP-positive host cells was measured by flow cytometry (Rengarajan *et al.*, 2016). The frequency of bacterial invasion was approximately four times higher in $\Delta\alpha$ E-catenin MDCK cells than in wild-type MDCK cells (Figure 1C). We also infected monolayers with Δ actA/ Δ inlA *L. monocytogenes* to determine whether host cell invasion was dependent on the E-cadherin/internalin A interaction (Schubert *et al.*, 2002). As anticipated, the number of host cells infected with Δ actA/ Δ inlA *L. monocytogenes* was significantly lower than the Δ actA strain for both wild-type and $\Delta\alpha$ E-catenin MDCK cells (Figure 1C).

Growth factors in serum-supplemented culture media can boost endocytosis in many cell types such as epithelial cells (Koivusalo *et al.*, 2010) and fibroblasts (Quinones *et al.*, 2010). Specifically, these growth factors enhance macropinocytosis, a nonspecific endocytic pathway that contributes to internalization of a variety of bacterial pathogens (Francis *et al.*, 1993; Garcia-del Portillo and Finlay, 1994; Weiner *et al.*, 2016) and viruses (Mercer and Helenius, 2008). Therefore we tested whether these growth factors played a role in facilitating *L. monocytogenes* invasion in MDCK cells, particularly in the absence of a physical link between the bacterium and F-actin. Following serum starvation for 1 h prior to infection, flow cytometry measurements showed that in the absence of serum, *L. monocytogenes* invasion increased reproducibly, but very modestly, in both wild-type and $\Delta\alpha$ E-catenin MDCK cells (Figure 1D). Thus we found no evidence that serum availability, which can regulate macropinocytosis, plays a major role in *L. monocytogenes* invasion in $\Delta\alpha$ E-catenin MDCK cells.

Taken together, our data show that *L. monocytogenes* invasion of MDCK cells is dependent on E-cadherin/internalin A binding but does not require α E-catenin and hence the canonical link between E-cadherin and F-actin. These observations raise the possibility that the minimal requirement for *L. monocytogenes* invasion in MDCK cells might be the adhesive properties provided by host cell E-cadherin, rather than the indirect association between the bacterium and F-actin via E-cadherin.

***L. monocytogenes* invasion of MDCK cells is largely driven by bacterial adhesion to the host cell surface and is dependent on actin polymerization**

Bacterial invasion requires several distinct steps: adhesion to the host cell surface, uptake, escape from the endosome, and replication in the cytoplasm (Tilney and Portnoy, 1989). To determine which step enhances *L. monocytogenes* invasion of $\Delta\alpha$ E-catenin MDCK monolayers, we analyzed the spatial pattern of adherent bacteria across the host cell monolayer early in the process of infection.

EGTA-treated wild-type and $\Delta\alpha$ E-catenin MDCK cells were cocultured with *L. monocytogenes* for 1 h, and then cells were stained for adherent bacteria. Fluorescence micrographs showed that bacteria adhered in clusters in wild-type MDCK monolayers and sometimes near cell-free gaps in the monolayer (Figure 2A, left). This is consistent with previous observations that *L. monocytogenes* binds to areas where E-cadherin is exposed to the apical side of the monolayer, such as sites of cell extrusion (Pentecost *et al.*, 2006). In contrast, adherent bacteria appeared to be more randomly dispersed across the $\Delta\alpha$ E-catenin MDCK monolayer (Figure 2A, right), consistent with a more uniform access of bacteria to E-cadherin. To quantify the distribution of bacteria in cell monolayers, we calculated the nearest neighbor index (NNI) for bacteria adhered to wild-type and $\Delta\alpha$ E-catenin MDCK cells (Clark and Evans, 1954), as described under *Materials and Methods*: An NNI value of 1 represents a completely random pattern, and a value of 0 represents a clustered pattern (Supplemental Figure S2). Our analysis showed that the pattern of adherent bacteria in $\Delta\alpha$ E-catenin MDCK monolayers had an average NNI = 1.02, while wild-type MDCK monolayers had an average NNI = 0.70 (Figure 2B). We also measured the magnitude of bacterial adhesion by calculating the ratio of adherent bacteria to the total number of host cells, counted using nuclear staining (Supplemental Figure S2). The level of bacterial adhesion in $\Delta\alpha$ E-catenin MDCK cells was approximately twofold higher than in wild-type MDCK cells (Figure 2C). Even though there was some day-to-day variability, bacterial adhesion to $\Delta\alpha$ E-catenin MDCK cells was always significantly higher than to wild-type MDCK cells.

Consistent with differences in adhesion levels, we found that, in nonpermeabilized host cells, surface E-cadherin was more accessible to anti-E-cadherin antibody in $\Delta\alpha$ E-catenin MDCK cells than in wild-type MDCK cells (Supplemental Figure S3). Maximum intensity projections of MDCK cells showed that *L. monocytogenes* adhered to both the apical and lateral surfaces of $\Delta\alpha$ E-catenin MDCK cells but only to the apical surface of wild-type MDCK cells that exhibited apical membrane labeling of E-cadherin (Supplemental Figure S3). These data imply that the structural integrity of cell–cell junctions and accessibility to E-cadherin have a strong effect on *L. monocytogenes* adhesion and invasion of MDCK cells, more so than the presence of a physical link between *L. monocytogenes* and F-actin through the E-cadherin/catenin complex.

To disrupt cell–cell junctions orthogonally without EGTA, we used a high-throughput integrated strain array (ISA) (Simmons *et al.*, 2011). Confluent MDCK cell monolayers were seeded in wells on collagen-I-coated flexible polydimethylsiloxane (PDMS) substrates,

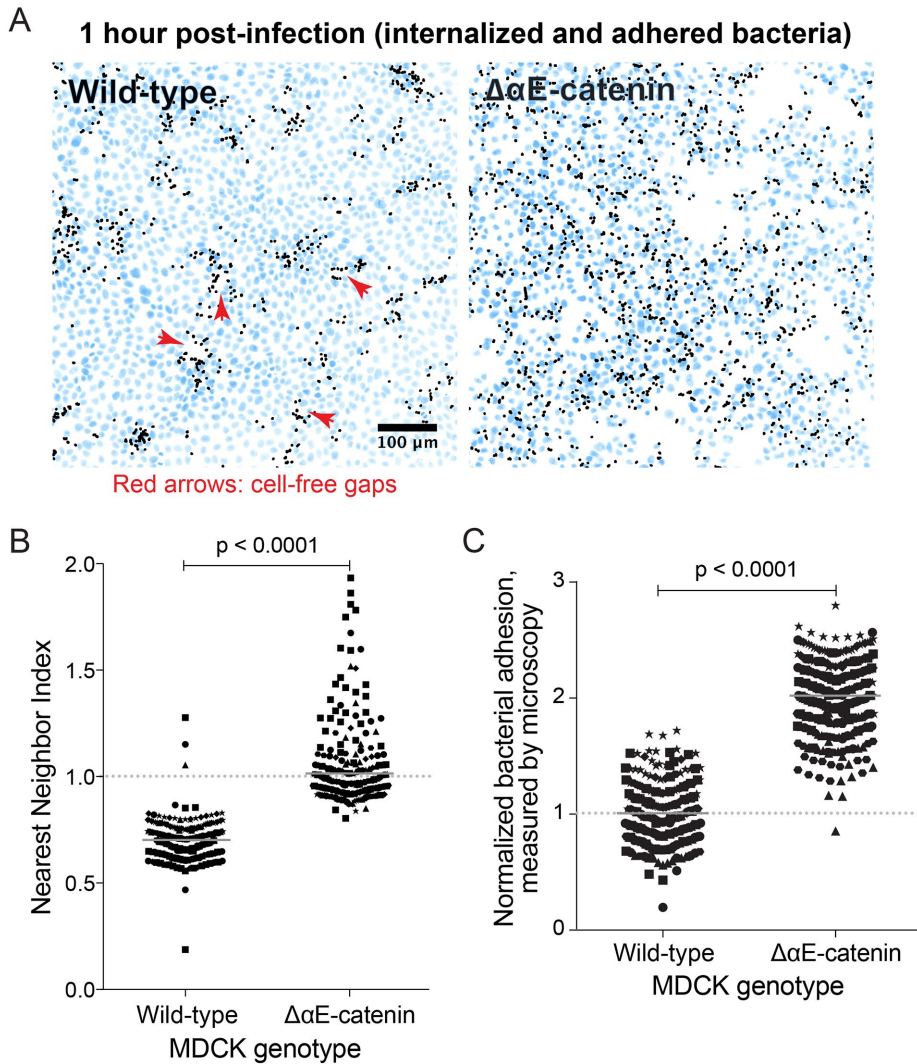


FIGURE 2: *Listeria monocytogenes* invasion of MDCK cells is largely driven by *L. monocytogenes* adhesion to the host cell surface. (A) Micrographs showing nuclei (DAPI, blue) and adherent bacteria (AlexaFluor488, black). Red arrows indicate cell-free gaps in the wild-type MDCK monolayer. (B) Nearest neighbor index (degree of clustering and randomness) for *L. monocytogenes* adhered to wild-type and $\Delta\alpha$ E-catenin MDCK monolayers. (C) Microscopy data, normalized to 1 for wild-type MDCK cells infected with wild-type *L. monocytogenes*, quantifying *L. monocytogenes* adhesion for wild-type and $\Delta\alpha$ E-catenin MDCK cells. For both B and C, each data point represents an individual image containing >1000 host cell nuclei. Each replicate (six total) is depicted as a different symbol. A total of 249 images were analyzed per condition. Horizontal bars indicate the mean. p values were calculated with the Wilcoxon rank sum test.

which were stretched through the application of vacuum pressure. We applied an in-plane 15% stretch across the MDCK monolayer for a total of 1 h, incubated host cells with *L. monocytogenes*, and measured the number of bacteria-associated host cells by fluorescence microscopy. Mechanical stretch resulted in a significant increase in the number of bacteria-associated host cells, compared with the nonstretched control cells, and this was dependent on E-cadherin/internalin A binding (Supplemental Figure S4).

Even though the α E-catenin-mediated link between E-cadherin and F-actin is dispensable for *L. monocytogenes* invasion in MDCK cells, it is possible that F-actin polymerization may still be required given its critical role in invasion of nonphagocytic epithelial cells (Wells *et al.*, 1998). To test this, we infected MDCK cells in the presence of Latrunculin B, which sequesters globular actin (G-actin) and

prevents F-actin assembly (Wakatsuki *et al.*, 2000). At baseline, wild-type MDCK cells had more prominent actin fibers than $\Delta\alpha$ E-catenin MDCK cells (Figure 3A), probably due to α E-catenin's actin-bundling activity (Rimm *et al.*, 1995). Latrunculin B depolymerized actin fibers in both wild-type and $\Delta\alpha$ E-catenin MDCK cells (Figure 3A). Quantifying bacterial invasion by flow cytometry showed a concentration-dependent decrease in invasion in both wild-type and $\Delta\alpha$ E-catenin MDCK cells on Latrunculin B treatment (Figure 3B). Interestingly, invasion of $\Delta\alpha$ E-catenin MDCK cells was more sensitive to Latrunculin B than in wild-type MDCK cells (Figure 3B). We confirmed that the Latrunculin B-mediated decrease in invasion cannot be explained simply by bacterial adhesion, since levels of adhesion remain comparable in both cell lines in the presence of Latrunculin B (Figure 3C).

Taken together, these results indicate that *L. monocytogenes* invasion of MDCK cells was dependent on access of bacteria to surface E-cadherin and on F-actin polymerization. Disrupting cell-cell junctions, by depleting α E-catenin or applying mechanical strain, increased bacterial adhesion and invasion perhaps due to increased surface exposure of E-cadherin. Our results indicate that a physical link between the E-cadherin/catenin complex and F-actin is not a requirement for *L. monocytogenes* invasion in MDCK cells.

E-cadherin extracellular domain is sufficient to mediate *L. monocytogenes* invasion in A431D epithelial cells

While α E-catenin is dispensable for *L. monocytogenes* invasion of MDCK cells, it is possible that other adherens junction proteins may couple E-cadherin to F-actin through the E-cadherin cytoplasmic domain. To explore this possibility, we used A431D cells, a human epithelial cell line that does not express endogenous E-cadherin but does express catenins (Lewis

et al., 1997). We generated A431D epithelial cells expressing human full-length E-cadherin, a truncated E-cadherin mutant lacking the β -catenin-binding site ($\Delta\beta$), and a membrane-attached truncated E-cadherin mutant with an external lipid anchor lacking both the transmembrane and cytoplasmic domains (GPI) (Figure 4A). Additionally, A431D cells overexpress epidermal growth factor receptor (EGFR) (Fabricant *et al.*, 1977), which increases the level of macropinocytosis (Nakase *et al.*, 2015). Therefore our use of A431D cells allowed us to also test whether there was a correlation between levels of macropinocytosis and *L. monocytogenes* invasion in epithelial cells for all E-cadherin constructs.

Levels of full-length and GPI E-cadherin surface expression were similar, and higher than $\Delta\beta$ E-cadherin, as indicated by increased staining with an antibody recognizing the E-cadherin extracellular

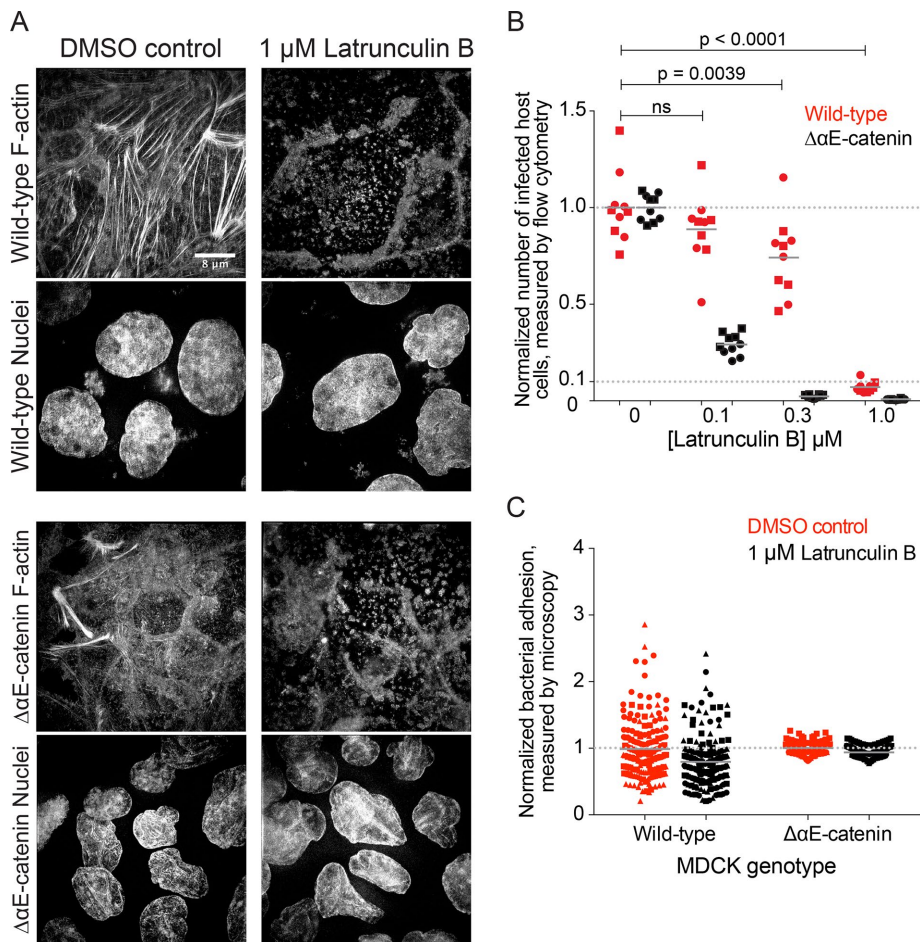


FIGURE 3: *Listeria monocytogenes* invasion of MDCK cells depends on F-actin polymerization. (A) Maximum intensity projections of three-dimensional structured illumination microscopy images showing the nuclei (DAPI) and F-actin (AlexaFluor488) of dimethyl sulfoxide (DMSO)-treated and Latrunculin B-treated MDCK cells. MDCK cells were incubated with 1 μ M Latrunculin B in DMEM alone for 1 h prior to fixing. (B) Flow cytometry data quantifying the number of *L. monocytogenes*-containing MDCK cells, and the effect of three concentrations of Latrunculin B on *L. monocytogenes* invasion of MDCK cells. Data were normalized to 1 for each cell line for DMSO-treated cells infected with Δ actA *L. monocytogenes* and pooled from two independent experiments, each depicted by a different symbol. Each data point represents an individual replicate where 10,000 host cells were analyzed. *p* values comparing DMSO-treated $\Delta\alpha$ E-catenin MDCK cells with those treated with any latrunculin concentration yielded *p* values less than 0.0001, and they were calculated with the Wilcoxon rank sum test. (C) Microscopy data, normalized to 1 for DMSO-treated cells for each cell line, quantifying the effect of 1 μ M Latrunculin B on *L. monocytogenes* adhesion to MDCK cells. Each data point represents an individual image containing >1000 host cell nuclei. A total of 192 images were analyzed per condition. Latrunculin treatment caused a very modest decrease in adhesion in both cell lines: adhesion to wild-type and $\Delta\alpha$ E-catenin cells decreased by 0.19 ± 0.04 and 0.06 ± 0.01 , respectively (difference between the means \pm SEM). For B and C, horizontal bars indicate the mean.

domain in nonpermeabilized cells (Figure 4B). Only expression of full-length E-cadherin caused F-actin to organize into prominent bundles (Supplemental Figure S5), probably due to E-cadherin's interaction with F-actin via catenins. All A431D cell lines were infected with *L. monocytogenes*, and bacterial adhesion and invasion levels were measured by fluorescence microscopy. A431D cells expressing full-length E-cadherin or $\Delta\beta$ E-cadherin had lower levels of bacterial adhesion than cells expressing GPI E-cadherin (Figure 4C). In general, levels of bacterial invasion measured directly by microscopy were statistically similar in A431D cells expressing full-length, $\Delta\beta$, or GPI E-cadherin (Figure 4D). Bacterial invasion in all cells was dependent

on E-cadherin/internalin A binding since parental A431D cells lacking E-cadherin expression exhibited levels of adhesion and invasion two orders of magnitude lower than those expressing any of the E-cadherin constructs (Figure 4D).

The $\Delta\beta$ mutant blocks binding of α E-catenin, and hence F-actin, to E-cadherin, but removing this site could also delete binding sites for other proteins. Therefore we used an E-cadherin mutant in which serine residues at positions 840, 846, and 847 were mutated to alanine (3S > A mutant) or aspartate (3S > D mutant) to selectively disrupt β -catenin binding to E-cadherin. These mutations were originally characterized in an A431D cell line background (McEwen et al., 2014). Bacterial adhesion and invasion levels in A431D cells expressing full-length E-cadherin, or the 3S > A or 3S > D E-cadherin mutants, were comparable and dependent on E-cadherin/internalin A binding (Supplemental Figure S6). These results reinforce our finding that binding of β -catenin and E-cadherin is largely dispensable for *L. monocytogenes* invasion of A431D cells.

Listeria monocytogenes can also invade epithelial cells using an E-cadherin-independent pathway involving binding of the bacterial surface protein Internalin B to the host cell receptor tyrosine kinase c-Met (Shen et al., 2000; Veiga and Cossart, 2005; Pentecost et al., 2010). To test whether Internalin B promotes bacterial invasion in the absence of the E-cadherin/catenin/F-actin interaction, we infected A431D cells with Δ inB *L. monocytogenes*. The absence of internalin B actually slightly increased the level of bacterial invasion in A431D cells expressing all E-cadherin constructs (Supplemental Figure S7A). We also blocked host cell c-Met activation with the highly selective and potent c-Met inhibitor SGX-523 (Buchanan et al., 2009). Treatment with high concentrations of SGX-523 led to a 50% decrease in *L. monocytogenes* invasion in cells expressing full-length E-cadherin and a 30% decrease in cells expressing GPI E-cadherin (Supplemental Figure S7B). Thus we can exclude the possibility that entry dependent on the internalin B/

cMet interaction is the major mechanism by which *L. monocytogenes* invades epithelial cells in the absence of the E-cadherin/catenin/F-actin interaction.

A431D cells overexpress EGFR, and we tested whether elevated levels of macropinocytosis induced by EGFR overexpression (Fabricant et al., 1977) also increased bacterial invasion. A431D cells were serum starved for 1 h before infection, and the number of *L. monocytogenes*-containing host cells was measured by flow cytometry. The level of bacterial invasion in serum-starved cells was ~50% higher in cells expressing full-length E-cadherin than cells expressing GPI E-cadherin, and two- to threefold higher than cells expressing

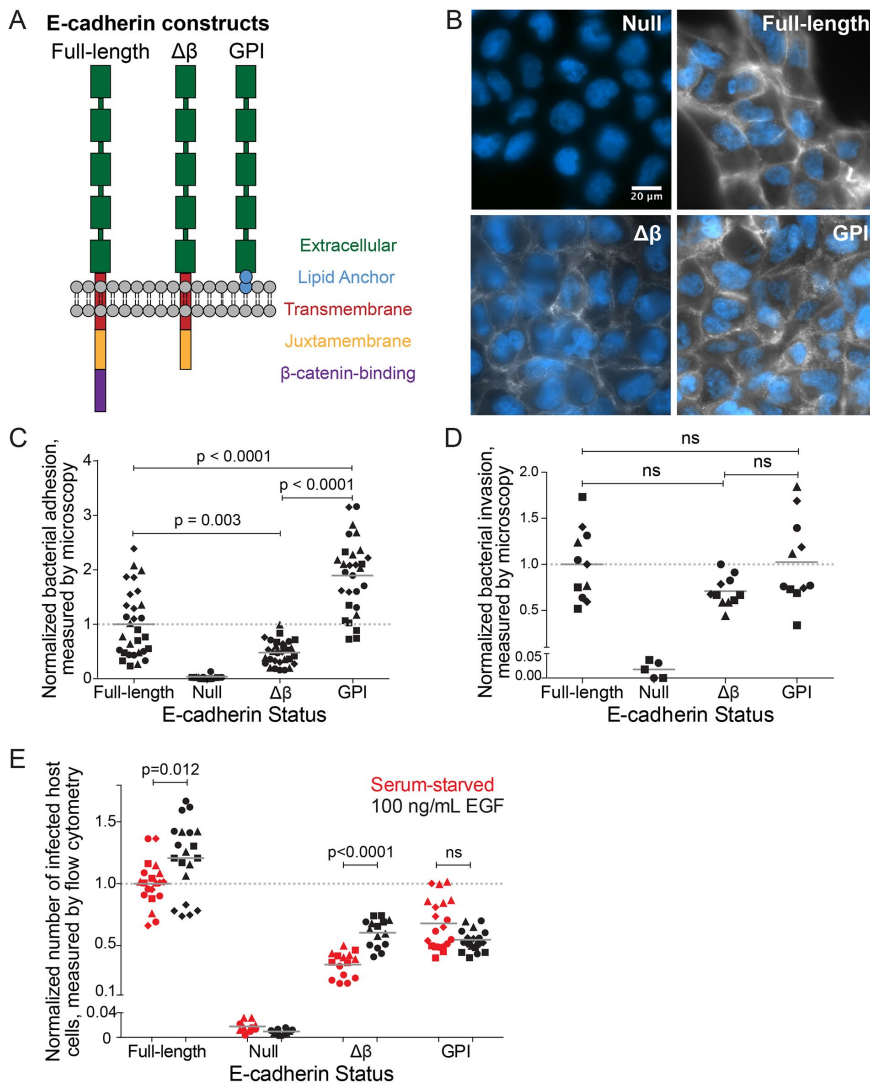


FIGURE 4: E-cadherin extracellular domain is sufficient to mediate *L. monocytogenes* invasion in A431D epithelial cells. (A) Schematic of E-cadherin constructs (full-length, $\Delta\beta$, and GPI) expressed in A431D cells. (B) Micrographs showing nuclei (DAPI, blue) and surface E-cadherin (white). (C) Microscopy data showing *L. monocytogenes* adhesion to A431D cells expressing full-length, $\Delta\beta$, or GPI E-cadherin, where each data point depicts an individual image. Comparing null E-cadherin A431D cells to cells expressing full-length, $\Delta\beta$, or GPI E-cadherin yielded p values of less than 0.0001. (D) Microscopy data showing *L. monocytogenes* invasion of null E-cadherin A431D cells, and A431D cells expressing full-length, $\Delta\beta$, or GPI E-cadherin. For both C and D, data were normalized to 1 for infection of A431D cells expressing full-length E-cadherin. Invasion data were pooled from four independent experiments (depicted by different symbols), and each data point represents the average of 15–20 images. Individual images contained 100–150 host cell nuclei and 30–50 bacteria. Comparing *L. monocytogenes* invasion of null E-cadherin A431D cells to cells expressing full-length, $\Delta\beta$, or GPI E-cadherin yielded p values equal to 0.0005. Bacterial adhesion and invasion were calculated for each image by dividing the number of adhered or internalized bacteria, respectively, by the number of host cell nuclei. (E) Flow cytometry data quantifying the number of *L. monocytogenes*-containing A431D cells, and the effect of either serum-starvation or addition of 100 ng/ml EGF on *L. monocytogenes* invasion of null E-cadherin A431D cells or cells expressing full-length, $\Delta\beta$, or GPI E-cadherin. Data were normalized to 1 for serum-starved A431D cells expressing full-length E-cadherin. Data were pooled from four independent experiments. Each data point represents an individual replicate where 10,000 host cells were analyzed. Horizontal bars indicate the mean. Comparing *L. monocytogenes* invasion of null E-cadherin A431D cells to cells expressing full-length, $\Delta\beta$, or GPI E-cadherin yielded p values less than 0.0001 (for both serum-starved and 100 ng/ml EGF conditions). Comparing *L. monocytogenes* invasion of A431D cells expressing GPI E-cadherin to cells expressing $\Delta\beta$ E-cadherin yields a p value less than 0.0001 for the serum-starved condition (p value is not significant when comparing these two cell lines in the presence of 100 ng/ml EGF). p values were calculated with the Wilcoxon rank sum test.

$\Delta\beta$ E-cadherin (Figure 4E). We also preincubated serum-starved A431D cells with 100 ng/ml purified epidermal growth factor (EGF), which increases macropinocytosis (Bryant *et al.*, 2007; Koivusalo *et al.*, 2010) and enhances bacterial adhesion by breaking up cell–cell junctions (Lu *et al.*, 2003). Compared to the serum-starved condition, the level of bacterial invasion in the presence of EGF was slightly greater in cells expressing full-length E-cadherin or $\Delta\beta$ E-cadherin and similar in cells expressing GPI E-cadherin. Thus we conclude that EGF-mediated changes in cell behavior cannot account for the efficient bacterial invasion we observe in epithelial cells where a direct connection between E-cadherin and the actin cytoskeleton is absent.

Our data in epithelial cells argue that *L. monocytogenes* adhesion to the host cell surface is a critical step in the infection process that determines the level of *L. monocytogenes* invasion. To test whether the “adhesion-centric” model of *L. monocytogenes* invasion is specific to epithelial cells, we retested whether the cytoplasmic tail of E-cadherin is necessary for *L. monocytogenes* invasion of fibroblasts (Lecuit *et al.*, 2000). We generated L-929 mouse fibroblasts expressing full-length, $\Delta\beta$, and GPI E-cadherin (Figure 4A). Full-length and GPI E-cadherin appeared to be expressed at higher levels than $\Delta\beta$ E-cadherin (Supplemental Figure S8A), similarly to A431D epithelial cells (Figure 4B). We found that *L. monocytogenes* adhesion was statistically similar in fibroblasts expressing full-length E-cadherin and $\Delta\beta$ E-cadherin (Supplemental Figure S8B), despite the lower level of $\Delta\beta$ E-cadherin expression (Supplemental Figure S8A). However, *L. monocytogenes* invasion in fibroblasts expressing full-length E-cadherin was an order of magnitude higher than $\Delta\beta$ E-cadherin cells (Supplemental Figure S8C), despite comparable levels of bacterial adhesion (Supplemental Figure S8B). We also used flow cytometry to measure the number of infected host cells and found that cells expressing $\Delta\beta$ or GPI E-cadherin had significantly lower levels of invasion than fibroblasts expressing full-length E-cadherin (Supplemental Figure S8D). These results are consistent with the previous report that E-cadherin lacking the β -catenin-binding domain resulted in a sevenfold decrease in bacterial invasion in fibroblasts compared with fibroblasts expressing full-length E-cadherin (Lecuit *et al.*, 2000).

Our data indicate that bacterial adhesion to the surface of fibroblasts increased invasion by about an order of magnitude relative to conditions of nonadherence

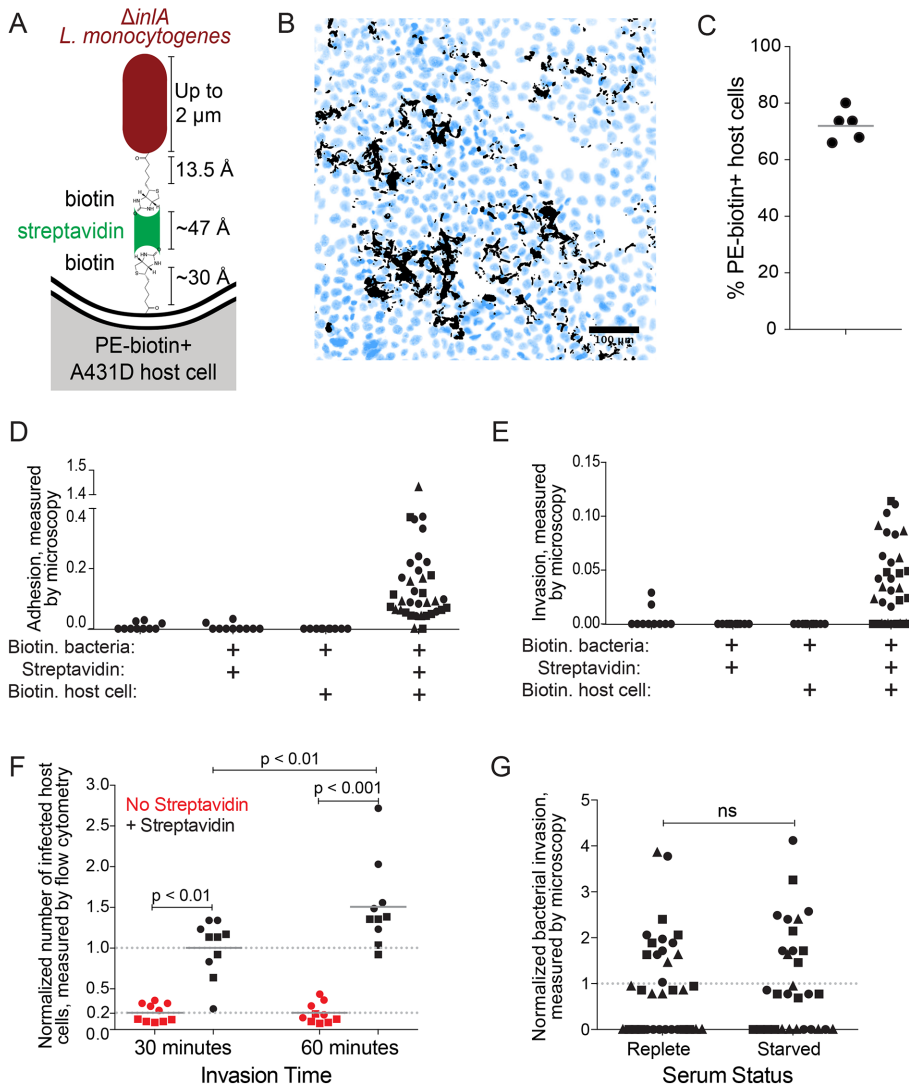


FIGURE 5: Artificial *L. monocytogenes* adhesion to the surface of A431D cells is sufficient to mediate *L. monocytogenes* invasion. (A) Schematic depicting the topology of the streptavidin/biotin-mediated *L. monocytogenes* invasion assay. (B) Fluorescence micrograph of null E-cadherin A431D cell nuclei (DAPI, blue) incubated with phosphatidylethanolamine-biotin (PE-biotin) stained with AlexaFluor546-conjugated streptavidin (black). (C) Flow cytometry data quantifying the number of null E-cadherin A431D cells containing PE-biotin. Each data point represents an individual replicate of 10,000 null E-cadherin A431D cells analyzed. (D) Microscopy data showing *L. monocytogenes* adhesion to null E-cadherin A431D cells in the presence or absence of different components of the biotin/streptavidin bridge. (E) Microscopy data showing *L. monocytogenes* invasion of null-E-cadherin A431D cells in the presence or absence of different components of the biotin/streptavidin bridge. For D and E, each data point represents an individual image analyzed containing 80–100 null E-cadherin A431D nuclei. Bacterial adhesion and invasion were calculated for each image by dividing the number of adhered or internalized bacteria, respectively, by the number of host cell nuclei. (F) Flow cytometry data quantifying the number of *L. monocytogenes*-containing null E-cadherin A431D cells. Data were normalized to 1 for host cells infected in the presence of streptavidin for 30 min. Data were pooled from two independent experiments (different symbols). Each data point represents an individual replicate from which 10,000 host cells were analyzed. (G) Microscopy data, normalized to 1 for serum-replete condition, showing the effect of serum on *L. monocytogenes* invasion of null E-cadherin A431D cells in the presence of all components of the biotin/streptavidin bridge. Each data point represents an individual image analyzed containing 80–100 null E-cadherin A431D nuclei. Horizontal bars indicate the mean. *p* values were calculated with the Wilcoxon rank sum test.

(no E-cadherin expression) and that the presence of the E-cadherin/catenin/F-actin link increased the level of invasion by an additional order of magnitude. Thus *L. monocytogenes* invasion of

epithelial cells and fibroblasts are strikingly different in the requirement for linkage to F-actin via E-cadherin.

Artificial adhesion to the surface of A431D cells is sufficient to mediate *L. monocytogenes* invasion

Our results raise the possibility that any type of adhesion to the surface of epithelial cells might be sufficient to mediate *L. monocytogenes* invasion. To test this hypothesis, we used an artificial biotin-streptavidin link to bind bacteria to the epithelial host cell surface. This biotin-streptavidin bridge resembles the topology of E-cadherin/internalin A-mediated binding without direct protein-protein associations between the bacteria and the host cell cytoskeleton via E-cadherin (Figure 5A). Null E-cadherin A431D cells were incubated with phosphatidylethanolamine covalently linked to biotin (PE-biotin) in the presence of lipofectamine. Incorporation of PE-biotin was confirmed by staining null E-cadherin A431D cells with fluorescent streptavidin (Figure 5B) and by flow cytometry (Figure 5C); there was some cell-to-cell variability in PE-biotin levels (Supplemental Figure S9). Surface-exposed proteins on *ΔinA* *L. monocytogenes* were biotinylated with Sulfo-NHS-biotin, and bacteria were incubated with PE-biotin labeled A431D cells in the presence or absence of streptavidin. Bacterial adhesion and invasion were measured by fluorescence microscopy.

Bacterial adhesion and invasion occurred only if all components of the bacteria-to-host cell biotin-streptavidin bridge were included (Figure 5, D and E). Significantly, the artificial biotin-mediated adhesion of bacteria to the surface of the host cell was sufficient to mediate *L. monocytogenes* invasion of A431D cells (Figure 5E). These results were confirmed by flow cytometry using biotinylated *ΔactA/ΔinA* *L. monocytogenes* strain expressing mTagRFP under the *actA* promoter. There was a time-dependent increase in bacterial invasion only in the presence of streptavidin-dependent bacteria-to-host cell adhesion (Figure 5F). *Listeria monocytogenes* invasion of A431D cells via the biotin-streptavidin bridge was unaffected by serum starvation (Figure 5G). These results indicate that high-affinity surface attachment of *L. monocytogenes* is sufficient for epithelial cell adhesion and invasion independently of expression of E-cadherin and linkage to F-actin.

DISCUSSION

While it is well established that E-cadherin/internalin A binding is necessary for *L. monocytogenes* invasion of epithelial cells in vitro

(Mengaud *et al.*, 1996) and in vivo (Wollert *et al.*, 2007), the importance of the physical link between E-cadherin and F-actin has not been tested directly in this cell type, the primary cell type for *L. monocytogenes* invasion in vivo. We were surprised to find that *L. monocytogenes* is able to invade $\Delta\alpha$ E-catenin MDCK cells more than wild-type MDCK cells. We suggest this is because 1) $\Delta\alpha$ E-catenin cells do not assemble robust cell–cell junctions, including a tight junction that normally blocks access of bacteria to E-cadherin localized on the lateral cell surface and 2) expression levels of E-cadherin in $\Delta\alpha$ E-catenin MDCK cells are similar to those in wild-type MDCK cells, and hence E-cadherin is available for binding bacteria. Mechanical disruption of cell–cell junctions by applying 15% stretch to an MDCK monolayer also increased *L. monocytogenes* invasion, which we suggest could be due to the disruptions of the barrier function of the tight junction (Samak *et al.*, 2014), allowing for the potential access of bacteria to lateral surface E-cadherin. Thus our results indicate that *L. monocytogenes* adhesion and invasion of an epithelial monolayer are highly sensitive to the accessibility of surface E-cadherin.

Listeria monocytogenes has been proposed to enter nonphagocytic host cells via an actin-dependent zipper mechanism (Cossart and Sansonetti, 2004). In this model, E-cadherin/internalin A binding provides a physical connection among the bacterium, E-cadherin, and F-actin (Lecuit *et al.*, 2000). This “catenin-centric” model argues that F-actin reorganization around sites of bacterium–E-cadherin adhesion are needed to internalize large, bacterium-sized cargo. Supporting evidence for this model is the requirement of the β -catenin-binding domain of human E-cadherin for efficient invasion of *L. monocytogenes* in mouse fibroblasts (Lecuit *et al.*, 2000). Indeed, we confirmed this result in mouse fibroblasts. However, we found that inclusion of the β -catenin-binding domain of E-cadherin or the entire cytoplasmic tail had only a modest effect on bacterial invasion in epithelial cells. More importantly, we showed that the canonical connection to F-actin via α E-catenin is dispensable using $\Delta\alpha$ E-catenin MDCK cells but that F-actin polymerization is still required for bacterial invasion in these cells. These data suggest that F-actin polymerization plays a critical, yet E-cadherin-independent, role in *L. monocytogenes* invasion of epithelial cells such as driving membrane invagination or aiding in vesicle scission. Our results highlight significant differences between the role of E-cadherin and the F-actin cytoskeleton in bacterial invasion of fibroblasts and epithelial cells.

Previous work implicated the importance of the intracellular domain of human E-cadherin in invasion of mouse fibroblasts by *L. innocua*, a nonpathogenic *Listeria* species, episomally expressing internalin A (Lecuit *et al.*, 2000). *Listeria innocua* differs from *L. monocytogenes*, which expresses other virulence factors such as Listeriolysin O (LLO), a hemolysin that boosts bacterial invasion in hepatocytes (Vadia *et al.*, 2011). One possibility is that LLO-secreting bacteria tightly adhered to the host cell membrane generate local membrane disruptions that are sufficient for invasion in our tissue culture-based assays. We also cannot rule out the possible involvement of other virulence factors specific to *L. monocytogenes*, except for internalin B, which we showed was not required for invasion in the absence of a link between E-cadherin and F-actin.

Our results in epithelial cells favor an “adhesion-centric” model, in which the most important determinant for invasion is strong binding between bacteria and the surface of the host cell plasma membrane, rather than a link to F-actin. This model is supported by our findings that *L. monocytogenes* invasion occurred in cells expressing 1) full-length E-cadherin, 2) truncated or mutated E-cadherin compromised in β -catenin/ α E-catenin binding to F-actin, and 3) E-

cadherin ectodomain anchored to the cell surface by a lipid anchor and lacking transmembrane and cytoplasmic domains. Significantly, artificial streptavidin-mediated binding of bacteria to biotinylated phospholipids in the outer leaflet of the host cell plasma membrane also facilitated *L. monocytogenes* invasion of epithelial cells. The common requirement in all cases, except the artificial streptavidin-mediated adhesion, is binding of bacterial internalin A to the ectodomain of E-cadherin. However, the mechanism downstream of E-cadherin/internalin A binding is less clear.

One possibility is macropinocytosis, a clathrin- and caveolae-independent endocytic pathway (Hewlett *et al.*, 1995) commonly employed by many cell types such as macrophages, fibroblasts, and epithelial cells (Lim and Gleeson, 2011). Macropinocytosis allows cells in the gut epithelium to sample luminal antigens and take up fluid and solid via endosomes of up to 5–10 μ m in diameter (Swanson and Watts, 1995). It is also well established that bacterial pathogens, such as *Salmonella typhimurium* (Francis *et al.*, 1993; Garcia-del Portillo and Finlay, 1994) and *Shigella flexneri* (Weiner *et al.*, 2016), as well as viruses such as vaccinia virus (Mercer and Helenius, 2008), can invade target host cells via macropinocytosis.

A431D cells express ~10 times higher levels of EGFR per cell than most other cancerous human cells grown in culture (Fabricant *et al.*, 1977), which raises the possibility that macropinocytosis mediates *L. monocytogenes* invasion in the absence of the E-cadherin/catenin/F-actin complex. However, we detected only modest differences in bacterial invasion levels between cells expressing several different E-cadherin constructs that were serum-starved (leading to low levels of macropinocytosis) or incubated with a high concentration of purified EGF (leading to high levels of macropinocytosis) prior to infection. Indeed, the modest effect of addition of EGF in increasing bacterial invasion may be largely due to the role of activated EGFR in disrupting cell–cell junctions (Lu *et al.*, 2003), which would result in increased bacterial adhesion. Even though these perturbations made little to no difference to *L. monocytogenes* invasion, we cannot rule out the possibility that basal levels of macropinocytosis inherent in tissue culture cells may contribute to this process.

An alternative to macropinocytosis is uptake triggered by activation of cMet by internalin B, which has been implicated in *L. monocytogenes* invasion of some types of epithelial cells (Veiga and Cossart, 2005; Pentecost *et al.*, 2010). However, we found that epithelial cell invasion by Δ inB *L. monocytogenes* was similar to wild-type *L. monocytogenes* and in fact exhibited reproducibly slightly higher levels of invasion in A431D cells expressing different E-cadherin mutants. Interestingly, blocking c-Met activation with high concentrations of the inhibitor SGX-523 caused a mild decrease in invasion of A431D cells expressing full-length and GPI E-cadherin. This suggests that while a cMet-dependent pathway may contribute to bacterial entry, albeit not requiring internalin B, this pathway seems unlikely to play a critical role in “adhesion-centric” invasion of epithelial cells in tissue culture.

Recent physical models suggest a third possible internalization mechanism, which does not rely on active engagement of the host cell cytoskeleton or endocytic machinery. In this scenario, membrane fluctuations are large enough to internalize particles with a radius of up to 2 μ m as long as the particle exhibits significant adhesion to the membrane surface, albeit with slightly slower kinetics than actin-driven phagocytic mechanisms (Tollis *et al.*, 2010). Our results showed that a lipid-anchored extracellular domain of E-cadherin-induced bacterial adhesion and internalization in both fibroblasts and epithelial cells and that a lipid-anchored biotin-streptavidin link without an E-cadherin backbone was sufficient for bacterial invasion; both results are consistent with such a physical model. Our observations that

Genotype	Strain designation	Promoter controlling mTagRFP	Parental strain designation	Parental strain reference
Wild-type	JAT1117	Hyper-SPO1 (constitutive)	10403S	Bishop and Hinrichs, 1987
$\Delta inIA$	JAT1240	Hyper-SPO1 (constitutive)	DP-L4405	Bakardjiev et al., 2004
$\Delta inIB$	JAT1241	Hyper-SPO1 (constitutive)	DP-L4406	Bakardjiev et al., 2004
$\Delta actA$	JAT610	ActA (intracellular expression)	DP-L3078	Skoble et al., 2000
$\Delta actA/\Delta inIA$	JAT1262	ActA (intracellular expression)	DP-L6053	This work

TABLE 1: *Listeria monocytogenes* strains used in this study.

“adhesion-centric” invasion requires actin polymerization suggest that actin dynamics may drive membrane fluctuations or may be involved in driving membrane invagination or pinching of the vesicle. Thus, in this model, the primary role of E-cadherin is the adhesive receptor for *L. monocytogenes* rather than a physical link to the actin cytoskeleton and its dynamic rearrangements.

MATERIALS AND METHODS

Bacterial strains and growth conditions

All 10403S *L. monocytogenes* strains used in this study are summarized in Table 1. Plasmid pMP74RFP was constructed by incorporating a codon-optimized mTagRFP (Zeldovich et al., 2011) in place of GFPmut2 in plasmid pMP74 (Pentecost et al., 2010). This established constitutive expression of RFP under the Hyper-SPO1 promoter fused to the 5' UTR of *hly* (Shen and Higgins, 2005). To incorporate fluorophores into *L. monocytogenes*, plasmid pMP74RFP was transformed into *E. coli* SM10 λ pir. Plasmids were transferred from SM10 λ pir to *L. monocytogenes* by conjugation and stable integration into the tRNA^{ARG} locus of the bacterial chromosome as previously described (Lauer et al., 2002). An identical approach was used to stably integrate plasmid pPL499 (Zeldovich et al., 2011; a gift from Peter Lauer, Aduro Biotech), which contains a codon-optimized mTagRFP open reading frame under the control of the ActA promoter, which becomes active under the reducing conditions of the host cell cytosol (Moors et al., 1999; Reniere et al., 2015). The $\Delta actA/\Delta inIA$ *L. monocytogenes* strain was generated, as previously described (Skoble et al., 2000), by an in-frame deletion in the *actA* open reading frame on the $\Delta inIA$ *L. monocytogenes* strain (Bakardjiev et al., 2004).

Prior to infection experiments, bacteria were streaked out from a frozen glycerol stock onto brain heart infusion (BHI) agar plates containing 200 μ g/ml streptomycin and 7.5 μ g/ml chloramphenicol and were grown at 37°C for 48 h. Several 2-ml BHI liquid cultures containing antibiotics were inoculated from plate colonies and grown overnight at room temperature in the dark without agitation for 16–18 h. Flagellated bacteria were grown to an OD₆₀₀ of 0.8 for all invasion assays.

Mammalian cell culture

MDCK type II G cells (Mays et al., 1995) and $\Delta\alpha$ E-catenin cells were grown in DMEM with low glucose (1.5 g/l) and low sodium bicarbonate (1.0 g/l) in the presence of 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. To generate confluent cell monolayers for invasion assays, 12-well plates (plastic-bottom for flow cytometry or glass-bottom for microscopy) were coated with 50 μ g/ml rat-tail collagen-I (diluted in 0.2 N acetic acid) for 1 h at 37°C, air-dried for 15 min, and UV-sterilized for 30 min in a tissue culture hood. MDCK cells were seeded at a density of 1×10^6 cells/well in low (5 μ M) calcium DMEM. After 2 h, media was replaced with DMEM with normal calcium levels (1.8 mM). Cells were grown for 48 h at 37°C to generate densely packed, quiescent cell monolayers. For ISA ex-

periments (Simmons et al., 2011), cells were handled similarly, except that they were seeded onto collagen-I-treated flexible PDMS substrates at a density of 80,000 cells/well.

A431D human epithelial carcinoma cells (gift from Cara Gottardi, Northwestern University) and L929 mouse fibroblasts (American Type Culture Collection) were grown in DMEM with high glucose (4.5 g/l) in the presence of 10% FBS and 1% penicillin-streptomycin. Transduced cell lines (described below) were cultured in media supplemented with 800 μ g/ml geneticin. For invasion assays, plastic-bottom 12-well plates (flow cytometry) or Micro90-washed 18-mm glass coverslips (microscopy) were incubated overnight at 37°C with 1 μ g/ml fibronectin (Sigma Aldrich). Cells were then seeded at a density of 10^5 cells/well and incubated at 37°C for 48 h.

Generation and validation of $\Delta\alpha$ E-catenin MDCK cells

We made clustered regularly interspaced short palindromic repeat-mediated knockouts of α E-catenin in MDCK cells. Constructs were designed to target CAS9-mediated DNA cleavage in exon 1, and genomic DNA in the region of the targeted sequences from monoclonal colonies was sequenced. Comparison of knockout with wild-type DNA sequence showed a frame shift in the α E-catenin amino acid sequence producing a stop codon, and loss of α E-catenin expression was confirmed by Western blots of $\Delta\alpha$ E-catenin cell lines; levels of E-cadherin and β -catenin were similar in $\Delta\alpha$ E-catenin MDCK cells and wild-type MDCK cells (Supplemental Figure S1). $\Delta\alpha$ E-catenin MDCK cells failed to form stable cell–cell adhesions, and any contacts that occurred were short lived compared with wild-type MDCK cells (Supplemental Videos 1 and 2).

For Western blot, 2.0×10^6 wild-type and $\Delta\alpha$ E-catenin MDCK cells were plated on 35-mm cell culture dish for 24 h. Cells were extracted with 1% SDS, 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, and boiled for 10 min at 90°C. Equal amounts of total protein were loaded on a 7.5% Tris-glycine polyacrylamide gel, and then transferred to Immobilon-FL membrane. Proteins were incubated with blocking buffer containing phosphate-buffered saline (PBS), 2% bovine serum albumin (BSA), and 1% goat and donkey serums for 1 h at room temperature. The following primary antibodies were used: β -catenin (ab32572; abcam), E-cadherin (24E10; Cell Signaling Technology), α -catenin (ALX-804-101-C100; Enzo), α -tubulin (15D9; Sigma), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ab8245; abcam). Secondary antibodies were IRDye 800CW goat anti-rabbit (925-32211; LI-COR) and IRDye 680RD donkey anti-mouse (925-68072; LI-COR). Blots were imaged using a LI-COR Odyssey scanner.

For immunofluorescence, 3×10^5 wild-type and $\Delta\alpha$ E-catenin MDCK cells were plated on 22×22 -mm rat-tail collagen-I-coated glass coverslip for 24 h. Cells were washed once with PBS, fixed with 4% paraformaldehyde for 15 min, and then permeabilized in PBS with 0.5% Triton X-100 for 3 min. Coverslips were incubated with blocking buffer containing PBS, 1% BSA, 1% goat serum, 1% donkey serum, 50 mM NH₄Cl for 30 min, and then incubated with the following primary antibodies: β -catenin (ab32572; abcam) and E-cadherin

(24E10; Cell Signaling Technology). The secondary antibody was goat anti-rabbit Rhodamine RedX (Jackson ImmunoResearch) secondary antibody; nuclei were stained with Hoechst 33342 (Thermo Fisher). All images were taken with a Zeiss Axiovert 200 M inverted wide-field epifluorescence microscope (Intelligent Imaging Innovations [3i]) and a Hamamatsu C11440 Digital camera (Orca-flash4.0OLT). The microscope is equipped with a motorized stage and harmonic drive z-focusing, an X-Cite 120 LED illumination source, and a quad filter cube for DAPI, fluorescein isothiocyanate (FITC), Cy3 and Cy5. Imaging was done with a 63× objective (NA 0.75) using Slidebook software (3i).

E-cadherin retroviral transduction

The full coding sequence for human full-length E-cadherin (882 amino acids, including the signal peptide) was cloned from the pcDNA3 human E-cadherin plasmid (gift from Cara Gottardi, Northwestern University). To create the $\Delta\beta$ -catenin-binding domain E-cadherin construct, amino acids 810–882 were deleted from the coding sequence. To create the GPI-anchored E-cadherin construct, the extracellular E-cadherin ectodomain was cloned upstream of the CH2 and CH3 domains of the immunoglobulin (Ig) F_C region followed by the human placental alkaline phosphatase signal sequence, which directs the formation of a glycosylphosphatidylinositol (GPI) anchorage (Whitehorn *et al.*, 1995).

E-cadherin phosphomutant cell lines used in this work have been described previously (McEwen *et al.*, 2014). Full-length, $\Delta\beta$, and GPI E-cadherin constructs were cloned into pLNCX2 Retroviral Vector (Clontech) by Epoch Life Sciences Inc. E-cadherin vectors (10 μ g) and an amphotropic packaging vector (10 μ g) were transfected into GP2-293 cells (Clontech) using calcium phosphate. Virus was concentrated 24 h after transfection, collected 48 h after transfection, passed through a 0.45- μ m cellulose acetate filter, and used immediately. A431D and L929 cells were transduced with virus and 8 μ g/ml polybrene for 24 h and selected with full media supplemented with 800 μ g/ml geneticin for 5–7 d.

Surface E-cadherin staining for wide-field microscopy

A431D and L929 cells were seeded onto fibronectin-treated coverslips as described above. Cells were washed once with PBS, fixed for 20 min with 4% paraformaldehyde at room temperature, washed three times with PBS, and incubated for 30 min with a mouse anti-E-cadherin antibody (ab1416; abcam), a monoclonal antibody (mAb) that binds to an extracellular domain of human E-cadherin (Ottenhof *et al.*, 2012) diluted at 1:1000 in PBS. Cells were stained for 30 min with a secondary goat anti-mouse antibody (Thermo Fisher) diluted at 1:250 in PBS. Nuclei were stained with DAPI. Images were collected with an inverted Eclipse Ti-E microscope.

Surface E-cadherin and F-actin staining for structured illumination microscopy

Wild-type and $\Delta\alpha$ E-catenin MDCK cells were cultured on collagen-I-coated high-performance cover glasses (Zeiss) as described above. Cells were preincubated with EGTA, infected with constitutively fluorescent *L. monocytogenes* (JAT1117) for 10 min, and washed three times with DMEM. Cells were fixed with 4% paraformaldehyde for 10 min followed by 50 mM NH₄Cl for 10 min (to quench paraformaldehyde autofluorescence), and incubated with blocking solution (PBS + 10% goat serum + 1% BSA) for 1 h at room temperature. Surface E-cadherin receptors were stained with mouse anti-E-cadherin antibody (RR1 clone, gift from the National Institutes of Health [NIH] mAb site, University of Iowa) diluted 1:200 in blocking solution for 1 h, and with a goat anti-mouse AlexaFluor488 secondary antibody (Thermo

Fisher) diluted 1:250 in blocking solution for 1 h. Host cell nuclei were stained with DAPI. To stain for F-actin, cells were fixed as described above and incubated with blocking/permeabilization (BP) buffer (PBS + 3% BSA + 0.1% Triton X-100) for 10 min at room temperature. Cells were then incubated with 0.2 μ M AlexaFluor488 phalloidin (Thermo Fisher), diluted in BP buffer, for 30 min at room temperature. Images were collected with OMX BLAZE 3D-structured illumination, super-resolution microscope (GE DeltaVision). Individual stacks were imaged 125 nanometers apart, and they were reconstructed and aligned with DeltaVision software.

Image analysis to quantify bacterial adhesion and NNI

MDCK monolayers, cultured in collagen-I-coated glass-bottom 12-well plates (Cellvis), were incubated with Δ actA *L. monocytogenes* for 1 h as described above, washed twice with DMEM alone, once with PBS, and fixed with 4% paraformaldehyde for 10 min. Monolayers were incubated with 50 mM NH₄Cl for 10 min and permeabilized with 0.3% Triton X-100 for 7 min. Cells were labeled with rabbit anti-*Listeria* polyclonal antibody (ab35132; abcam) diluted 1:100 in PBS + 0.1% Triton X-100 + 3% BSA for 1 h. Cells were washed three times with PBS, and labeled with DAPI and goat anti-rabbit AlexaFluor488 secondary antibody (Thermo Fisher) diluted 1:250 in PBS + 0.1% Triton X-100 + 3% BSA for 1 h. Cells were washed three times with PBS.

Images were randomly collected with an inverted Eclipse Ti-E microscope using a motorized stage controlled by μ Manager's High Content Screening Site Generator plugin. A total of 49–64 images were collected per well using a 20× air objective (NA = 0.75). This image acquisition method covered 6–8% of the total area of each well. Images were exported as TIFF files and processed using MATLAB's image processing toolbox (MathWorks). Images containing adherent *L. monocytogenes* and DAPI-stained nuclei were processed separately.

Bacteria and nuclei were thresholded using Otsu's method (Sezgin and Sankur, 2004) and segmented using the distance transform (Maurer and Raghavan, 2003). Object segmentation was used to determine the number of bacteria or nuclei per image. To calculate the nearest neighbor index (Clark and Evans, 1954), the following formula was used:

$$NNI = \frac{D}{0.5\sqrt{\frac{A}{N}}}$$

where D is the average of the distances of each bacterium to its nearest neighbor, A is the area occupied by the host cells, and N is the number of bacteria adhered to the monolayer. To calculate D , the Euclidean distance between each bacterium's centroid and its closest neighbor was measured and averaged for all the bacteria in a single image. To measure bacterial adhesion per image, the number of adherent bacteria was divided by the number of host cell nuclei.

Time-lapse microscopy

Wild-type and $\Delta\alpha$ E-catenin MDCK cells were cultured on rat-tail collagen-I-coated glass-bottom 12-well plates (Cellvis) for 48 h as described above. Cells were washed with L-15 twice, and medium was replaced with L-15 + 10% FBS. Cells were imaged every 5 min with an inverted Eclipse Ti-E microscope in an environmental chamber set to 37°C.

Standard invasion assays

Flagellated bacteria (OD₆₀₀ of 0.8) were washed twice with PBS to remove soluble bacterial products and diluted in DMEM. Host cells were washed once with DMEM, and bacteria were added at a

multiplicity of infection (MOI) of 200–300 bacteria per host cell. MOI was determined by immediately plating colony-forming units onto BHI agar plates containing antibiotics from the bacterial mix used to infect host cells. For some experiments, host cells were serum starved in DMEM for 1 h before infection. For A431D cell flow cytometry assays, serum-starved cells were also cultured in 100 ng/ml recombinant EGF (Sigma Aldrich) or c-Met inhibitor SGX-523 (5356; Tocris) for 1 h before infection. For SGX-523 experiments, A431D cells were infected with *L. monocytogenes* in the presence of the compound. For MDCK cell invasion assays, adherens junctions were disrupted with 1 mM EGTA for 15 min before infection (except for strain array experiments). For latrunculin experiments, MDCK cells were preincubated with 1 μ M Latrunculin B (428020; Calbiochem) for 1 h before EGTA treatment and were infected in the presence of the compound. Bacteria and host cells were incubated together in calcium-containing media at 37°C for 10 min, at which time host cells were washed four times with DMEM to remove nonadherent bacteria. Host cells and adherent bacteria were incubated at 37°C for 30 min for A431D cells, or 1 h for MDCK cells and fibroblasts, to allow for the adherent bacteria to invade host cells.

For inside/outside staining (using constitutively fluorescent *L. monocytogenes*), host cells were fixed with 5% formaldehyde as previously described (Yam and Theriot, 2004), and surface-exposed bacteria were labeled using a rabbit anti-*Listeria* polyclonal antibody (ab35132; abcam) at a 1:100 dilution in PBS. Host cell nuclei were stained with DAPI. Images were collected randomly with an inverted Eclipse Ti-E microscope using a 60 \times oil objective (NA = 1.40) until 1500–2000 host cell nuclei had been imaged. Bacterial adhesion and invasion were calculated for each image by dividing the number of adhered or internalized bacteria, respectively, by the number of host cell nuclei.

For flow cytometry (using *L. monocytogenes* expressing fluorophore under the ActA promoter), media was removed from host cells and replaced with DMEM supplemented with 10% FBS and 20 μ g/ml gentamicin for A431D and L929 cells; a concentration of 50 μ g/ml gentamicin was used initially and then decreased to 10 μ g/ml after 30 min for MDCK cells. Gentamicin kills extracellular bacteria and prevents additional invasion events (Portnoy et al., 1988). Host cells were incubated at 37°C for 5 h after addition of gentamicin to allow sufficient time for bacteria to replicate and express the fluorophore. Host cells were washed with room-temperature PBS and detached with ~100 μ l/well 0.25% trypsin/EDTA without phenol red. After a 5- to 10-min incubation at 37°C, trypsin was neutralized with ice-cold PBS + 10% FBS. Cells were passed through a 35- μ m nylon filter by centrifugation for 4 min into a polystyrene FACS tube and kept on ice until analyzed with a BD FACScan.

Mechanical strain invasion assay

The ISA and validation of stretch by bead displacement has been described previously (Simmons et al., 2011). Two days after seeding MDCK cells onto the PDMS substrate, the wells were positioned over a pillar on an acrylic baseplate; the width of the pillar varied between the wells so different levels of in-plane stretch (0–15%) can be applied in the same experiment. Vacuum pressure applied through a high-precision vacuum regulator (Parker Hannifin Corporation; Part Number P3RA17132NNKN) resulted in biaxial stretching of the PDMS substrate and biaxial in-plane stretching within the cell monolayers. Experiments were conducted at 0% (no stretch) and 15% stretch levels.

Static and constant equibiaxial and in-plane stretch were maintained on the cell monolayers for 30 min, at which point *L. monocyto-*

genes (OD₆₀₀ of 0.8) was added at an MOI of 200–300 bacteria per host cell, followed by incubation for 30 min at 37°C. Host cells were washed twice with DMEM and once with PBS and were then fixed with 4% paraformaldehyde for 10 min at 37°C. Vacuum pressure was turned off, and host cells continued to be fixed for an additional 10 min at room temperature. *Listeria monocytogenes* adhesion and invasion were quantified by inside/outside staining as described above.

Biotin/streptavidin invasion assay

Phosphatidylethanolamine covalently linked to a biotin group (cat # B1616; Thermo Fisher), was mixed with Lipofectamine 3000 (Thermo Fisher) and incubated at room temperature for 2 h. The lipid/lipofectamine mixture was then sonicated for 4 min before addition to A431D cells for a final lipid concentration of 6 μ M. Host cells, which had been seeded 24 h before addition of lipid, were incubated at 37°C for an additional 24 h.

The day of the infection, *L. monocytogenes* was grown to an OD₆₀₀ of 0.8 (as described above), washed twice with PBS, resuspended in 200 μ l of PBS, and mixed with 4 μ l of 5 mg/ml Sulfo NHS-biotin (Thermo Fisher). Bacteria were mixed by pipetting up and down several times and incubated at room temperature for 2 min. Bacteria were then washed three times with PBS and diluted in DMEM. Host cells were washed once with DMEM and incubated with 100 μ g/ml streptavidin solution (Thermo Fisher) in DMEM for 10 min at 37°C. Host cells were then washed twice with DMEM and infected with biotinylated bacteria at an MOI of 200–300 bacteria per host cell. Invasion assay was then carried out as described above. Streptavidin removal served as a negative control in microscopy and flow cytometry experiments.

To measure PE-biotin incorporation into A431D cells, cells were prepared for flow cytometry as described above. Once in the FACS tube, cells were incubated with FITC-conjugated streptavidin (Amersham) diluted to 1 μ g/ml in PBS. Cells were incubated on ice for 20 min, washed once with PBS, and analyzed with a BD FACScan.

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