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Listeria Monocytogenes can Utilize both M Cell Transcytosis and InlA-Mediated Uptake to Cross the Epithelial Barrier of the Intestine during an Oral Infection Model of Listeriosis

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LISTERIA MONOCYTOGENES CAN UTILIZE BOTH M CELL TRANSCYTOSIS AND INLA –
MEDIATED UPTAKE TO CROSS THE EPITHELIAL BARRIER OF THE INTESTINE DURING AN
ORAL INFECTION MODEL OF LISTERIOSIS

THESIS

A thesis submitted in partial fulfillment of the requirements of Master of Science in the
College of Medicine at the University of Kentucky

By

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Lexington, Kentucky

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Medicine

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2014

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ABSTRACT OF THESIS

LISTERIA MONOCYTOGENES CAN UTILIZE BOTH M CELL TRANSCYTOSIS AND INLA – MEDIATED UPTAKE TO CROSS THE EPITHELIAL BARRIER OF THE INTESTINE DURING AN ORAL INFECTION MODEL OF LISTERIOSIS

The invasive pathways, InlA- and InlB-mediated uptake and M cell transcytosis, that *Listeria monocytogenes* uses to invade the intestine have mainly been studied using infection models that do not truly replicate what occurs during a natural infection. Recently, our lab has developed an oral infection model that is more physiologically relevant to what occurs during food borne listeriosis. We have sought to evaluate the relative roles of the previously defined invasive pathways, in our oral model of infection. We have done this by utilizing an InlA^{mCG} *Lm* strain that is able to bind murine E-cadherin, knockout *Lm* strains, $\Delta inlA$ *Lm*, and $\Delta inlA\Delta inlB$ *Lm*. We also took advantage of a knockout mice strain CD137^{-/-} that has M cells that are deficient in M cell transcytosis. We were able to show that these invasive pathways are relevant in our oral infection model, that M cell transcytosis is a compensatory pathway for InlA-mediated uptake, and that there might be another mechanism that *L. monocytogenes* uses to invade the intestines. To confirm this, it is necessary though that the M cell transcytosis deficiency be confirmed in the CD137^{-/-} mice.

KEYWORDS: *Listeria monocytogenes*, oral infection model, InlA – mediated uptake, M cell transcytosis, invasion

Hilary Lynn Denney

May 7, 2014

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Introduction

Listeria monocytogenes is a facultative intracellular bacterium that causes food borne disease in humans. Clinically, an infection caused by *L. monocytogenes* can present as a mild, self-limiting gastroenteritis or can present as severe septicemia and meningoccephalitis [1,4]. Even with the use of antibiotics to treat listeriosis the average mortality rate reaches 20-30% [1,4]. *L. monocytogenes* can also cause severe fetoplacental infections that eventually lead to abortion of the fetus. Most reported cases of listeriosis in adults involves the CNS and in immunocompromised patients *L. monocytogenes* is the most common form of bacterial meningitis [1]. It is not well understood what differs in the infection and the immune response between the self-limiting gastroenteritis and the severe systemic infection.

The intestine is a hollow tube with epithelial cells lining the lumen acting to provide a barrier between the lumen and lamina propria [10]. These epithelial cells also serve to process nutrients absorbed from the gut lumen. On top of the epithelial cells exists a layer of mucus, which is made up of mucin proteins. The mucus layer serves as a protective barrier to prevent easy access of the epithelial cells to potentially harmful bacteria that are in the lumen. Below the epithelial cells is the lamina propria. Within the lamina propria there are some immune cells, lymph, and lymphatic vessels that connect the lamina propria to the mesenteric lymph nodes. In order for *L. monocytogenes* to establish an infection, the bacteria must be able to cross the mucus layer, bind to epithelial cells and become endocytosed, and then invade past the

epithelial cells to the lamina propria. Once *L. monocytogenes* reaches the lamina propria it can then disseminate to secondary organs such as the mesenteric lymph nodes, liver, spleen and the brain.

Through previous research it has been shown that *L. monocytogenes* use internalin A (InIA) protein, an invasin, to bind to E-cadherin expressed on epithelial cells of the gut [2,3]. E-cadherin becomes transiently available for *L. monocytogenes* to bind when epithelial cells are extruded at the villus tips as a part of the normal regeneration of the intestinal lining [4]. *L. monocytogenes* has also been shown to bind to the hepatocyte growth factor receptor, c-Met, through internalin B (InIB) [5]. The receptor c-Met is found at the multicellular junction between epithelial cells of the intestinal lining. This receptor can become transiently available when epithelial cells are extruded at villus tips. It has been shown that InIB assists invasion of *L. monocytogenes* by working in conjunction with binding of InIA to E-cadherin [5]. It is thought that InIA provides the specificity to epithelial cells by binding its receptor E-cadherin first, then InIB-binding to c-Met acts to promote and quicken endocytosis of *L. monocytogenes* at the cell extrusion site [5].

A second way that *L. monocytogenes* can invade the intestine is through M cell transcytosis [6,7]. M cells are a unique cell type that are part of the follicle-associated epithelium that lies on top of Peyer's patches [8]. Peyer's patches are lymphoid follicles found throughout the intestines, existing in the most concentrated numbers in the distal small intestine, that can elicit a quick immune responses to pathogens invading the intestine. The M cells on top of the Peyer's patches cells are unique in their ability to

transcytose microparticles from the lumen to their basolateral side [8]. They are also distinct in morphology in comparison to epithelial cells that make up the rest of the follicle associated epithelium and the epithelial barrier of the gut; a hallmark of a fully functional M cell is the presence of a basolateral pocket below the M cell that contains B cells, dendritic cells, as well as other immune cells [8,11]. The M cell's normal function is to sample the contents of the lumen of the intestine and present antigen to the immune cells residing in the Peyer's patch [9,10]. Enteric pathogens such as *Salmonella typhimurium* and *Shigella flexneri*, as well as *L. monocytogenes*, can take advantage of the natural immune surveillance function of M cells to translocate across the epithelial barrier of the intestine [8].

A significant amount of research has focused on how *L. monocytogenes* is able to invade the gut and spread to secondary organs. However, studying *L. monocytogenes* in animal models has been difficult because the internalin proteins that *L. monocytogenes* uses to bind and invade cells, and the binding of these to their receptors have a species tropism. Focusing on how *L. monocytogenes* is able to invade the intestine could help delineate if there are differences in the early invasive characteristics of a mild *L. monocytogenes* infection versus a severe *L. monocytogenes* infection.

These aspects of *L. monocytogenes* invasion have mostly been characterized in infection models, such as intravenous infection, intragastric inoculation, ligated loop assay, and cell culture systems. While these model systems have shed light onto the invasive routes that *L. monocytogenes* can utilize, they are not truly reflective of what occurs in a natural infection of *L. monocytogenes*. Recently, our lab has developed a

food borne infection model that can be used to physiologically replicate what occurs during human listeriosis [12]. We sought to evaluate these routes of invasion in our food borne model of infection. Our hypothesis was that, while the internalin invasion pathways might contribute to the majority of infection seen in the intestines, other pathways, such as M cell transcytosis, would also significantly contribute to establishment of infection. We also thought that even without one of the pathways available, if the other were present, than *L. monocytogenes* would still be capable of colonizing the intestines. To do this we used a murinized strain of *L. monocytogenes*, InIA^mCG *Lm*; this strain has a modified internalin A protein that enables it to bind to mouse E-cadherin [13]. We also used a deletion strain, *ΔinIA Lm*, that lacks the internalin A protein (provided by Cormac Gahan: University College Cork, Ireland), and a double deletion strain *ΔinIAΔinIB Lm* strain that was created in our lab, which lacks both the internalin A and internalin B proteins. These strains of *L. monocytogenes* were used to evaluate the role internalin-mediated uptake during invasion after a food borne infection. To evaluate the role of M cell transcytosis in the establishment of infection, we used a CD137-deficient mouse that had M cells that were deficient in M cell transcytosis (provided by David Lo: Univ. of California Riverside) [14]. During the development of M cells, crypt stem cells at the bottom of intestinal villi receive signals, such as lymphotoxin, that commits the stem cell to an M cell fate [14]. These committed M cells begin to express CD137 and CCR6+ B cells that are migrating to the CCL20+ lymphoid follicle interact with the CD137 expressing M cell [14]. Through signaling between CD137/CD137L the M cell and B cell establish a “bond”[14]. This bond causes

an induction of cytoskeletal changes in the M cell that causes the formation of the basolateral pocket and the development of transcytosis capability [14]. Because the CD137^{-/-} mice are deficient in CD137, M cells in the intestine develop to be structurally normal, but have a significantly reduced capability of transcytosing microparticles from the lumen to their basolateral side [14]. In using these strategies to study the role of the invasion pathways during a food borne infection model, we were able to show that *L. monocytogenes* does utilize internalin-mediated and M cell transcytosis as pathways for invasion in a food borne model of infection. It appears that M cell transcytosis can compensate for not having InIA-mediated uptake during colonization. When internalin-mediated pathways of invasion are knocked down, InIA/InIB, as well as M cell transcytosis, *L. monocytogenes* is still capable of invading the intestine. This suggests that while the internalin-mediated and M cell transcytosis pathways are predominantly responsible for establishment of infection, there may be other pathways that *L. monocytogenes* can use to colonize the intestines.

Results

InIA^mCG infects the colon and ileum of BALB/c/J and CD137^{-/-} mice at the same rate

Because it has been shown in various model systems of *Listeria monocytogenes* infection that *L. monocytogenes* can utilize InIA – mediated uptake and M cell transcytosis to invade the gut, we wanted to determine M cell transcytosis was required for *L. monocytogenes* to establish an infection in the colon and ileum using our food borne model of infection. To do this, groups of BALB/c/J (BALB) mice and CD137-deficient mice (CD137^{-/-}) were infected InIA^mCG (InIA^m). The colons and ileums of these mice were harvested three days post infection and the tissues were treated to collect the luminal contents and whole tissue. The total number of *L. monocytogenes* was nearly the same in colon and ileum contents (Figure 1A), using an un-paired t test, there was no significant difference between the mean values, in both the BALB and CD137^{-/-} mice. This trend was also observed when looking at the total cell-associated *L. monocytogenes*. There was no significant difference between the total number of cell-associated *L. monocytogenes* in the colon and ileum of BALB and CD137^{-/-} mice (Figure 1B). It was noted that more *L. monocytogenes* was recovered in the colon contents and tissue samples than in the ileum contents and tissue samples for both groups of mice (Figures 1A and 1B). While there appeared to be no significant effect on the ability of *L. monocytogenes* to infect and colonize the colon and ileum when M cell transcytosis was not available for an invasion pathway, it was thought that it might affect the ability of *L. monocytogenes* to localize to the different intestinal fractions and that by collecting whole tissue samples a phenotype within the fractions might be masked.

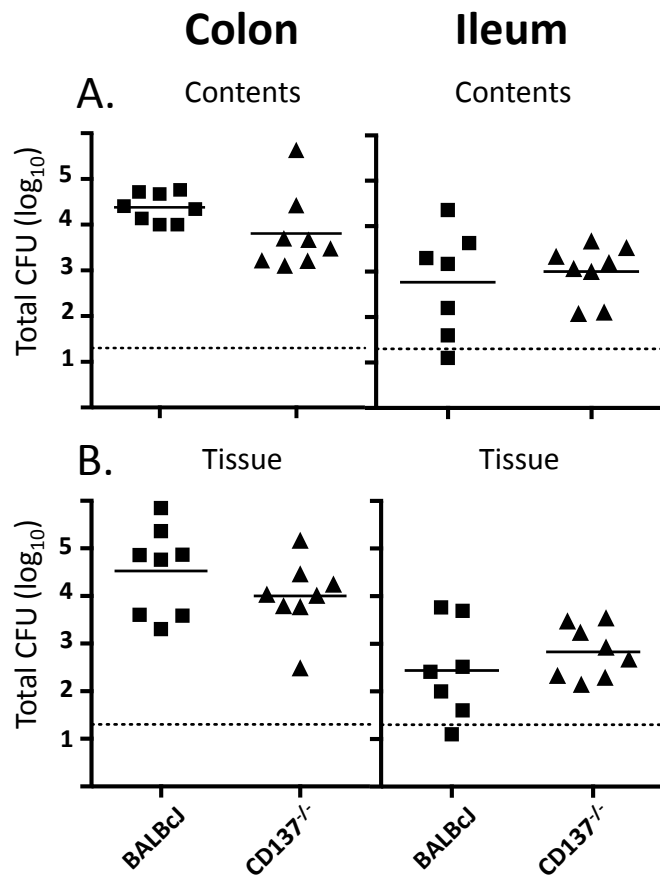


Figure 1. InIA^m colonizes the colon and ileum of CD137^{-/-} and BALB mice at the same rate. BALB and CD137^{-/-} mice were infected with 2-3 x 10⁸ CFU/mouse of InIA^m by food borne infection. The total number of *L. monocytogenes* CFU (Log₁₀) in the luminal contents (A) and whole tissue (B) in the colon and ileum was determined 3 days post infection. Symbols represent the values for individual mice; horizontal lines indicate the mean for each group. Pooled data from 2 experiments is shown. Dashed horizontal lines indicate the limit of detection for each intestinal sample.

*When M cell transcytosis is not available as a pathway for invasion, there is no effect on the ability of *L. monocytogenes* to localize to the mucus layer, epithelial cell layer, and lamina propria of the colon and ileum*

While there was no observed difference in the ability of *L. monocytogenes* to colonize the intestinal tissues in the absence of M cell transcytosis, we wanted to determine if there was an effect of the ability of *L. monocytogenes* to localize to the different intestinal fractions. This is important to understand because *L. monocytogenes* must pass through the epithelial cell layer and invade to the lamina propria in order to disseminate to other organs in the body. We decided at this point, to harvest the colon and ileum at 20 hours post infection, so that we could study the early invasion events. To do this, we orally infected BALB and CD137^{-/-} mice with InIA^m *Lm*. The colons and ileums of the mice were harvested at 20 hours post infection and treated to separate and collect the epithelial cell layer, lamina propria layer and mucus layer. It was observed that there was no difference in the ability of InIA^m to localize to the epithelial cell layer of the colon in BALB and CD137^{-/-} mice (Figure 2A). There was also no difference in the localization of InIA^m to the lamina propria or mucus layer in the BALB and CD137^{-/-} mice (Figure 2B and C). Therefore, while M cell transcytosis was not accessible as a means of invasion, this did not affect the ability of *L. monocytogenes* to establish infection in the mucus layer, epithelial cell layer, and lamina propria.

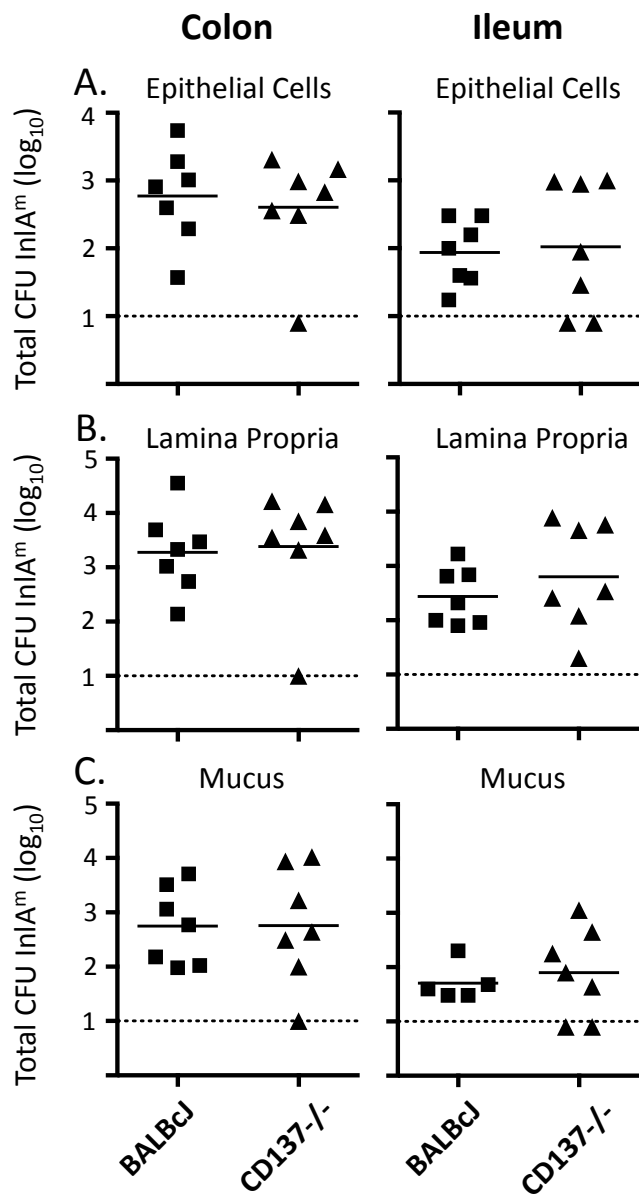


Figure 2. There is no defect in the ability of InIA^m to colonize the mucus layer, epithelial cell layer and lamina propria in BALB or CD137^{-/-} mice. BALB and CD137^{-/-} mice were infected with $5-6 \times 10^8$ CFU/mouse of InIA^m by oral infection. The total number of *L. monocytogenes* CFU (Log₁₀) in the epithelial cells (A), lamina propria (B) and mucus (C) in the colon and ileum was determined 20 hours post infection post infection. Symbols represent the values for individual mice; horizontal lines indicate the mean for each group. Pooled data from 2 experiments is shown. Dashed horizontal lines indicate the limit of detection for each intestinal sample. When the data of the two groups was compared using a unpaired t-test, there was no statistical difference between the two groups in either the colon or ileum mucus layer, epithelial cell layer, or lamina propria.

Without InIA-mediated uptake and M cell transcytosis, L. monocytogenes has a defect in colonizing the colon, but not the ileum

In the first experimental studies we wanted to determine how removing a single pathway of invasion, M cell transcytosis, would affect the ability of *L. monocytogenes* to invade the colon and ileum. Previously, it had also been shown that when InIA-mediated uptake is unavailable, this causes a defect in the ability of *L. monocytogenes* to colonize the intestines [12]. Considering that we had shown *L. monocytogenes* is capable of establishing infection when M cell transcytosis is unavailable, and can establish an infection when InIA-mediated is not unavailable, but to a lesser extent, we thought that removing both of these invasion pathways would have an effect on colonizing the colon and ileum. To answer this question we orally co-infected CD137^{-/-} mice with InIA^m-Kan *Lm* and Δ *inIA*-Tet *Lm* at a 1:1.4 ratio. The colon and ileums were dissected from infected mice at 20 hours post infection and the processed samples were plated on both BHI 1mM IPTG Tet-10 and BHI 1mM IPTG Kan-50 plates to differentially isolate InIA^m-Kan *Lm* and Δ *inIA*-Tet *Lm*. There was a significant difference observed in the ability of Δ *inIA*-Tet *Lm* to colonize the colon compared to InIA^m-Kan *Lm* and Δ *inIA*-Tet *Lm* invaded to a lesser extent than did InIA^m-Kan *Lm* (Figure 3A). While there was no significant difference between the Log₍₁₀₎ CFUs recovered of Δ *inIA*-Tet *Lm* and InIA^m-Kan *Lm* in the ileum, it is noted that there is a bi-modal distribution of data observed (Figure 3A). Also, in the competitive index (Figure 3B), there was a 13.8 fold decrease in the ability of Δ *inIA*-Tet *Lm* to colonize the colon and a 4.6 fold decrease in the ileum, compared to InIA^m-Kan *Lm*.

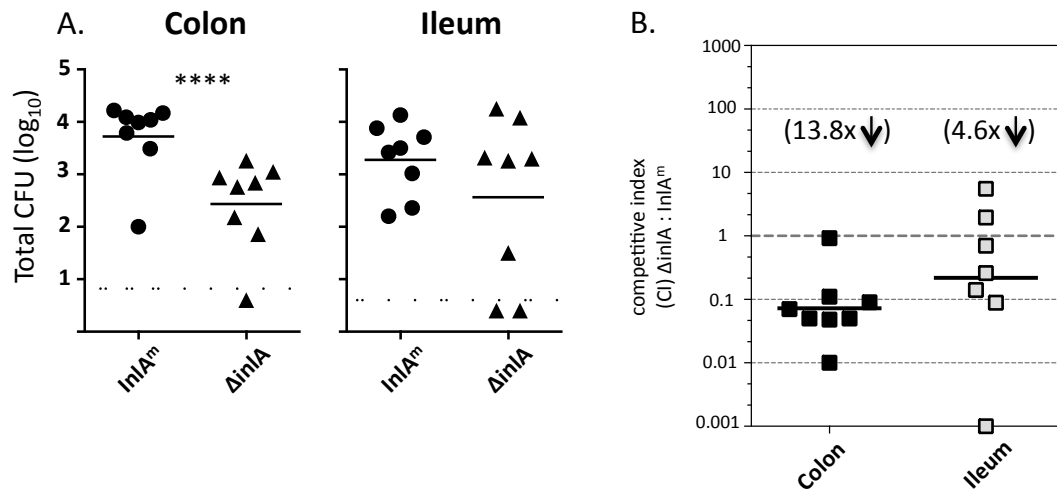


Figure 3. ΔinA *Lm* has a disadvantage in colonizing the colon and ileum of $CD137^{-/-}$ mice.

$CD137^{-/-}$ mice were infected with 1.3×10^8 CFU/mouse of $InIA^m$ -Kan *Lm* and 1.81×10^8 CFU/mouse of ΔinA -Tet *Lm* by food borne infection. The total cell-associated number of *L. monocytogenes* CFU (\log_{10}) in colon and ileum (A) was determined 20 hours post infection. The competitive index ratio of ΔinA -Tet: $InIA^m$ -Kan for colon and ileum is shown in (B). The geometric mean was compared for each group was compared to a theoretical value of 1.0 and the fold difference is shown in the parenthesis above. Symbols represent the values for individual mice; horizontal lines indicate the mean for each group. The pooled data from 2 separate experiments is shown. In A, dashed horizontal lines indicate the limit of detection for each intestinal sample. In A, statistical significance was determined by student's t test; asterisks indicate significant differences.

When InIA-mediated uptake and M cell transcytosis cannot be accessed by L. monocytogenes, the ability of Lm to localize within the mucus layer, epithelial cell layer and lamina propria of the colon and ileum is affected

Because it was observed that removing two pathways of invasion, InIA-mediated and M cell transcytosis, did indeed affect the ability of *L. monocytogenes* to infect the colon and ileum, we wanted to determine how the defect observed translated to localization of *L. monocytogenes* to the mucus layer, epithelial cell layer and lamina propria layer of the intestine. We thought that because we observed a defect in colonizing whole tissue, that this would affect the ability of *L. monocytogenes* to establish an infection within the mucus, epithelial cells, and would cause a defect in reaching the lamina propria. To study this, we orally co – infected CD137^{-/-} mice with InIA^m-Kan *Lm* and Δ *inIA*-Tet *Lm* at a 1:1.35 ratio. The colon and ileums were dissected from infected mice at 20 hours post infection and the processed samples were plated on both BHI 1mM IPTG Tet-10 and BHI 1mM IPTG Kan-50 plates to differentially isolate InIA^m-Kan and Δ *inIA*-Tet. It was observed in all of the colon intestinal fractions (Figure 4A,B,C) that Δ *inIA*-Tet had significantly fewer CFUs than InIA^m-Kan recovered. However, in all of the ileum fractions there was no significant difference between the mean Log₍₁₀₎ CFU recovered of Δ *inIA*-Tet and InIA^m-Kan (Figure 4A,B,C). There was an interesting bi-modal distribution of data for the Log₍₁₀₎ CFU of Δ *inIA*-Tet in the ileum lamina propria (Figure 4B). There was a group of 11 mice, in which Δ *inIA*-Tet was able to reach similar Log₍₁₀₎ CFU as InIA^m, but in 7 mice no CFUs were recovered for Δ *inIA*-Tet. The competitive index ratios also demonstrate the observed phenotypes of the recovered Log₍₁₀₎ CFU in the colon cellular fractions

(Figure 4D). In the colon mucus, $\Delta inIA$ -Tet *Lm* has a 5 fold decreased ability to colonize compared to $InIA^m$ -Kan *Lm*, 12 fold decreased ability in the epithelial cell layer, and a 11 fold decreased ability in the colon lamina propria.

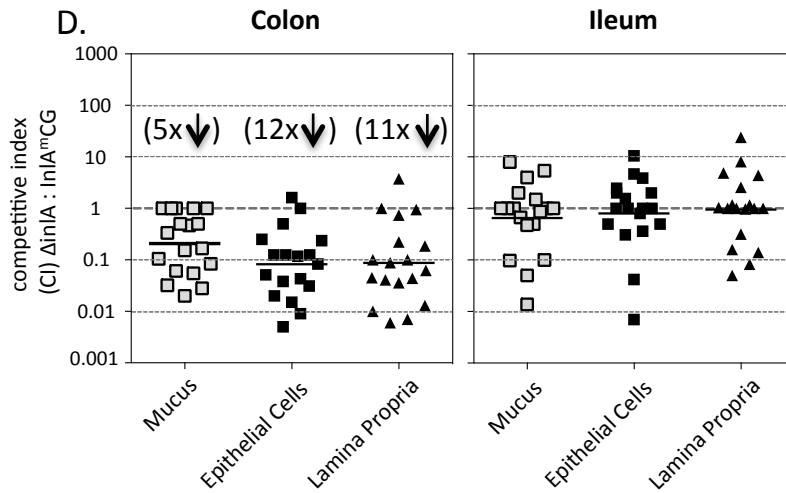
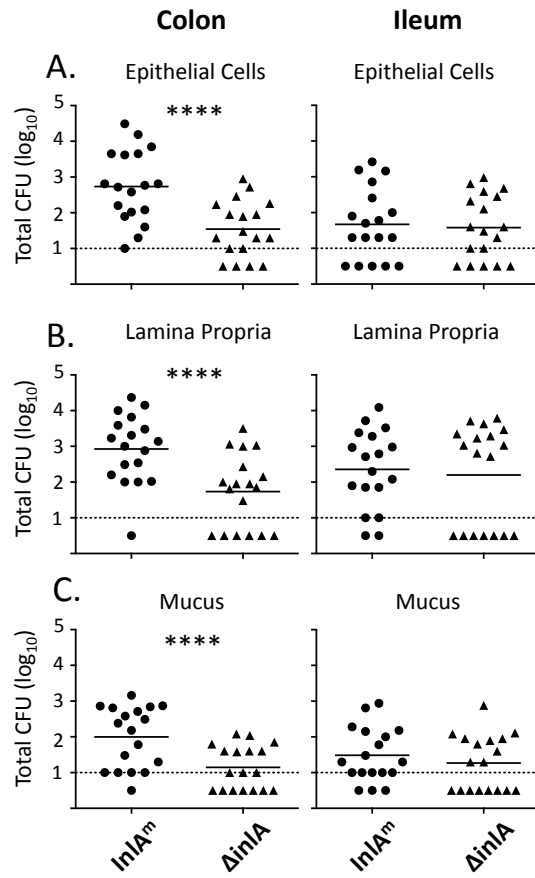


Figure 4. *ΔinIA Lm* has a disadvantage in colonizing the mucus layer, epithelial cell layer, and lamina propria of the colon and has a defect in reaching the lamina propria of the ileum of CD137^{-/-} mice. CD137^{-/-} mice were infected with 3-5 x 10⁸ CFU/mouse of InIA^m-Kan *Lm* and 5-7 x 10⁸ CFU/mouse of *ΔinIA*-Tet *Lm* by food borne infection. The total of *L. monocytogenes* CFU (Log₁₀) in the epithelial cell layer (A), lamina propria layer (B), and mucus (C) of the colon and ileum was determined 20 hours post infection. The competitive index ratio of *ΔinIA*-Tet: InIA^m-Kan for each intestinal fraction in the colon and ileum is shown in (D). The geometric mean for each group was compared to a theoretical value of 1.0 and the fold difference is shown in the parenthesis above. Symbols represent the values for individual mice; horizontal lines indicate the mean for each group. This is pooled data from 2 separate experiments. In A- C, dashed horizontal lines indicate the limit of detection for each intestinal sample. In A-C, statistical significance was determined by student's t test; asterisks indicate significant differences.

When InIA-mediated uptake and M cell transcytosis cannot be utilized by L. monocytogenes, the intracellular and extracellular localization of L. monocytogenes within the mucus, epithelial cells and lamina propria layer is altered in the colon and ileum

Because we observed that removing two pathways of invasion affected the ability of *L. monocytogenes* to colonize the mucus, epithelial cells, and lamina propria of the colon and caused a bottleneck to reach the lamina propria of the ileum, we were curious how this might affect the intracellular/extracellular localization of *L. monocytogenes*. To determine if the intracellular/extracellular localization was affected, we orally co-infected CD137^{-/-} mice with InIA^m-Kan *Lm* and Δ InIA-Tet *Lm* at a 1:1.8 ratio. The colon and ileums were dissected from infected mice at 20 hours post infection. After fractionation of the tissues into the mucus layer, epithelial cell layer, and lamina propria layer, half of each fraction sample was treated with gentamycin to kill any extracellular bacteria. The processed samples were plated on both BHI 1mM IPTG Tet-10 and BHI 1mM IPTG Kan-50 plates to differentially isolate InIA^m-Kan *Lm* and Δ InIA-Tet *Lm*. In both the colon and ileum, Δ InIA-Tet *Lm* reached lower total CFU numbers in the epithelial cells and lamina propria (Figure 5A and B). In particular the colon lamina propria, while in 4 out of 5 mice InIA^m-Kan *Lm* could be detected intracellularly, Δ InIA-Tet *Lm* could not be detected intracellularly (Figure 5B). This suggests that removing InIA-mediated uptake and M cell transcytosis as invasive pathways that *L. monocytogenes* can utilize, might decrease the ability *L. monocytogenes* to exist intracellularly and bacteria that are able to establish an infection are predominantly extracellular. This is important because

the localization of *L. monocytogenes* within a cell or extracellularly, could potentially impact its ability to disseminate from the intestines. To demonstrate that the results observed for $\Delta inIA$ -Tet *Lm* in these experiments were not due to InIA^m-Kan *Lm* assisted colonization, meaning that damage or uptake initiated by InIA^m-Kan *Lm* helps $\Delta inIA$ -Tet *Lm* to colonize, we performed this same experiment again, but instead infected with only $\Delta inIA$ -Tet *Lm*. CD137^{-/-} were orally infected, by food borne infection, with $\Delta inIA$ -Tet *Lm*. The colon and ileums were harvested and processed as describe above. Very few $\Delta inIA$ -Tet *Lm*, total or intracellular, were able to be recovered in the colon and ileum epithelial layer (Figure 5C). $\Delta inIA$ -Tet *Lm* was able to be recovered in the lamina propria of the colon and ileum, and as observed before (Figure 5B), very few $\Delta inIA$ -Tet *Lm* were able to be found intracellularly in the colon lamina propria (Figure 5D).

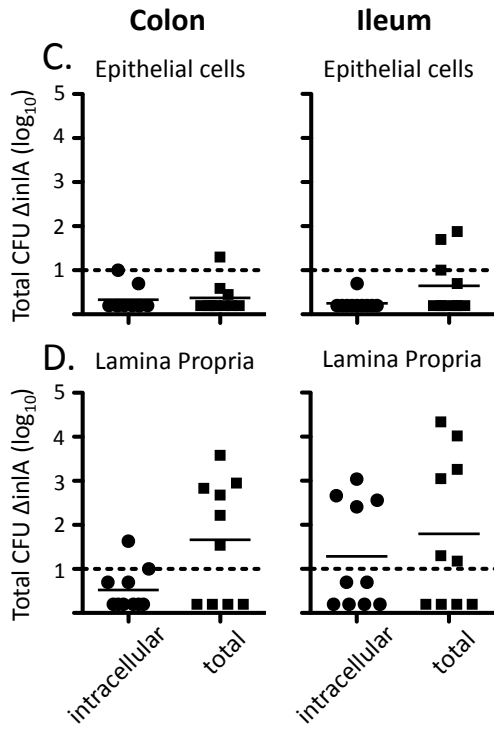
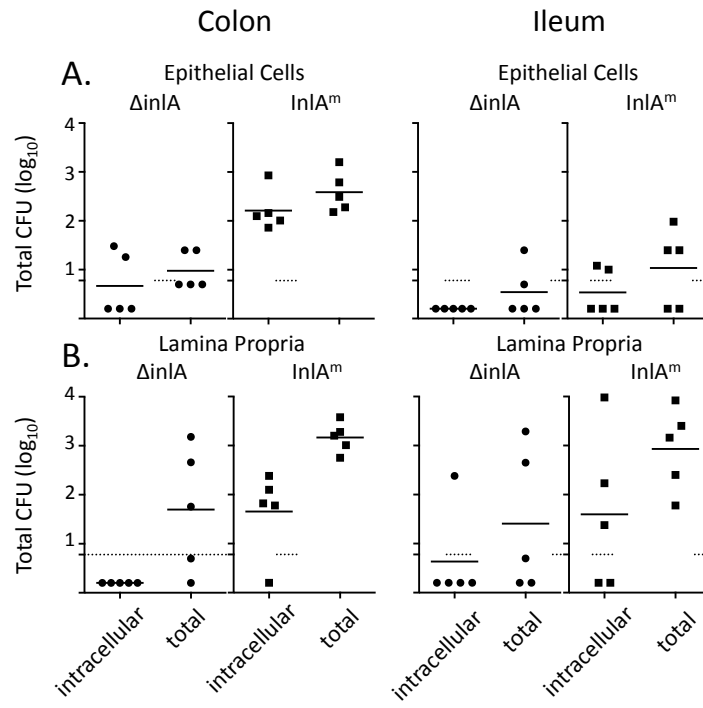


Figure 5. *ΔinIA Lm* is less capable of intracellular localization within intestinal fractions of the colon and ileum of CD137^{-/-} mice. For A-B, CD137^{-/-} mice were infected with 2.17×10^8 CFU/mouse of InIA^m-Kan *Lm* and 4×10^8 CFU/mouse of *ΔinIA*-Tet *Lm* by food borne infection. The intracellular and total number of *L. monocytogenes* CFU (Log₁₀) in the epithelial cell layer (A), and lamina propria layer (B), of the colon and ileum was determined 20 hours post infection. This data is from 1 experiment. For C-D, CD137^{-/-} mice were infected with 5-7 CFU/mouse of *ΔinIA*-Tet *Lm* by food borne infection. The intracellular and total number of *ΔinIA*-Tet *Lm* CFU (Log₁₀) in the epithelial cell layer (C), and lamina propria layer (D), of the colon and ileum was determined 20 hours post infection. Symbols represent the values for individual mice; horizontal lines indicate the mean for each group. This is pooled data from 2 separate experiments. Dashed horizontal lines indicate the limit of detection for each intestinal sample.

Knocking out InIA and InIB – mediated uptake, and reduced M cell transcytosis affects the ability of Lm to invade the colon more so than the ileum

Since we were able to recover $\Delta inIA$ *Lm* in the intestines of $CD137^{-/-}$, we thought that InIB – mediated uptake, which normally serves to promote uptake once InIA has bound to E-cadherin, could be allowing residual $\Delta inIA$ *Lm* to bind and be endocytosed. We hypothesized that by also knocking out InIB mediated uptake, residual $\Delta inIA$ *Lm* might no longer be recovered. To test this, we orally infected $CD137^{-/-}$ and BALB mice with $\Delta inIA\Delta inIB$ –Tet *Lm*. The colon and ileums were harvest from infected mice at 20 hours post infection, treated to collect the intestinal fractions, and the processed samples were plated on both BHI 1mM IPTG Tet-10. $\Delta inIA\Delta inIB$ *Lm* was recovered in the colon and ileum mucus layer, epithelial cells, and lamina propria layer of both the BALB and $CD137^{-/-}$ mice (Figure 6A,B,C). There was significantly less $\Delta inIA\Delta inIB$ *Lm* recovered in all of the colon fractions compared to the bacterial numbers recovered in the same fractions of the colon in the BALB mice (Figure 6A,B,C). There appears to be a trend for there to be less $\Delta inIA\Delta inIB$ *Lm* in the ileum epithelial cells and the mucus, but the difference is not significant (Figure 6A,B,C).

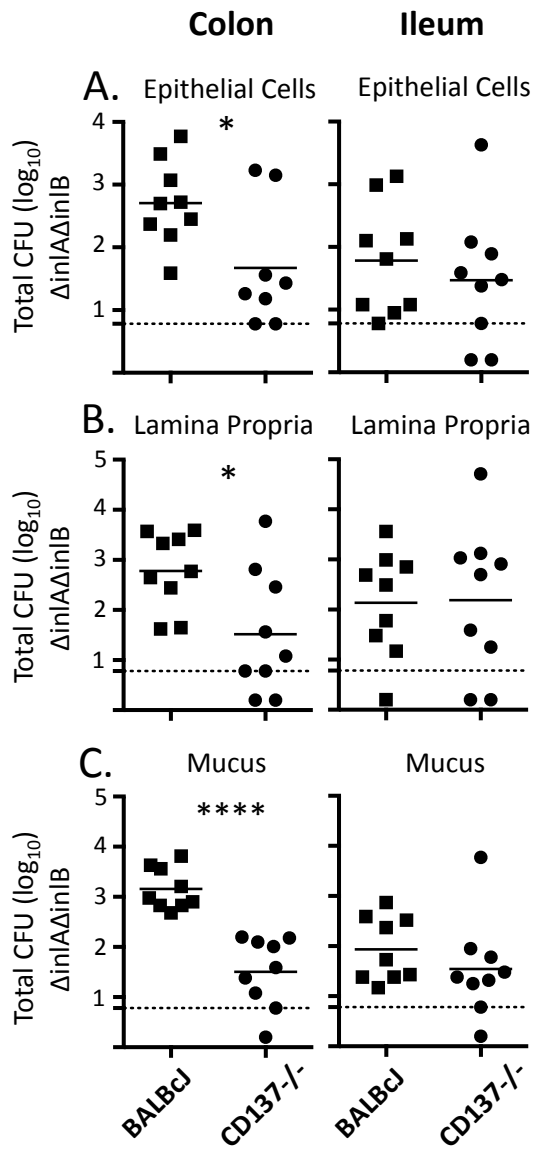


Figure 6. Without InIA and InIB-mediated uptake and M cell transcytosis, *L.*

***monocytogenes* is deficient in colonizing the intestinal fraction of the colon.** BALB and CD137^{-/-} mice were infected with $2-5 \times 10^8$ CFU/mouse of $\Delta inIA\Delta inIB$ *Lm* by food borne infection. The total number of *L. monocytogenes* CFU (Log₁₀) in the epithelial cells (A), lamina propria (B) and mucus (C) in the colon and ileum was determined 20 hours post infection post infection. Symbols represent the values for individual mice; horizontal lines indicate the mean for each group. Pooled data from 2 experiments is shown. Dashed horizontal lines indicate the limit of detection for each intestinal sample. Statistical significance was determined by student's t test; asterisks indicate significant differences.

Discussion

Previous work has demonstrated that *L. monocytogenes* uses multiple pathways to invade and cross the intestinal barrier [1-7]. The role of *L. monocytogenes* internalin proteins InIA and InIB has been described as mediating entry into epithelial cells of the intestine. InIA binds to E-cadherin and InIB binds to c-Met, together they promote endocytosis of *L. monocytogenes* at sites where epithelial cells are being extruded. In vivo and in vitro studies have also demonstrated that *L. monocytogenes* can cross the intestinal barrier through M cell transcytosis [6,7]. While the infection models used to determine the invasive pathways of *L. monocytogenes* have provided insight into how *L. monocytogenes* is able to infect the intestines, they do not replicate what occurs during a natural infection with *L. monocytogenes*. Our purpose was to evaluate the relative roles of each of these pathways in our food borne infection model of Listeriosis.

We were able to show that when BALB and CD137^{-/-} are challenged with InIA^m *Lm*, this strain is able to reach similar bacterial numbers in the colon and ileum whole tissue and within the mucus layer, epithelial cell layer, and lamina propria of the colon and ileum. In consideration of previous work that demonstrated that $\Delta inIA$ *Lm* was able to colonize the intestines BALB mice, this suggests that M cell transcytosis is a compensatory invasive pathway to InIA-mediated uptake. Demonstrating that *L. monocytogenes* is able to colonize the intestines in the absence of InIA-mediated uptake, contradicts beliefs that InIA-mediated endocytosis is required for invasion of the intestinal epithelium. It seems that other pathways such as M cell transcytosis, can be used by *L. monocytogenes* to establish a similar infection as when only InIA-mediated

uptake is available. It is possible that internalin mediated pathways of uptake may be most efficient during invasion, but, this does not necessarily mean that they are required for *L. monocytogenes* to invade the intestines.

We next hypothesized that if these pathways contribute to the majority of the establishment of infection, that making both InIA-mediated uptake and M cell transcytosis unavailable to *L. monocytogenes*, this would cause a defect in colonization of the intestines. By knocking out InIA-mediated uptake and M cell transcytosis, we demonstrated that when *L. monocytogenes* is not able to access either of these invasive pathways it causes a defect in its ability to colonize the colon whole tissue and localize within the mucus layer, epithelial cell layer, and lamina propria of the colon. We also observed that there was a bottle-neck in reaching the lamina propria of the ileum. *L. monocytogenes* either cannot reach the ileum lamina propria at all, but if it does, it can reach similar bacterial numbers as bacteria that can access InIA-mediated uptake. The fact that there is a phenotypic difference in the defects observed in the colon and ileum and the amount of bacteria recovered in colon and ileum, is somewhat puzzling. Currently, we have not been able to determine why this difference exists between the colon and ileum. It is important to note that the composition of the cells and immune structures differ between the colon and ileum. For one, it is thought that there are more Peyer's Patches within the ileum compared to the colon, suggesting that there may be more Peyer's patch M cells within the ileum as well. Recently though, another type of M cell has been characterized known as a villous M cell [15]. This type of M cell is not associated with Peyer's Patches, develops in Peyer's Patch null mice, and has a different

gene expression pattern than Peyer's Patch M cells; so it is possible villous M cells develop along a different developmental pathway than follicle-associated M cells [15,16]. It has also been shown that these villous M cells are capable of taking up *Salmonella*, *Yersinia*, and *Escherichia coli*-expressing invasin [15]. Because the Peyer's Patch M cell transcytosis defect relies on the defect of the CD137/CD137L signaling step during development, and the villous M cells have been shown to have a different gene expression pattern, it could be possible that these M cells retain their transcytosis capabilities in the CD137^{-/-} mice. If this is the case, and if there were more villous M cells in the ileum, it could be why there are not significant differences between the colonization of Δ *inlA* *Lm* and InlA^mCG in the ileum of CD137^{-/-} mice. There are also more goblet cells, which have also been demonstrated as cells that *L. monocytogenes* can invade, in the colon compared to the ileum. Since this is the case, it could be allowing more *L. monocytogenes* to invade the colon epithelium and therefore be responsible for the higher CFU recovered in the colon compared to the ileum.

We also demonstrated that removing InlA-mediated uptake and M cell transcytosis, has an effect on whether *L. monocytogenes* localizes intracellularly or extracellularly during colonization of the colon and ileum epithelial cells and lamina propria. This defect was most clearly observed in the colon lamina propria, in which *L. monocytogenes* that could not access InlA-mediated uptake or M cell transcytosis could not be recovered intracellularly. This observation is important because if *L. monocytogenes* needs to exist intracellularly to disseminate from the intestines, not

having these two pathways available could inhibit the ability of *L. monocytogenes* to disseminate.

In all of the experiments involving food borne infection with $\Delta inIA$ *Lm*, the bacteria were able to be recovered in both the ileum and colon of CD137^{-/-} mice. As mentioned before, a second internalin protein, InIB, can promote invasion of *L. monocytogenes* through binding to c-Met and accelerating clathrin mediated endocytosis. Because we were still recovering bacteria that were not able to utilize M cell transcytosis or InIA-mediated uptake to infect the gut, we thought that InIB binding to c-Met could allow for the residual colonization of the intestines. When we infected CD137^{-/-} mice with a double deletion mutant, $\Delta inIA\Delta inIB$ *Lm*, we were able to recover $\Delta inIA\Delta inIB$ *Lm* in the colon and ileum of the CD137^{-/-} mice, but at a lesser extent in the colon compared to BALB mice. While we thought that we would see a more significant decrease in the recovered CFU of $\Delta inIA\Delta inIB$ *Lm*, it is possible that InIB does not play as large of a role in the establishment of infection within the intestines as we thought. It would seem from our data, that InIB plays a much lesser role than InIA in colonization of the intestines, and primarily serves to accelerate endocytosis of the pathogen once *L. monocytogenes* has bound to E-cadherin on the surface of epithelial cells through InIA. While it seems that during food borne infection the internalin-mediated, InIA/InIB, pathways of uptake and M cell transcytosis, contribute to a large portion of establishment of infection in the intestines, this data suggests that there could be another pathway that *L. monocytogenes* can utilize to invade the intestine. While this would be a novel idea to pursue, it also suggests that the M cell transcytosis phenotype

may not be as deficient as we assume. However, in personal communication with our collaborator David Lo, his lab has demonstrated through microparticle uptake assays that Peyer's Patch M cells have nearly a complete defect in their ability to transcytose microparticles from the lumen to their basolateral surface [14]. His next step is to show in a ligated loop assay that Peyer's Patch M cells from CD137^{-/-} mice are defective in transcytosing GFP-expressing *L. monocytogenes*. If he is able to demonstrate this defect using *L. monocytogenes*, it would further point to the existence of other pathways that *L. monocytogenes* can use to colonize the colon and ileum during a food borne infection.

Materials and Methods

Bacterial Strains

The modified strain InIA^mCG was made by the lab from an InIA^m provided by W.D. Schubert (Braunschweig, Germany). The *inIA* deletion mutant used, is an *inIA* deletion on the EGDe background and was a gift of Cormac Gahan (University College Cork, Ireland). A *inIA* and *inIB* deletion mutant was created in our lab through the use of a pKSV7 site-specific integrative plasmid [19]. A 5' up stream primer of the InIB region was created (5' ACGGATAAAGGATCCACAAATGACATCAATTTATATGCA), and included a BamHI site. A 5' down stream primer of the InIB region was created (5' TCGGTAGTCGACTAAACAACATTAAAGTTAGAAACAATTA), and included a Sall site. These two sites (BamHI and Sall) were used to integrate the pKSV7 plasmid into the InIB region and then excise a portion of the InIB gene. The resulting gene contains the first 19 amino acids and the last 48 amino acids, with a PstI site where the 20-582 amino acids were excised. Antibiotic resistant version of these *Listeria monocytogenes* strains were generated to be used in co-infection studies using site-specific pIMC3 plasmids encoding kanamycin and tetracycline resistance [20]. Each strain was intestinally passaged by orally infection a BALB mouse. Bacteria that were recovered from the small intestine of the infected mouse were grown to stationary phase in Brain Heart Infusion (BHI) broth shaking at 37 °C, and then aliquots were prepared and stored at -80 °C. To prepare the bacteria for an infection, an aliquot was thawed on ice, cultured standing in BHI broth for 1.5 h at 30°C, washed once with PBS, and then suspended PBS and butter. Tissues

samples collected from a harvest were plated on BHI agar (Difco) that was supplemented with 15g/L LiCl and 10g/L glycine (BHI/L+G). This agar was used as a selective medium to inhibit the growth of most intestinal bacteria. Colonies were allowed to grow on BHI/L+G plates for 48 h at 37 °C, any colonies where there was uncertainty if the colony was *L. monocytogenes* were plated on CHROMagar *Listeria* plates.

Mice

Male and Female BALB/c/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 4 weeks of age and used in experiments when they were 6-8 weeks old. The CD137 – deficient mice (CD137^{-/-}) were a gift from David D. Lo (University of California, Riverside) and were bred in the University of Kentucky animal facility in specific – pathogen free conditions. All mice were maintained in a specific-pathogen free facility at the University of Kentucky with a 14 h light cycles (7 AM – 9 PM) and a 10 h dark cycle (9 PM – 7 AM). The mice were then moved at least 2 weeks prior to an experiment to a room with a reversed light cycle.

Oral Infection

Two weeks prior to infection, mice were moved to a reversed light cycle room to have time to acclimate. For the oral infection, mice were placed in cages that contained wire floors (#3 mesh) that were raised (1 inch) to prevent coprophagy. Mice were starved for 16-24 h prior to the infection unless otherwise mentioned. The *L. monocytogenes* strain

used for the infection was resuspended in 3 μ l of PBS plus 2 μ l of salted butter for each mouse in the experiment. This inoculum was then used to saturate a 2-3 mm piece of white bread in microcentrifuge tube, unless otherwise noted. During the infection, each mouse was placed in an empty cage without bedding and the contaminated bread piece was presented to the mouse, using forceps, then placed on the bottom of the cage. After the mice ate the bread piece, they were returned to their original cages and provided normal mouse chow and water.

Luminal and cell-associated *Listeria* in the intestines

The colon and small intestine were dissected from the infected mice at either 3 days or 20 h post infection, unless otherwise noted. The small intestine was measured and the distal third of the small intestine was kept and labeled the ileum. The ileum and colon were then processed separately. The contents of the intestines were removed by squeezing with sterile forceps, then each section was flushed with either PBS or HB-2 solution (contains 1x Hanks balanced salt solution (HBSS), Ca^{2+} - and Mg^{2+} - free and FBS) through a 25g needle. The contents and flushes of the lumen were then pooled and centrifuged for 20 min. at 12,000 x *g*. If the contents were not to be collected for CFUS, the intestines were flushed as describe above but the luminal contents was discarded. The bacterial pellet was then resuspended in 0.25 – 1.0 ml of sterile water and serial dilutions were plated on BHI/L+G agar to determine the number of bacteria in the lumen. The washed intestinal tissues were cut longitudinally with a sterile scalpel blade, placed in 2ml sterile water, and then homogenized for 1 minutes using a PowerGen

1000 homogenizer (Fisher) at 60% power. The total number of cell-associated (includes adherent extracellular and intracellular) bacteria was determined by plating serial dilutions on BHI/L+G agar.

Co-infections with InIA^m and Δ inIA *L. monocytogenes*

For the co – infection studies, bacterial suspensions of each strain were mixed prior to saturation of a single bread piece. To determine the number of bacteria in the intestines from each strain, each strain was tagged with antibiotic resistance genes. The tissue homogenates and fractions were differentially plated on BHI with 1mM IPTG plus 50 μ g/ml kanamycin (to detect *Lm* InIA^mCG) or 10 μ g/ml tetracycline (to detect *Lm* Δ inIA). Competitive index (CI) ratios were determined by dividing the number of Δ inIA *Lm* CFU by the number of *Lm* InIA^mCG recovered from each tissue. If no CFU were recovered for one of the strains, than a value equal to the limit of detection was used for that strain.

Fractionation of intestinal tissues

To separate and collect the fractions of the intestinal tissues, flushed colon and ileum were treated following a similar protocol as one described in [12]. In brief, the tissues were first treated with N-acetylcystein (NAC; Sigma) to remove the mucus layer, next treated with RPMI 1640 (Invitrogen) containing 5% FBS (RP-5), 5mM EDTA, and 1mM DTT to remove the epithelial cell layer, and then the remaining tissue was treated with type IV collagenase (1mg/ml, 150units/ml or 100units/ml; Worthington) and DNase I (40 μ g/ml; Worthington) to isolate the lamina propria cells. After each treatment the washed tissue was filtered over an Oakridge tube and the pooled filtered washes were

centrifuged for 20 min. at 12,000 x *g* and the pellets from the washes were resuspended in sterile water and then plated to determine the total number of bacteria. The DTT/EDTA treatment was modified to include one 20 min. incubation, two 10 min. incubation and then a final 5 min. wash with RP5/HEPES. The collagenase IV treatment was modified to include two 30 min. incubations. In studies that determined the intracellular or extracellular localization of the bacteria, half of the supernatants and cellular pellets from each fractionation treatment were kept to plate for total bacterial number. The other half was incubated for 30 min. at 37 °C with 7% CO₂ in RP-5 containing 50 µg/ml gentamycin to kill any extracellular or adherent *L. monocytogenes*. After the gentamycin treatment, the cells were washed twice with PBS, lysed with sterile water, then diluted and plated to determine the number of intracellular *L. monocytogenes*.

Statistics

All statistical analysis was performed using Prism6 for Macintosh (Graph Pad). *P* values less than 0.05 were considered to be significant and are indicated in the figures as follows: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001.

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