Lmo1656 is a secreted virulence factor of Listeria monocytogenes that interacts with the sortin nexin 6-BAR complex

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Running title: Lmo1656 interacts with the SNX-BAR complex

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ABSTRACT

Listeria monocytogenes (Lm) is a facultative intracellular bacterial pathogen, the causative agent of listeriosis, a rare but fatal disease. During infection, Lm can traverse several physiological barriers; it can cross the intestine and placenta, and in immunocompromised individuals, the blood-brain barrier. With the recent plethora of sequenced genomes available for Lm, it is clear that the complete repertoire of genes used by Lm to interact with its host remains to be fully explored. Recently, we focused on secreted Lm proteins, since they are likely to interact with host cell components. Here, we investigated a putatively secreted protein of Lm, Lmo1656, that is present in most sequenced strains of Lm, but is absent in the non-pathogenic species Listeria innocua. Lmo1656 gene is predicted to encode a small, positively-charged protein. We show that Lmo1656 is secreted by Lm. Furthermore, deletion of the lmo1656 gene (Δlmo1656) attenuates virulence in mice infected orally but not intravenously, suggesting that lmo1656 plays a role during oral listeriosis. We identified sorting nexin 6 (SNX6), an endosomal sorting component and BAR-domain containing protein, as a host cell interactor of Lmo1656. SNX6 colocalizes with wild-type Lm during the early steps of infection. This colocalization depends on Lmo1656 and RNAi of SNX6 impairs infection in infected tissue culture cells, suggesting that SNX6 is utilized by Lm during infection. Our results reveal that Lmo1656 is a novel secreted virulence factor of Lm that facilitates recruitment of a specific member of the sorting nexin family in the mammalian host.

INTRODUCTION

The foodborne pathogen, Listeria monocytogenes (Lm), can cross several physiological barriers and infect multiple cell types. The pathogenic potential of Lm relies on the ability of this bacterium to cross multiple physiological barriers, as well as its ability to enter and replicate within a wide variety of host cell types (reviewed recently (1, 2)). Upon binding to host cell surface receptors, Lm induces its internalization into both professional phagocytes and nonphagocytic cells (reviewed recently (2)). From there, Lm escapes into the cytosol by rupturing its vacuole. Lm is able to evade host cell immune responses (reviewed recently (3)) and subvert the host cell actin
cytoskeleton to drive intra- and intercellular motility (reviewed recently (4–6)).

Secreted and surface exposed Lm proteins can encounter host components and serve as virulence factors. For example, the secreted pore forming toxin Listeriolysin O (LLO) is one of the most well-characterized and potent virulence factors of Lm (reviewed by (7)). Secretion of LLO occurs prior to Lm entry into the host cell. It inserts into the host plasma membrane and makes large pores. The resulting ion flux drives a diverse array of responses within the cell, from global deSUMOylation (8) to mitochondrial fragmentation (9). Upon entry, Lm can escape into the host cytosol by lysing the phagosomal membrane through the combined actions of secreted LLO and the phospholipases A and B (PlcA and PlcB) (10–12).

Recent work has uncovered novel secreted Lm virulence factors and their binding partners in the host cell. The secreted protein Listeria nuclear targeted protein A (LntA) targets the host epigenetic regulator BAHD1, altering host cell transcription (13). The small secreted protein Internalin C (InlC) sequesters Tuba, a Cdc42 guanine exchange factor, to induce relaxation of membrane cortical tension thereby facilitating increased bacterial cell-to-cell spread (14, 15). InlC also directly binds to host IKKα, interfering with host innate immunity (16).

The recent plethora of genomics data and the rise of bioinformatics pipelines have enabled the rapid comparison of multiple bacterial strains and species (17–19). It is clear that the complete repertoire of proteins with which Listeria infects its host, and targets host cell functions, remains to be fully explored. Many intracellular bacteria co-opt endomembrane trafficking to promote replication and spread. The sorting-nexins (SNXs) are conserved proteins that play a role in endomembrane trafficking. Their defining feature is the phox homology (PX) domain which allows binding to different phosphoinositides (reviewed (20)). The SNX-BAR subfamily of proteins is composed of SNX1/2/5/6/32 that contain, in addition to a PX domain, a Bin/amphiphysin/Rvs (BAR) domain thought to sense or induce membrane curvature and tubulation as well as mediate dimerization. Heterodimers of either SNX1/2 with either SNX5/6/32 then form a complex with the core retromer components (20). The SNX-BAR – Retromer complex captures endosomal cargo for retrograde trafficking to the Golgi network.

To search for novel putative virulence factors of Listeria, we performed a bioinformatics screen for genes present in Lm but absent in the closely-related, but non-pathogenic Listeria innocua (13). Here, we uncover the predicted secreted protein Lmo1656 as an additional—virulence factor of Lm. We show that Lmo1656 is indeed a secreted protein. Lmo1656 plays a role during a murine model of infection, revealing that lmo1656 is a bona fide virulence factor of Lm. We show that lmo1656 contributes to virulence in mice infected via the oral, but not by intravenous route, suggesting a role during the gastrointestinal phase of infection. Furthermore, we uncover the endosomal sorting protein sorting nexin 6 (SNX6) as a eukaryotic host target protein of secreted Lmo1656. The related sorting nexins SNX5 and SNX6 are recruited to Lm entry sites. Recruitment of SNX6 is abrogated when cells are infected with Lm Δlmo1656, suggesting that lmo1656 contributes to SNX recruitment. Other members of the SNX-BAR – retromer complex, SNX1 and SNX2, are not recruited to Lm entry sites, suggesting a possible differential recruitment and role of SNX-BAR proteins during infection. Together, these results uncover lmo1656 as a secreted Lm virulence factor that leads to the recruitment of distinct members of the SNX-BAR retromer complex.

**RESULTS**

_Lmo1656 is conserved in clostridia and bacilli._ To identify novel virulence factors of Lm, we performed a bioinformatics screen to identify putative secreted proteins that are present in Lm but absent in the closely-related but non-pathogenic Listeria innocua. One such candidate gene, _lmo1656_, is conserved in 59/70 (84.3%) of sequenced Lm strains (Figure 1A) (17) and absent mainly in lineage III which is itself poorly represented in clinical isolates. _Lmo1656_ is conserved in several other bacterial
species, mainly the *clostridia* and *bacilli* class of Gram-positive bacteria (Figure 1B). Interestingly, a homolog of *Lmo1656*, a *Salmonella enterica* serovar Agona hypothetical protein (NCBI Reference WP_085417617.1), is the only homolog found from a Gram-negative bacterium. However, in all cases, the function or functions of these hypothetical proteins are unknown.

The N-terminus of Lmo1656 harbors a predicted signal peptide for Sec-dependent secretion. The predicted mature Lmo1656 protein is 12.5 kDa and 113 residues in length with an isoelectric point of 10.0. However, no other significant domains or motifs are apparent (SMART/Pfam). Together, these data suggest that Lmo1656 is a putative secreted, small, positively-charged *Lm* candidate virulence factor.

**Lmo1656 is a secreted protein.**

To assess whether Lmo1656 is secreted, we assayed for its presence in growth medium supernatant. We created an *Lm* strain stably overexpressing full-length Lmo1656 tagged at the C-terminus with 2xFLAG (Lmo1656-FLAG) under the constitutive pHyper promoter using the integrative plasmid pAD (21). Overexpressed Lmo1656-FL, but not EF-Tu (a cytosolic non-secreted *Lm* protein used as a control) (22), is detected and can be immunoprecipitated from the growth medium supernatant of exponentially-growing *Lm* using anti-FLAG resin or an antibody we generated against Lmo1656 (Figure 2A, 2B). However, we were unable to detect endogenous Lmo1656 from the bacterial pellet or the growth medium supernatant from *Lm* WT, likely due to levels of expression below the sensitivity of the antibody.

To address whether Lmo1656-FLAG can be secreted into the cytosol of infected mammalian cells, we infected human choriocarcinoma JEG3 cells with either WT or *Lm* overexpressing Lmo1656-FLAG. Using an anti-FLAG antibody, Lmo1656-FLAG can be immunoprecipitated from the soluble fraction of JEG3 cell lysate infected with *Lm* overexpressing Lmo1656-FLAG but not WT *Lm* expressing Lmo1656 at endogenous levels (Figure 2C). Together, these results show that overexpressed Lmo1656 can be secreted as a soluble protein from bacteria grown in BHI and from infected mammalian cells.

We next sought to examine the localization of Lmo1656 in the cytoplasm of host cells. We transiently transfected HeLa cells with plasmids encoding Lmo1656-GFP and analysed its localization by confocal microscopy. Notably, overexpressed Lmo1656-GFP localized to puncta scattered throughout the cytoplasm with enrichment around the perinuclear region (Figure 2D). This pattern is reminiscent of proteins involved in endomembrane trafficking, suggesting that Lmo1656 might regulate host membrane trafficking during Listeria infection. Altogether, our results demonstrate that Lmo1656 can be secreted from bacteria and localizes to endomembranes.

**Lmo1656 is a virulence factor.**

To test whether *lmo1656* plays a role during infection, we infected tissue culture cells and mice. We first complemented the Δ*lmo1656* mutant by chromosome integration of a plasmid encoding the entire *Lmo1656* ORF preceded by the putative *Lmo1656* promoter region. We then infected tissue culture cells with the *Lm* WT, deletion mutant, and its complemented strain and assayed for recovered bacteria surviving the standard gentamicin assay for internalized bacteria. We found that there were fewer *Lm* Δ*lmo1656* at early time points of infection in HeLa cells as compared to infection with *Lm* WT. In contrast, there was no difference in bacterial counts between *Lm* WT and Δ*lmo1656* in Caco-2 cells and bone-marrow-derived macrophages (Figure 3A, 3B). Since entry into HeLa cells is mainly dependent on the host receptor Met, whereas entry into the other assayed cell types can utilize both the Met receptor and E-Cadherin (2), our results suggest that *lmo1656* may play a role during early Met-dependent infection. Lmo1656 could potentially promote adherence to cells and/or endocytosis of *Lm* or the escape from primary vacuole, two early steps in *Lm* infection.

To assess whether *lmo1656* is a virulence factor *in vivo*, we infected mice with *Lm* WT, Δ*lmo1656*, and its complemented strain. Deletion of *lmo1656* had no effect on bacterial burden in the liver or the spleen 72 hpi in
intravenously-infected BALB/c mice (Supplemental Figure 1A). To assess whether lmo1656 contributes to oral listeriosis, we infected mice that express humanized E-Cadherin_E16P via oral gavage. This point mutation in E-Cad mimics the docking site of human E-Cad with the Lm surface protein Internalin A and renders mice more susceptible to Lm oral infection (23). Whereas intravenously-infected mice displayed no difference in infection, orally-inoculated mice infected with Δlmo1656 had a reduction in bacterial burden in the liver 48 hours post infection (hpi) and 72 hpi (Figure 3C, 3D). Notably, there is also a decrease 72 hpi in spleens of mice infected with LmΔlmo1656. There is no difference in bacterial burden in the mesenteric lymph nodes or the intestinal content between the three Lm strains either at 48 or 72 hpi (Supplemental Figure 1B). While we see no difference in intracellular Lm WT and Lm Δlmo1656 in the small intestine, there is a significant difference between LmΔlmo1656 compared to its complemented strain (Figure 3D). Therefore, lmo1656 contributes to Lm virulence during oral, but not intravenous, infection in vivo.

Listeria entry recruits distinct members of the SNX-BAR family.

To assess the mechanisms by which Lmo1656 contributes to virulence, we sought possible eukaryotic binding proteins of Lmo1656. Using a human placental cDNA library as bait, we performed a yeast two-hybrid screen against the predicted mature form of Lmo1656. We identified the sorting nexin BAR protein SNX6 as a predicted high-confidence direct interaction partner of Lmo1656. To test whether Lmo1656 and SNX6 do interact, we transiently transfected HeLa cells with plasmids encoding GFP-SNX6 and plasmids encoding either Lmo1656-FLAG, empty vector or GFP alone. With an anti-FLAG immunoprecipitation, we were able to co-immunoprecipitate GFP-SNX6 along with Lmo1656-FLAG (Figure 4A). These results indicate that Lmo1656 and SNX6 can biochemically interact.

Immunofluorescence analysis using Lmo1656-GFP showed colocalization of Lmo1656 and endogenous SNX6 in a subset of vesicular structures scattered throughout the cytoplasm (Figure 4B). However, when Lmo1656 was tagged with FLAG, its localization appeared more diffuse (Supplemental Figure 2). In order to ensure that the vesicular localization of Lmo1656-GFP was not due to the GFP, we expressed GFP alone. GFP showed a diffuse staining through the cytoplasm and did not colocalize with endogenous SNX6 (Figure 4B). It is possible that the positive charge of the FLAG tag at least partially altered Lmo1656 localization.

We subsequently tested whether SNX6 contributes to Lm infection. We had previously performed a genome-wide RNAi screen and found SNX6 as one of the top candidate genes contributing to Lm infection (24). However, the bacterial strain Lm EGD-e PrfA* was used in this screen, which is more invasive than the reference strain Lm EGD-e. When we used Lm EGD-e PrfA*, we independently confirmed these results (Figure 4C and Figure 4D). We then sought to determine the role of SNX6 during infection. Since lmo1656 contributes to early infection of certain types of cultured cells (Figure 3A, 3B), we first assessed whether the subcellular localization of its putative host target SNX6 is affected during infection. Using differentially-labelled surface-exposed versus internalized Lm (25), we found that endogenous SNX6 was recruited to invading Lm in HeLa and Caco-2 cells (Figure 5A, 5C). We then assessed if lmo1656 contributes to the recruitment of SNX6 to internalizing Lm. Endogenous SNX6 colocalizes with surface-localized and some internalized Lm WT, but not with Lm Δlmo1656 in HeLa cells (Figure 5A, 5B). Colocalization analysis revealed that 93% of extracellular Lm colocalize with SNX6, whereas only 7% of extracellular Lm Δlmo1656 colocalize with SNX6. However, no such difference is convincingly displayed when infecting Caco2 cells (Figure 5C, 5D). In addition, we found that SNX6 transiently associates with invading Lm, as the colocalization between Lm and SNX6 drops to 40% once bacteria are internalized. These results suggest that SNX6 is transiently recruited to internalizing Lm in an lmo1656-dependent manner, in cell types which utilize...
InlB/Met entry, a finding which parallels the effect of Lmo1656 on early step of infection (Figure 3A, 3B).

Since the SNX-BAR proteins assemble as a heterodimer of either SNX1/2 with SNX5/6 as part of the SNX-BAR – retromer complex (20), we wondered whether other components of the SNX-BAR complex are also recruited to internalizing Lm. We transiently-transfected HeLa cells with GFP-SNX constructs (26, 27) to assess their subcellular localization upon infection. GFP-SNX6 colocalizes in HeLa cells with WT adherent and internalizing Lm, but not with Δlmo1656 Lm (Figure 6E, 6E’), similarly to endogenous SNX6 (Figure 6A). GFP-SNX5 (Figure 6D, 6D’) is also recruited to Lm entry sites in an lmo1656-dependent manner. We found 91.5% of Lm colocalized with SNX5 at 2 hpi. Interestingly, the association of SNX5 with Lm seems to have different dynamics compared to SNX6. Indeed, SNX5 colocalized with internalized Lm to greater extent (82%) compared to SNX6 (40%), suggesting a differential role of the two proteins during infection. In contrast, neither GFP-SNX1 nor GFP-SNX2 are recruited to internalizing Lm (Figure 6A, 6B). This is somewhat surprising since either SNX1 or SNX2 are known to form a heterodimer with either SNX5 or SNX6 (20, 28). Additionally, an unrelated sorting nexin, GFP-SNX3 (Figure 6C), is not recruited to Lm entry sites, suggesting a specific recruitment of SNX5/6 to internalizing Lm.

Together, our results have uncovered Lmo1656 as a novel secreted virulence factor in Lm infection. They suggest a role of Lmo1656 in recruiting some members of the SNX-BAR subfamily of proteins to Lm entry sites.

**DISCUSSION**

In this report, we describe the secreted protein Lmo1656 as a novel virulence factor during *Listeria monocytogenes* infection. This small positively-charged protein is secreted from bacteria and interacts with the SNX-BAR subfamily protein SNX6. Deletion of *lmo1656* lowers infection levels in certain cell types during early infection *in vitro* and reduces bacterial load in the liver following oral infection *in vivo*. Certain members of the SNX-BAR subfamily proteins are recruited to sites of Lm entry suggesting that Lm infection may assemble an infection-specific sorting nexin complex. In addition, SNX6 is required for effective Lm infection.

We used comparative genomics to identify putative virulence factors absent in non-pathogenic *Listeria* species but present in Lm. Our data revealed Lmo1656 as a bona fide Lm virulence factor. Homologs of *lmo1656* are found in most sequenced strains of Lm but are mainly absent in lineage III strains of Lm, which are poorly represented in clinical isolates (29, 30). Strikingly, we found homologs of *lmo1656* in other pathogenic bacterial species, mainly in the Gram-positive *bacilli and clostridia*. One notable exception is the *lmo1656* homolog in the Gram-negative *Salmonella enterica* subsp. *enterica* serovar Agona, which has recently been implicated in human disease (31–33). This homolog in *Salmonella*, while sharing a high degree of identity with *lmo1656*, is predicted to encode a smaller protein and could thus interact with different cellular partners than Lmo1656. Whether Lmo1656 homologs have conserved functions in these other species remains to be explored.

We found Lmo1656 to play a role during early step of infection in HeLa cells, a cell line that relies on Met receptor for Lm invasion. Since deletion of *lmo1656* lowers bacterial load in HeLa cells at early time points, this suggests that Lmo1656 might be implicated either in the interaction of *Listeria* with target cells (by modulating adhesion and/or internalization) or in the escape from primary vacuole via a still unidentified mechanism. Lm entry is known to recruit, in addition to Met, a number of host cell components including EEA1, Cbl, clathrin, clathrin adaptor protein-1 (AP-1), dynamin (34, 35), and was recently found to recruit components of the exocytic machinery (36). We have uncovered that certain members of the SNX-BAR subfamily of proteins are recruited to sites of Lm entry. Since the SNX-BAR complex is thought to be composed of a heterodimer of SNX1/2 and SNX5/6 (20), differential
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recruitment could constitute novel infection-related SNX-BAR complexes (24). We determined that Lmo1656 interacts with SNX6, which we had previously identified as a human gene modulator of Lm infection through a genome-wide RNAi screen (24). Notably, SNX6 is the only member of the SNX-BAR subfamily that significantly contributes to Lm infection of HeLa cells, as the results from the genome-wide screen for the other components of the SNX-BAR – retromer complex were less clear. Interestingly, the distinct effects of different sorting nexins on infection parallel the non-canonical recruitment of SNX5/6 to sites of Lm entry. Recently, an unrelated sorting nexin, SNX10, was implicated in controlling Lm infection in mouse macrophages (37) through an effect on phagosomal maturation. In contrast, SNX6 is required for productive infection in epithelial cells and lmo1656 deletion does not alter bacterial growth in macrophages. It is tempting to speculate that distinct sorting nexins, which have different roles during endomembrane trafficking, have distinct effects during bacterial infection. We had initially hypothesized that Lmo1656 could disrupt the retromer complex, but since we did not find evidence to support that hypothesis, we cannot exclude that sorting nexins 5 and 6 could in some way act as sensors of Listeria infection. Future studies will elucidate which of these potential mechanisms is at play during Listeria infection.

Interestingly, SNX-BAR proteins are recruited to Chlamydia trachomatis inclusion bodies (38) via direct interaction with a secreted virulence factor (38–41). In this case, all members of the SNX-BAR family (SNX1/2/5/6) are recruited to the inclusion bodies and induce membrane tubulation. SNX-BAR proteins then reduce Chlamydia infectivity (38), possibly by promoting lysosomal function, though this is currently unclear (40). Here, we show an interaction between a secreted Lm protein and the sorting nexin-BAR family proteins. Notably, only a distinct subfamily of the SNX-BAR proteins appear recruited by Lm to sites of invasion. It will be interesting to test whether Lm perturbs, in addition to lysosomal integrity (42), lysosomal trafficking during early infection.

Sorting nexins are also targeted by Salmonella enterica (reviewed recently (43)). SNX1 is recruited during early Salmonella infection to Salmonella-containing vacuoles (SCVs), where SNX1 facilitates the removal of CI-MPR from SCVs (44). SNX3 is also recruited to SCVs and plays a role in the recruitment of host factors and thus plays a role in SCV maturation (45). Other work has further implicated the disruption of CI-MPR trafficking (46). A recent proteomics approach of SCVs has also revealed the core retromer component VPS26B as a member of SCVs (47). It would be appealing to determine if the homolog of lmo1656 in Salmonella plays a role in SNX1 or SNX3 recruitment during infection.

Notably, lmo1656 plays a role during infection in vivo. The lack of a detectable effect in intravenously infected mice, compared to orally-infected mice is intriguing. The observation that after oral infection there is no difference in bacterial load in intestinal content or in intestinal cells despite a significant difference in bacterial load in the liver, strongly suggests that Lmo1656 positively affects transcytosis in the intestine across the goblet cells. We propose that transcytosis of bacteria may be controlled by an Lmo1656-dependent effect on SNX proteins.
Experimental Procedures

**Molecular cloning**

Deletion of *lmo1656* was created as previously described (13). Briefly, PCR products of ~600 bp upstream and downstream of the *lmo1656* open reading frame (ORF) were fused via splicing by overlap extension (SOE) into the pMAD vector with appropriate restriction sites. To create a plasmid for complementation of deletion mutants, a pAD plasmid (21) was created with the full-length *lmo1656* ORF with the predicted promoter (200 bp upstream of the detected transcription start site) to generate the plasmid pEndo-1656.

To create a plasmid for the overexpression of full-length *Lmo1656*-FLAG, the entire *lmo1656* ORF, with a 2X FLAG at the C-terminus, was synthesized as a gBlock (Integrated DNA Technologies) and subcloned into the integrative plasmid pAD (21) using the appropriate restriction sites.

To create a plasmid for the overexpression of *Lmo1656*-GFP in mammalian cells, the cDNA encoding the predicted mature form of *Lmo1656* was codon optimized for human expression and synthesized (GeneCust) with 2X FLAG at either the N- or C-terminus of *Lmo1656*. The resulting construct was then subcloned into pCDNA3.1 using the appropriate restriction sites.

**Listeria monocytogenes mutant construction**

Electrocompetent *Lm* was transformed using standard methods (48). Briefly, a culture of *Lm* was grown overnight in Brain-Heart Infusion growth medium (BHI). This overnight culture was diluted in fresh BHI supplemented with 500 mM sucrose (sterile-filtered), then later supplemented with 10 μg/mL of ampicillin and grown shaking at 37°C to exponential phase (*OD600nm = 0.8 – 1*). The bacterial pellet was washed several times in cold electroporation buffer (10% glycerol, 500 mM sucrose pH 7, sterile-filtered) then snap frozen in aliquots. *Listeria* was electroporated with 1 μg of plasmid.

To verify the lack of off-target mutations, the chromosomes of EGDe and two independent strains of EGDe-*Δlmo1656* were sequenced (Genopole, Institut Pasteur).

**Generation of antibodies**

Peptide fragments encoding 17 residues near the N-terminus and C-terminus of *lmo1656* were generated (RRAVNGATNGKYHSLNK and EKAMDWYTVKIEGTISN, respectively) and coupled to KLH were created as antigens (GeneCust). Two separate rabbits were injected with each antigen supplemented with Freund’s adjuvant (Covalabs). The resulting affinity-purified antibodies were then pooled per antigen and dialyzed to 1mg/mL in PBS/50% glycerol.

**Secretion assays**

*Lm* overexpressing the full-length *lmo1656* and tagged at the C-terminus with 2XFLAG were grown in BHI to exponential phase. After centrifugation, the BHI supernatant was kept on ice then sterile filtered (0.2 μm). ~2μg of protein (Bradford assay) were subjected to immunoprecipitation overnight at 4 ºC with either 20 μL of wet packed beads of M2-agarose (Sigma) or rabbit antibodies raised against *Lmo1656* (0.5 μg of affinity-purified antibody) followed by precipitation with Protein A-agarose beads (GE).

For intracellular secretion assays, exponentially-growing *Lm* were washed 3x in PBS then used to infect JEG3 cells at a multiplicity of infection (MOI) of 20 for a total of 4 hours post infection (hpi). Cells were lysed in 50 mM Tris HCL pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, phosphSTOP (Roche), and complete protease inhibitor cocktail (Roche). The crude cell lysate was centrifuged at 5000g for 15 minutes at 4 ºC to remove cellular debris, nuclei, and bacteria. ~2μg of protein from the soluble cell fraction was then subjected to immunoprecipitation overnight at 4 ºC with M2-agarose beads (Sigma).

**Animal Infections**

Animal experiments conformed to the Council Directive of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes (86/609/Eec). Experiments that relied on
laboratory animals were performed in strict accordance with the Institut Pasteur's regulations for animal care and use protocol, which was approved by the Animal Experiment Committee of the Institut Pasteur (approval no. 03-49).

Inocula for intravenous and oral infections were prepared as described previously (49). Briefly, an overnight Lm culture was diluted in fresh BHI and grown shaking at 37°C until OD_{600nm} = 0.8 – 1. Bacteria were centrifuged several times and washed with cold saline, resuspended with saline, then snap frozen into aliquots with liquid nitrogen.

For intravenous infections, 8-10 week old BALB/c female mice inoculated with 10^7 CFU and dissected 72 hpi. For oral infections, BALB/c were gavaged with 5 x 10^9 CFU in saline supplemented with PBS/CaCO_3. Transgenic mice expressing E-CadE16P (50) were orally-infected by gavage with 10^9 CFU in saline supplemented with PBS/CaCO_3 and dissected 24, 48, and 72 hpi. For all infections, inocula were plated to control for the number of Lm. Small intestines were washed 5x in DMEM and incubated in DMEM with 100 µg/mL gentamicin for 2 h at room temperature, then washed again 5x in DMEM prior to sonication (49). Serial dilutions of organ homogenates were plated onto either BHI-agar plates (pancreas and liver), BHI-agar plates supplemented with 50 µg/mL of nalidixic acid (mesenteric lymph nodes and small intestines), or selective Oxford plates (intestinal contents).

**Cell culture and infection**

Cells were grown in appropriate medium. HeLa and JEG3 cells were transiently transfected with Lipofectamine 2000 (plasmids, Invitrogen) or Lipofectamine RNAiMAX (RNAi, Invitrogen) using the manufacturer instructions.

Infection of cultured cells was performed as described previously (25). Briefly, exponentially-growing Lm strains were washed three times with PBS and used to infect HeLa (MOI 40), JEG3 (MOI 10), Caco2 (MOI 10), and bone marrow-derived macrophages (BMDM, MOI 5). After 1 h of infection in MEM, cells were washed 1x with complete cell culture medium supplemented with 50 µg/mL of gentamicin, then incubated with complete cell culture medium supplemented with 50 µg/mL of gentamicin. At the desired timepoints, cells were washed 2x with MEM then lysed with 0.1% TX100. Cell lysates were serially diluted in saline then spread onto BHI-agar plates. CFUs were counted either manually or with an automatic colony counter (Scan 500, Interscience).

**Identification of binding partners of Lmo1656**

A yeast two-hybrid screen was performed (Hybrigenics) using the predicted mature form of Lmo1656 (aa 31-143; fusion N-LexA-lmo1656-C) as the bait with a human placental cDNA as the source of prey. After screening 56.8 million interactions, two proteins were identified as “very high confidence” interactors of Lmo1656: SIPA1L1 (Genbank AF090990.1) and SNX6 (Genbank NM_152233.2).

**Immunofluorescence preparation and analysis**

**Sample preparation and image acquisition**

Samples were prepared as described previously (25). Briefly, tissue culture cells on glass coverslips were fixed in PBS/4% paraformaldehyde for 20 minutes, washed twice in PBS and twice in PBS/1% BSA. Samples were stored in PBS/1% BSA/0.03% sodium azide at 4°C for at least overnight until further processing.

Antibodies at the appropriate dilutions (Table 2) in PBS/1% BSA (surface staining) or PBS/1% BSA/0.1% TX100 (permeabilized/total staining) were incubated with fixed samples for 20 minutes at room temperature, washed 3x in PBS/1% BSA then incubated with the appropriate Alexa-Fluor-conjugated goat secondary antibodies (Sigma). Cells were permeabilized with PBS/1% BSA/0.1% TX-100 when required. Samples were mounted in Aqua-Poly/Mount (Tebu-Bio) and allowed to clear for at least overnight at 4°C.

**Image acquisition and analysis**

Confocal z-stacks (0.3 µm step size) were acquired on a Zeiss AxioObserver.Z1 inverted fluorescence microscope equipped with an Evolve EM-CCD camera (Photometrics) and a Yokogawa CSU-X1 spinning disk confocal system. Images were
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acquired using MetaMorph with a 100× oil objective with a N.A of 1.4.

Images were visualized using Icy Software (51). When necessary, comparisons of experiments and controls were acquired on the same session with the same image acquisition parameters and data analysis. 3D surface reconstructions of Lm with SNX6 was assembled using Imaris (Bitplane), using identical processing and thresholding when appropriate.

For assembly of figures, images were assembled using Photoshop and Illustrator (Adobe) and resized when necessary by bicubic interpolation with minimal changes at normal magnifications. Images for the same sets of experiments and controls were adjusted using the same settings to fill in the signal range over full output grayscale over the entire image.

Immunoprecipitation
HeLa cells in 10cm Petri dishes were transfected with 10µg of Lmo1656-FLAG and 7µg of SNX6-GFP or 7µg of GFP (amount per dish) by using Fugene (Promega) according to manufacture’s instruction. Twenty four hours after transfection, the cells were washed twice in phosphate-buffered saline (PBS) and lysed for 30 min with 1 ml lysis buffer/10cm dish (20 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Triton X-100) supplemented with protease and phosphatase inhibitors. Lysis and all subsequent steps were performed at 4 °C. The lysate was clarified (13000xg, 10 min) and the protein concentration of the supernatant was determined by Bradford assay (Pierce). 1 mg of lysate was incubated overnight with 30µl of anti-FLAG agarose beads (Sigma). Immune complexes were retrieved by centrifugation (500x g, 5 min). After four washes with lysis buffer, bound protein was eluted from the beads by boiling for 10 min in 30 µl Laemmli buffer. The eluate was analyzed by gradient SDS-PAGE (Biorad), and subjected to western blotting via wet transfer to 0.45µm nitrocellulose membrane (Millipore).

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Conflict of Interest
The authors declare that they have no conflict of interest with the contents of this article.

Author Contributions
DJD and AP designed and performed the experiments the in vitro and tissue culture experiments. DJD and MAN designed and performed the animal infection experiments. DJD, AP and LR prepared the figures and analyzed the data. DJD, LR, and PC wrote the manuscript and incorporated comments from all authors.
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Figures

Figure 1: Lmo1656 is a predicted secreted protein of Listeria monocytogenes.

A: Synteny of the lmo1656 locus. Lmo1656 is conserved in most sequenced strains of L. monocytogenes but absent in the closely-related but non-pathogenic L. innocua. Epidemic Lm strain F2365 is shown as an example of a clinical isolate. B: Homologs of Lmo1656 are predicted in other bacterial species, most of which are Gram-positive. Multiple sequence alignment (CLUSTALX2) of the predicted proteins,
excluding the putative Sec-dependent signal peptide. The mature form of Lmo1656 is predicted to have a MW of 12.49 kDa and an isoelectric point of 10.61.

Figure 2: Lmo1656 is a secreted protein

A. Overexpressed Lmo1656-FLAG is secreted into the growth medium. Either wild type (LmWT) or Lmo1656-FLAG-overexpressing (Lm1656-FL+) bacteria were grown to exponential phase in broth media. The sterile-filtered supernatant was immunoprecipitated against FLAG. Lmo1656-FL is immunoprecipitated in the sterile-filtered supernatant of the growth medium from Lm1656-FL+ but not LmWT bacteria. Data are representative of three independent experiments. (Pt: bacterial pellet; In: Input fraction; IP: immunoprecipitated fraction). B. Secreted Lmo1656-FLAG can be immunoprecipitated by antibodies raised against Lmo1656. Either wild type (LmWT), lmo1656 deletion mutant (LmΔ1656), or Lmo1656-FLAG overexpressing (Lm1656-FL+) bacteria were grown to exponential phase in broth media. The sterile-filtered supernatants were immunoprecipitated with a pooled mix of affinity-purified anti-Lmo1656 polyclonal rabbit antibodies (1µg Ab/50 µg protein). Samples were subjected to Western blotting with the same anti-Lmo1656 antibody pool (1:500 in TBST/5% milk). (Pt: bacterial pellet; In: Input fraction; Un: unbound fraction post immunoprecipitation; IP: immunoprecipitated fraction). C. Overexpressed Lmo1656-FLAG is secreted into infected cells. JEG3 cells were infected (MOI20) with either wild type (LmWT) or Lmo1656-FLAG overexpressing (Lm1656-FL+) bacteria. The cells were lysed 4 hpi and subjected to immunoprecipitation against FLAG. Lmo1656-FLAG is immunoprecipitated in the soluble lysate from cells infected with Lm1656-FL+ but not LmWT bacteria. Data are representative of three independent experiments. (Pt: insoluble pellet post lysis; In: input fraction; IP: immunoprecipitated fraction). D. Overexpressed Lmo1656-GFP localizes to endomembranes. HeLa cells were transiently transfected with Lmo1656-GFP for 24 h. Actin and nuclei were stained with phalloidin (phall) and DAPI, respectively. Images were acquired with a spinning disk confocal microscope. Data are representative of at least 3 independent experiments.

Figure 3: Lmo1656 is a bona fide virulence factor of Listeria monocytogenes

A-B. Lmo1656 contributes to early infection in certain cell types. LmΔlmo1656 have decreased bacteria at early timepoints (A: t=2hpi) but not later (B: t=4 or 24hpi) of infection in HeLa but not Caco2 nor mouse bone marrow-derived macrophages. Results are normalized to the mean colony forming units (CFU) for WT per replicate. (n=3 wells per replicate over at least 2 independent replicates; *, p=0.0339 ANOVA). C-D. Lmo1656 contributes to oral infection in vivo. Knock-in mice expressing a “humanized” E-CadE16p were infected with LmWT, LmΔlmo1656, or the complemented LmΔlmo1656-C via oral gavage. Mouse livers have decreased bacterial burden both (C) 48 hpi and (D) 72 hpi when infected by LmΔlmo1656, whereas spleens have decreased bacterial burden (D) 72 hpi. (n=7-8 mice per Lm genotype; *, p=0.0261 Mann-Whitney U test).

Figure 4: SNX6 and Lmo1656 interact biochemically and genetically

A. GFP-SNX6 and Lmo1656-FLAG biochemically interact. HeLa cells were transiently transfected with GFP-SNX6 or GFP and either empty vector (-) or Lmo1656-FL (+). Anti-FLAG immunoprecipitation was performed on clarified lysate 48 h post transfection. GFP-SNX6 can be co-immunoprecipitated along with Lmo1656-FLAG from cells transfected with Lmo1656-FLAG but not with empty vector. Results are representative of two independent experiments. B. Lmo1656-GFP colocalizes with SNX6. HeLa cells were transiently transfected with Lmo1656-GFP (upper panel) or GFP (lower panel) and stained for endogenous SNX6. Inset: magnification of colocalizing Lmo1656-GFP and SNX6. C. SNX6 contributes to Lm infection. HeLa cells were transiently transfected with either non-targeting siRNA pool (Control) or an siRNA pool targeting SNX6. 72 h post transfection, cells were infected with Lm EGDe-PrfA* and lysed 2, 3 and 5 hpi. (*, p<0.05). Results are in triplicate from two independent experiments.
D. Knock down of SNX6 in HeLa cells. HeLa cells were either treated with scramble siRNA (-) or siRNA against SNX6 (+). After 72h, protein levels were analyzed by Western blotting with the indicated antibodies.

**Figure 5: SNX6 colocalizes with Lm entry sites in an lmo1656-dependent manner**

A-B. Endogenous SNX6 is recruited to internalizing Lm in an lmo1656-dependent manner in HeLa cells. HeLa cells infected with either Lm WT (A) or Lm Δ1656 (B) (MOI 40, 2 hpi) and stained for external and internalized bacteria. 3D surface reconstruction (A,ii) of endogenous SNX6 recruited to Lm WT entry sites (Imaris).  

C-D. Endogenous SNX6 is recruited to internalizing Lm in an lmo1656-independent manner in Caco2 cells. Caco2 cells were infected with either Lm WT (C) or Lm Δ1656 (D) (MOI 10, 2 hpi) and stained for external bacteria. Internalized Lm WT (C’) or Lm Δ1656 (D’) Lm colocalize with SNX6. Magnification of bacteria (purple squares) in adjacent images. Data are representative of at least three independent experiments; controls (Lm WT or Lm Δ1656) for each cell type were imaged at the spinning disk confocal microscope during the same session using identical settings and, where necessary, identical adjustments for brightness and contrast. (A-B vs C-D).

**Figure 6: Certain GFP-SNX-BAR proteins are recruited to Lm entry sites in an lmo1656-dependent manner**

A-C. Lm entry sites do not recruit a subset of GFP-SNX proteins. HeLa cells transiently transfected with GFP-SNX constructs and stained for external Lm (MOI 20, 2 hpi), (A) GFP-SNX1, (B) GFP-SNX2, (C) GFP-SNX3.  

D-E. Lm entry sites recruit some GFP-SNX-BAR family proteins. HeLa cells transiently transfected with GFP-SNX-BAR constructs and stained for external Lm WT; (D, D’) GFP-SNX5 with Lm WT or Lm Δ1656; (E, E’) GFP-SNX6 with Lm WT or Lm Δ1656 respectively. Magnification of bacteria (purple squares) in adjacent images. Data are representative of at least three independent experiments; controls (Lm WT or Lm Δ1656) were imaged at the spinning disk confocal microscope during the same session using identical settings and, where necessary, identical adjustments for brightness and contrast.
ASNKIVALMARAKAFKEHNLNMTKINNSAAGFNSK--NSTVHIHEKFLGDYFGRVSLS-------FNYKYKEFFTKANA-KKKKKKAEAA-MDWTYKIEKISK---
ASNKIVALMARAKAFKEHNLNMTKINNSAAGFNSK--NSTVHIHEKFLGDYFGRVSLS-------FNYKYKEFFTKANA-KKKKKKAEAA-MDWTYKIEKISK---

Salmonella_enterica_Ser. Agona

VSQNVNISMLHEETNGAKNNKYHKLSKGYANLKLSVAASGNAA--PTVTVSLMKEKFGFDSSYGKRNFTP----------GKKWSYKTTTHQYYVDGNN---SSYYLVAEKS-GRYYEVRATGTLKN--

VSQNVDISMQHEVTNGAKNNKYHKLSKGYANLKLSVAASGAAT--PTVTVSLMKEKFGFDTSYGKRNFIP----------GKKWSSKTTTHQYYVDGNN---SSYYLVAEKS-GRYYEVRATGTLKN--

Listeriaceae_bacterium

VSQNVNIKFQHEVVNGAKNGKYHKLKKGYANLQLIVSAEGKVA--TPVTITLKKERFGFDSSYGTRTFIP----------GKSYTGKTTTHQYYIDGD---SSKYYLIAKDN-RYYYYWITA--

Listeria newyorkensis

--SSYSYNAVY-IVDGKANGIYHTLNKGTATIDGHAFYNGSKEN-WADGITPPGETVTYCLYREKTGFDT---------------------SYGCVNHYVSKNNDKSNVSKIKESFPSKLDSDSSKYYL

--SAYSFTMEFRVVNGKNNGIYHSLSSGSPTISGSTSVVSSKPG-AFDPYDIKYTLYKDN-------------------------------------------------------FFGDTGYGTV----

Anoxybacillus_flavithermus

--SNFSWTMDYRIVDGEKNGQIHTLHDGTIQLDGNIMTVSQDSGWTPTPTSVKIAVWEQDGALSADDVGTVQPSSTLSGSTTSFSKNGWFATDGSDSLNGETQYHSHDNNSNGCDEKTY

Paralibococcus_ryukyuensis

--SNFQWALKGNSNKTSKEFLYINSFARRNLSSKSGPCT-ACQHYNTAVKGFTTVSSTT----------GNKFPTKQDGTNYLILFSGK--SNNTTVSLS--

Bacillus_simplex

--SAYSFTMEFRVVNGKNNGIYHSLSSGSPTISGSTSVVSSKPG-AFDPYDIKYTLYKDN-------------------------------------------------------FFGDTGYGTV----

Clostridium-cellulosi

--SNFQWALKGNSNKTSKEFLYINSFARRNLSSKSGPCT-ACQHYNTAVKGFTTVSSTT----------GNKFPTKQDGTNYLILFSGK--SNNTTVSLS--

David et al, 2017

Figure 1
A

| Input                         | IP FLAG       |
|-------------------------------|---------------|
| GFP                          | - - +         |
| GFP-SNX6                     | + + -         |
| 1656-FLAG                    | - + +         |

GFP-SNX6
GFP-Lmo1656 SNX6 (endog) merge

B

GFP-Lmo1656  SNX6 (endog)  merge

GFP  SNX6 (endog)  merge

C

% CFU relative to EGDe

2 hours  3 hours  5 hours

D

D

SNX6 siRNA

50- SNX6
37- GAPDH

David et al, 2017
Figure 4
-Lmo1656 is a secreted virulence factor of Listeria monocytogenes that interacts with the sortin nexin 6-BAR complex
Daryl Jason David, Alessandro Pagliuso, Lilliana Radoshevich, Marie-Anne Nahori and Pascale Cossart

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