Extending the Host Range of *Listeria monocytogenes* by Rational Protein Design

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**SUMMARY**

In causing disease, pathogens outmaneuver host defenses through a dedicated arsenal of virulence determinants that specifically bind or modify individual host molecules. This dedication limits the intruder to a defined range of hosts. Newly emerging diseases mostly involve existing pathogens whose arsenal has been altered to allow them to infect previously inaccessible hosts. We have emulated this chance occurrence by extending the host range accessible to the human pathogen *Listeria monocytogenes* by the intestinal route to include the mouse. Analyzing the recognition complex of the listerial invasion protein InlA and its human receptor E-cadherin, we postulated and verified amino acid substitutions in InlA to increase its affinity for E-cadherin. Two single substitutions increase binding affinity by four orders of magnitude and extend binding specificity to include formerly incompatible murine E-cadherin. By rationally adapting a single protein, we thus create a versatile murine model of human listeriosis.

**INTRODUCTION**

The constant interaction of humans with countless microorganisms has led to the evolution of an intricate, multifaceted, and remarkably efficient immune system. Pathogens have, however, coevolved, refining their arsenal of pathogenicity factors to exploit loop holes in the host’s defenses (Akira et al., 2006). This dedicated specialization of pathogens restricts their choice of alternative hosts and limits the usefulness of animal models to study human pathogens in vivo. Molecular mechanisms defining host tropism have been identified for a limited number of pathogens, including the food-borne human pathogen *Listeria monocytogenes* (Hamon et al., 2006). These include two listerial invasion proteins, internalin (InlA) and InlB, that promote uptake of bacteria into distinct sets of nonphagocytotic cells (Cossart et al., 2003).

InlA promotes listerial uptake into intestinal epithelial cells (Gaillard et al., 1991) by targeting the N-terminal domain of human E-cadherin (hEC1) (Mengaud et al., 1996), the dominant adhesion molecule of adherens junctions (D’Souza-Schorey, 2005). InlA similarly recognizes E-cadherin from guinea pig and rabbit but fails to bind the corresponding domain of murine E-cadherin (mEC1), despite a sequence identity of 90% to hEC1 (Lecuit et al., 1999) and a pronounced structural similarity (Schubert et al., 2002). Among other targets, the second listerial invasion protein InlB recognizes the HGF or Met receptor (Shen et al., 2000) inducing uptake into a wide range of mammalian cells including hepatocytes and endothelial cells. InlB recognizes both murine and human Met receptor but not that of the guinea pig and rabbit (Khelef et al., 2006). The lack of a small-animal model to study the interplay of InlA and InlB in human listeriosis in vivo was first addressed by the generation of a transgenic mouse producing human alongside murine E-cadherin. By rationally adapting a single protein, we thus create a versatile murine model of human listeriosis.

Animal models for other pathogens have similarly been created by the genetic humanization of mice (Lecuit and Cossart, 2002). However, because pathogens typically abuse host molecules of complex biological function, humanizing such molecules may incur unexpected secondary effects. Humanized mouse lines, furthermore, need to be crossed with existing knockout strains (Yap and Sher, 2002), thereby adding another level of complexity.

In nature, pathogens occasionally switch their host specificity through spontaneous changes in their molecular arsenal (Gamblin et al., 2004), causing in high mortality...
rates in the naive host population (Weiss, 2003). Zoonotic pathogens eliciting recent pandemics in the global human population (Lewis, 2006) include HIV (Heeney et al., 2006) and SARS (Li et al., 2005), whereas influenza A subtype H5N1 may be poised to do the same (Stevens et al., 2006).

We imitate this natural route of pathogen evolution by extending the host spectrum of *Listeria monocytogenes*. Our approach is based on the rational structure-based re-engineering of InlA such that modified InlA is able to recognize the previously incompatible receptor mEC1. By adapting the bacterium to the mouse ("murinization") rather than humanizing the mouse, we introduce the human route of infection in mice and hence provide a versatile animal model to investigate listeriosis in vivo.

**RESULTS**

**Structure-Based Virulence-Factor Design**

Our working hypothesis was that the weak binding affinity of InlA for hEC1 is due to imperfect surface complementarity and hence numerous bridging water molecules between the two proteins (Schubert et al., 2002). To increase binding affinity, we proposed individual amino acid substitutions in InlA (Figure 1A) to create additional, stabilizing contacts to hEC1, thus improving complementarity and binding affinity.

Although Ser192 of InlA (Ser192Wild) faces hEC1 in the complex InlA/hEC1, its side chain is too short for a direct hydrogen bond to hEC1. Replacing it by asparagine could allow such a direct interaction. Solving the crystal structure of InlA<sup>Ser192N</sup>/hEC1 confirms that engineered Asn192<sub>Wild</sub> creates a direct hydrogen bond to the main-chain carbonyl of Phe17<sub>hEC1</sub> (Figure 1B). Correspondingly, isothermal titration calorimetry (ITC) indicates the dissociation constant (K<sub>D</sub>) of InlA<sup>Ser192N</sup>/hEC1 to be 200 ± 50 nM (Figure 2), a 40-fold increase in binding affinity relative to InlA<sup>Wild</sup>/hEC1 (K<sub>D</sub> = 8 ± 4 μM).

To improve the unfavorable interaction of bulky Tyr369<sub>InlA</sub> to hEC1, we chose to replace it by the small hydrophilic amino acid serine. The crystal structure of the double variant InlA<sup>Ser192N-Tyr369S</sup> (henceforth denoted InlA<sup>Mod</sup>) in complex with hEC1 confirms that the interaction of Tyr369<sub>InlA</sub> has been replaced by a water-mediated hydrogen bond from Ser369<sub>InlA</sub> to Asn27<sub>hEC1</sub> (Figure 1C). The binding affinity of InlA<sup>Mod</sup> for hEC1 is found to be K<sub>D</sub> = 1.2 ± 0.3 nM by ITC. The substitution Y369S<sub>Wild</sub> thus increases binding affinity by a factor of 170 (Figure 2), whereas both substitutions together increase affinity 6700-fold compared to InlA<sup>Wild</sup>/hEC1. Although separated by 34 Å, the substitutions convert a weak interaction to a tight recognition. Note that a mere eight nonhydrogen atoms within the recognition interface of InlA and hEC1 have been altered.

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**Figure 1. Re-engineered Variants of Internalin (InlA) in Complex with the N-Terminal Domain of Human E-Cadherin**

(A) Superposition of InlA/hEC1 (violet) and InlA<sup>Ser192N-Tyr369S</sup>/hEC1 (gray).
(B) Critical residues near mutation site S192N<sub>Wild</sub> are shown as ball and stick. Asn192<sub>Wild</sub> adds a direct H bond to the carbonyl oxygen of Phe17<sub>hEC1</sub>.
(C) The mutation Y369S<sub>Wild</sub> introduces a water-bridged interaction to Asn27<sub>hEC1</sub>.

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892 *Cell* 129, 891–902, June 1, 2007 ©2007 Elsevier Inc.
Effect of Improved Affinity in Human Cells

To analyze the biological effects of improved affinity of InIA\textsuperscript{m} on the adhesion to and invasion of human epithelial cells in vitro, we modified wild-type \textit{Listeria monocytogenes} EGD (Lmo-EGD) by exclusively replacing the gene \textit{inlA} by \textit{inlA}\textsuperscript{S192N-Y369G} to produce the mutant strain Lmo-InlAm. Our strategy of minimal intervention circumvents \textit{inlA} and \textit{inlB} expression-level changes caused by disrupting the bicistronically transcribed \textit{inlAB} locus (Lingnau et al., 1995). Both listerial invasion proteins are thus expressed at native levels, ensuring that valid biological conclusions may be drawn from the infection process (Figure S1 in the Supplemental Data available online).

Binding of InIA to human E-cadherin induces listerial uptake into human epithelial cells by the zipper mechanism (Cossart and Sansonetti, 2004), requiring localized rearrangements of the cytoskeleton as well as a physically tight interaction between bacterium and eukaryotic cell membrane. To establish whether improved affinity of InIA\textsuperscript{m} for its receptor results in stronger adhesion of Lmo-InlAm, we analyzed its adhesion to the E-cadherin expressing human epithelial cell line Caco2. We observe a 2-fold increase in the adhesion efficiency of Lmo-InlAm compared to wild-type bacteria (Figure 3A).

We investigated the link between improved adhesion of Lmo-InlAm\textsuperscript{m} and bacterial uptake by using...
Gentamicin-protection-invasion assays (Eisinghorst, 1994). We observe a doubling in the number of internalized bacteria when expressing InlAm compared to InlA WT (Figure 3B). Increased invasion thus appears to be predominantly caused by improved adhesion of Lmo-InlAm to Caco2 over Lmo-EGD.

After phagocytosis, bacteria need to escape from the phagosome to avoid lysosomal degradation. To rule out that factors after uptake affect the observed increase in intracellular bacteria, we analyzed intracellular growth rates of Lmo-EGD and Lmo-InlAm. A plot of colony-forming units (cfu) against time (Figure 3B) indicates a similar exponential increase for both Lmo-EGD and Lmo-InlAm. A logarithmic plot (Figure 3C) reveals the replication time of both strains to be 50 ± 4 min (gradient). The re-engineering of InlA therefore predominantly affects the process of listerial adhesion, whereas uptake, phagosomal escape, intracellular replication rates, and cell-to-cell spread appear unaltered.

As a control, we compared intracellular growth curves of both strains in the professionally phagocytic, macrophage-like cell line J774, where uptake is InlA independent (Drämsli et al., 1997). We observe indistinguishable invasion efficiencies and intracellular replication times of 46 ± 3 min for both strains (Figure 3D). InlA-independent pathophysiological characteristics of Lmo-EGD thus remain unaltered in Lmo-InlAm.

Modifying Binding Specificity

Although E-cadherin and in particular the N-terminal domain EC1 is highly conserved among mammals, the differences in amino acid sequence vary sufficiently to disallow binding of InlA to murine EC1 (mEC1). As a consequence, E-cadherin-based invasion of murine epithelial cells does not occur (Lecuit et al., 1999). Genetically replacing Glu16mEC1 by proline in mice has been proposed as a route to create a new animal model, rendering all E-cadherin expressing cells susceptible to InlA-mediated entry (Lecuit, 2005). Analyzing the affinity of InlA for mEC1E16P biophysically, we find the interaction to be exceedingly weak (Kd > 40 μM), preventing the binding affinity from being quantified unambiguously. We estimate the binding affinity as follows: InlAm binds mEC1E16P with a Kd = 14 ± 4 nM (Figure 2) and thus confirms the dominant effect (factor of 700) of the Glu16Pro substitution. The ten remaining substitutions in mEC1 (Figure 4A) contribute a factor of ~12 (Figure 2). Provided the changes are largely additive, the Kd of InlA/mEC1E16P would be ~12 × Kd = 8 μM (InlA/hEC1) or ~96 μM (Figure 2). In InlA-coated latex beads, this low binding affinity could be offset by a high density of InlA molecules leading to recognition of mEC1E16P-expressing cells (Lecuit et al., 1999). On the surface of Listeria, physical and physiological constraints would limit the density of InlA, preventing efficient adhesion and hence uptake of Lmo-EGD.

The crystal structure of InlAm/mEC1 reveals that a second substitution in mEC1 is involved in InlAm recognition. In InlA/hEC1, Glu64hEC1 forms a salt bridge to Arg85InlA (Figures 4A and 4B). In mEC1, this glutamate is replaced by glutamine, substituting the salt bridge by a hydrogen bond to Arg85inlA. Reverting these two substitutions in mEC1 produces mEC1WT-G64E with an affinity of Kd = 4 ± 2 nM for re-engineered InlAm (Figure 2) or Kd = 11 ± 4 μM for InlA WT — similar to that of InlA/hEC1 (Kd = 8 μM). A transgenic mouse bearing both the substitutions Glu16-Pro and Gln64Glu in murine E-cadherin could therefore represent a system more closely resembling the human situation than the singly substituted protein.

Altered Host Tropism In Vivo

To establish whether increased binding affinity of InlAm for murine E-cadherin will allow infection of the murine intestine comparable to that of humans, we infected C57BL/6J mice intragastrically with both Lmo-EGD and Lmo-InlAm. Challenging mice with 5 × 10⁷ to 5 × 10¹⁰ Lmo-InlAm results in dose-dependent mortality rates (Figure 5A). The median lethal dose is inferred to be ~5 × 10⁷. By contrast, the highest achievable inoculum of 5 × 10¹⁰ Lmo-EGD is lethal only for ~20% of infected mice (Figure 5A, dashed line). Lmo-InlAm is thus at least three orders of magnitude more virulent in mice than wild-type Lmo-EGD.
To establish the route of infection of Lmo-InlAm in mice, we analyzed the load of Lmo-InlAm and Lmo-EGD in affected organs, after intragastric challenge with $1 \times 10^{10}$ bacteria (Figure 5 and Figure S2). In stomach and intestine, loads of Lmo-InlAm and Lmo-EGD (Figures 5B and 5C) are largely comparable until day 2 post infection (p.i.). Thereafter, Lmo-InlAm loads increase strongly (Figures 5B and 5C), whereas loads of Lmo-EGD remain constant.

In mesenteric lymph nodes, the spleen, and the liver, bacterial loads of both strains increase until day 2 post infection (p.i.). Thereafter, Lmo-InlAm loads increase strongly (Figures 5B and 5C), whereas loads of Lmo-EGD remain constant.

To compare bacterial-growth kinetics at a lower infection dose, we challenged mice intragastrically with $5 \times 10^7$ of Lmo-EGD and Lmo-InlAm (Figure S3). Again, we observe enhanced virulence for Lmo-InlAm relative to Lmo-EGD. Although the effect in terms of bacterial numbers is not as pronounced as for the higher dose, slower kinetics of bacterial dissemination allow later time points to be investigated. In the stomach and small intestine, differences in bacterial loads become significant by day 5 p.i. In the

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**Figure 4. Comparison of InlA/hEC1 and InlAm/mEC1 Complexes**

(A) Sequence alignment of the N-terminal, extracellular domains (EC1) of human (hEC1, violet), and murine (mEC1, yellow) E-cadherin. Sequence differences involving charged residues are marked by red and blue boxes, and all others were marked by green boxes.

(B) Superposition of both protein complexes. LRRs are numbered. Critical residues are shown as ball and stick, and solvent-exposed substitutions are shown as spheres (colors are as shown in [A]).

(C) Hydrophobic Pro16 of hEC1 (violet) is accommodated in a hydrophobic pocket of InlA. The carboxylate of Glu16 of mEC1 (yellow), well defined in the 2Fo-Fc difference electron density (green, contoured at 1σ), occupies the same pocket.

(D) Lys19 of hEC1 forms a favorable salt bridge to InlA. Trans Glu16 of mEC1 repositions Lys19 of mEC1, trapping its side-chain through an intramolecular H-bond (arrow).
spleen, liver, and mesenteric lymph nodes, Lmo-InlAm loads are consistently higher than those of Lmo-EGD.

**Histological Analysis of InlA-Dependent Infection Mechanisms**

Histological and immunohistochemical studies of Peyer’s patches of infected mice indicate that both Lmo-EGD and Lmo-InlAm remain restricted to the dome and germinal centers, where they induce neutrophil infiltration and necrosis (Figures S4A–S4D) with gradually increasing severity along the small-intestinal axis. Colonization is, however, essentially indistinguishable until day 4 p.i. Whereas the Lmo-EGD infection subsides after day 4 p.i., no such remission is observed for Lmo-InlAm.

Immunohistochemical analyses of the intestinal mucosa clearly demonstrate that Lmo-EGD do not invade epithelial tissue (Figure 6). Instead, bacteria are exclusively observed in the lumen or occasionally adhere to the surface of individual villi (Figures 6B, 6F, and 6J) as late as day 4 p.i. (Figure 6N). Similarly, the intestinal tissue is fully intact without any signs of inflammation until day 3 p.i. (Figures 6A and 6E). Transient inflammatory response with enhanced mucus secretion and mild, local erosion of epithelium is present at day 4 p.i. (Figure 6I). The inflammation, however, subsides by day 5 p.i. (Figure 6M).

In Lmo-InlAm-infected mice, colonization of epithelia at villous tips are observed 24 hr p.i. in the ileum (Figure 6D), rapidly spreading to extended areas of the epithelium and accumulating within the lamina propria (subepithelial tissue) (Figures 6H, 6L, and 6P). On day 4 p.i., high bacterial loads cause erosion of the epithelium and fusion of villi (Figures 6K and 6O). Lesions are more pronounced in the ileum than in the duodenum and jejunum.

**Role of InlA in Systemic Spread**

To analyze the role of InlAm in systemic infections of internal organs independently of intestinal uptake, we inoculated mice intravenously (i.v.) with both low ($5 \times 10^3$) or high ($2.4 \times 10^4$) doses of Lmo-EGD or Lmo-InlAm. In contrast to the oral route of infection, the survival rates for both strains are found to be indistinguishable for both bacterial doses (Figures 7A and 7B). Similarly, bacterial loads in mesenteric lymph nodes, the spleen, and the liver at different time points after i.v. inoculation demonstrate...
comparable virulence of Lmo-EGD and Lmo-InlAm (Figures 7C–7E).

Blood-Placental Barrier

Lmo-EGD remains problematic to humans inter alia because of its ability to cross the blood-placental barrier, thereby resulting in sepsis or miscarriage. Ex vivo experiments have shown that InIA-mediated entry into human placental trophoblasts is important in placental infection (Lecuit et al., 2004). Studies of Lmo-EGD-infected pregnant BALB/c mice (functional InIB, nonfunctional InIA) (Le Monnier et al., 2007) and guinea pigs (functional InIA and nonfunctional InIB) (Bakardjiev et al., 2004) indicate that InIA and InIB individually are insufficient at inducing vertical transmission in vivo. They, however, confirm trophoblasts to be the primary target of Lmo-EGD. To ascertain whether the combination of functional InIA and InIB facilitates vertical transmission, we analyzed the ability of Lmo-InlAm to breach the murine blood-placental barrier. Pregnant BALB/c mice were infected orally with $5 \times 10^9$ cfu of Lmo-EGD or Lmo-InlAm at embryonic day (E13.5) or E14.5 of gestation. We find bacterial loads of fetal-placental units at day 2, 3, and 4 p.i. to be indistinguishable (Figure S5). Histopathological analysis of placentas and embryos confirm that lesions and inflammatory responses are similar in mice infected with either strain (Figure S6). Equivalent bacterial loads were also observed in fetal-placental units in female mice challenged with $5 \times 10^4$ cfu.
Lmo-InlAm and Lmo-EGD intravenously at day E14.5 (data not shown). Crossing of the blood-placental barrier is thus not InlA dependent in BALB/c mice.

DISCUSSION

Rationally Redesigning the InlA/hEC1 Complex

By using minimal, structure-derived modifications of a single pathogenicity factor, we have rationally redesigned the interface of InlA/hEC1 to increase its binding affinity and, in the process, modify the binding specificity of InlA to include murine E-cadherin. Incorporating these modifications into the original bacterium, we create a new listerial strain that mimics the uptake of wild-type Lmo-EGD in the human intestine in the mouse instead. Our approach circumvents the limitations of existing models of listeriosis in that both early and late responses are accessible and by uniquely providing a system in which both InlA and InlB are fully functional and expressed at wild-type levels. Because intestinal uptake depends only on murine E-cadherin, all mouse strains may be analyzed with Lmo-InlAm. It should, furthermore, allow both host responses to food-borne pathogens crossing the intestinal barrier and the role of individual listerial factors during infection to be analyzed in an in vivo setting. To our knowledge, this is the first time that a virulence factor has been rationally modified, without relying on known mutations from related strains, or that a novel strain of a pathogen has been created with an extended host range.

Implications of InlAm affinity for EC1

To induce uptake into epithelial cells by the zipper-mechanism, L. monocytogenes must adhere tightly to the eukaryotic-cell membrane. Accordingly, tighter adhesion of Lmo-InlAm to Caco2 cells increases the efficiency of uptake compared to wild-type Lmo-EGD. Surprisingly,
however, the 6700-fold increased binding affinity merely causes a doubling in adhesion (Figure 3A). Because low binding affinity may be counteracted by high protein concentration, the efficient adhesion of Lmo-EGD to Caco2 cells is probably due to the high amount of E-cadherin on these cells.

In the intestinal epithelium, the amount of E-cadherin accessible to *L. monocytogenes* apically is limited because of its basolateral localization. The exception being multicellular junctions in epithelial monolayers where E-cadherin is transiently exposed (Pentecost et al., 2006). The low abundance of such junctions and the low affinity of InlA for its receptor E-cadherin would severely limit uptake of *L. monocytogenes* in vivo. By increase of binding affinity, the available E-cadherin could be utilized more efficiently, thus allowing a higher proportion of Lmo-InlAm to invade the intestinal epithelium. Although pathogenicity would be increased, higher rates of uptake of InlAm may prove to be advantageous therapeutically as in oral vaccination (Guimaraes et al., 2005), bacterial gene therapy (Dietrich et al., 1998), or drug delivery (Sleator and Hill, 2006). Because regulation of E-cadherin is, intimately linked to cell transformation and to the development of cancer (Wheelock and Johnson, 2003), InlAm may, further, prove to be useful in the study of E-cadherin-mediated signal transduction.

**InlA-Dependent and -Independent Routes of Listerial Infection**

In mice, Lmo-EGD primarily target the Peyer’s patches (Marco et al., 1997). Specialized, epithelial-like M cells (Clark and Hirst, 2002) covering these centers of mucosal immunity actively transport antigens and viable bacteria from the intestinal lumen to the underlying immune cells (Kraehenbuhl and Neutra, 2000). Food-borne pathogens such as *Salmonella* or *Shigella* exploit this indiscriminate uptake by reinvading M cells from the basolateral side, spreading to cells of the adjoining epithelium (Cossart and Sansonetti, 2004). E-cadherin on the basolateral side of M cells potentially allows Lmo-EGD to employ a similar strategy in humans. Our analyses, however, indicate that colonization of M cells and Peyer’s patches by *L. monocytogenes* apically is limited because of its basolateral localization. The exception being multicellular junctions in epithelial monolayers where E-cadherin is transiently exposed (Pentecost et al., 2006). The low abundance of such junctions and the low affinity of InlA for its receptor E-cadherin would severely limit uptake of *L. monocytogenes* in vivo. By increase of binding affinity, the available E-cadherin could be utilized more efficiently, thus allowing a higher proportion of Lmo-InlAm to invade the intestinal epithelium. Although pathogenicity would be increased, higher rates of uptake of InlAm may prove to be advantageous therapeutically as in oral vaccination (Guimaraes et al., 2005), bacterial gene therapy (Dietrich et al., 1998), or drug delivery (Sleator and Hill, 2006). Because regulation of E-cadherin is, intimately linked to cell transformation and to the development of cancer (Wheelock and Johnson, 2003), InlAm may, further, prove to be useful in the study of E-cadherin-mediated signal transduction.

The courses of infection after intravenous inoculation are, perhaps surprisingly, indistinguishable for the two listerial strains, implying that InlA is not essential for the later stages of listerial infections. Transport of *Listeria* by immune cells may be more important for systemic spreading than previously anticipated (Neuenhahn et al., 2006). A tendency of slightly higher virulence of Lmo-InlAm indicates that InlA may nevertheless impart a slight advantage during this stage of the infection as well. Overall, InlA is crucial for establishing the infection of the intestinal epithelium. InlB and potentially other virulence factors then take over, leading to spread and systemic infection (Hamon et al., 2006).

**L. monocytogenes Infection in Pregnant Mice**

Pregnancy in humans increases susceptibility toward *L. monocytogenes* (Vazquez-Boland et al., 2001), and InlA is reportedly crucial in crossing the blood-placental barrier (Lecuit et al., 2004). Both pregnant mice (Le Monnier et al., 2006; Le Monnier et al., 2007) and guinea pigs (Bakardjiev et al., 2005) have been used to investigate this link. The usefulness of these models is, however, limited by the respective host specificities of InlA and InlB (see above). Lmo-InlAm overcomes these limitations by providing active InlA and InlB for the murine system. Infecting pregnant mice orally with Lmo-InlAm mimics the human course of infection and vertical transmission. We, however, do not observe a significant difference between Lmo-EGD and Lmo-InlAm both by intravenous and oral infection of pregnant mice. This would indicate that the natural route of placental infection in mice is not dependent on either functional InlA or InlB. The uniquely invasive form of trophoblast-cell-mediated hemochorial placentaion in humans (Moffett and Loke, 2006) could imply distinct susceptibilities for listerial infections. Alternatively, vertical transmission may occur by the “Trojan horse” strategy.
via infected monocytes or macrophages as discussed for the blood-brain barrier (Drevets, 1999; Bakardjiev et al., 2005). In this case, InLA-mediated entry would play a subordinate role and would only be detectable in vitro or ex vivo where the "Trojan horse" mechanism is absent.

**EXPERIMENTAL PROCEDURES**

**Protein Expression, Purification, and Crystallization**

For structural and biophysical studies, functional fragments of InLA (residues 36–496) and E-cadherin (EC1, residues 1–105), were used. Murine EC1 (mEC1) was cloned from a cDNA library (German Resource Centre for Genome Research, clone ID IMAPq998A095932Q1) into the pGEX-6P-1 expression vector as previously described for hEC1 (Schubert et al., 2002). Site-directed mutations were introduced by QuikChange Mutagenesis (Stratagene). Proteins were expressed and purified as described (Schubert et al., 2002). Crystals were grown by vapor diffusion with 5 mM protein and a stoichiometric ratio of 1:1. The reservoir solution for InLA-variant/mEC1 crystals was 20–25% PEG4000, 100 mM MES/Tris buffer (pH 7.0–7.5), 100 mM Na acetate, and 20–100 mM CaCl2 for InLA*S192N-Y369S/mEC1, the reservoir solution was 20% PEG6000, 0.1 M Na citrate (pH 5.2), and 0.5 M LiCl, whereas 18% PEG4000 (v/v) was added for cryoprotection.

**Structure Determination**

Data were collected at BL1, Protein Structure Factory, BESSY (Emsley and Cowtan, 2004) and validated with COOT (Emsley and Cowtan, 2004). The structural model was built, analyzed, and validated with COOT (Emsley and Cowtan, 2004) and refined with REFMAC5 (Murshudov et al., 1997). Figures were prepared with PYMOL (www.pymol.org).

**Isothermal Titration Calorimetry**

Thermodynamic data were obtained with a Microcal ITC (MicroCal). All samples were dialyzed against 50 mM HEPES (pH 7.5) and 20 mM CaCl2, and 5–10 μl aliquots of InLA variants were injected into the ITC cell containing 1.35 ml of mEC1 or hEC1 variants, respectively. After correction for heat of dilution (ΔHₒ) data were analyzed with the “single set of independent sites” model (Micro Cal Origin 2.9).

**Construction of the Isogenic Mutant Stain of Listeria monocytogenes**

Full-length inLA was amplified from genomic DNA with the primers 5’-AGGAGGATCCATGCTGACGAGCGACACGGCAACCTG3’ and 5’-AGGAGGGCCGCCGCTCTAGATGCGGTGGACGGCT3’ and cloned into the vector pPL2 (Lauer et al., 2002) with BamHI and NotI. We incorporated the desired mutations by excising a 1026 nucleotide BamHI/BstXI fragment from pPL2 and replacing this with an equivalent fragment. The mutated part of inIB was amplified with primers 5’-AGGAGGATCCATGCTGACGAGCGACACGGCAACCTG3’ and 5’-AGGAGGGCCGCCGCTCTAGATGCGGTGGACGGCT3’ and cloned into the vector pPL2 (Lauer et al., 2002) with BamHI and NotI. The resulting plasmid bears full-length, mutated inLA, whereas the 5’ inIB fragment is fused to gfp. The inLA*S192N-Y369S-inIB-gfp fragment was excised from pETM11-GFP-FUS-InLA*S192N-Y369S_inIBp by digesting with NotI, filling in by Klenow polymerase and dNTP (NEB), heat inactivation, and cutting with BamHI. The fragment was cloned into the pAUL-A shuttle vector (Chakraborty et al., 1992) cut with HindIII, filled in with Klenow polymerase and dNTP’s, heat inactivated, and digested with BamHI. This vector pAUL-A-InLA-inIB was integrated (Chakraborty et al., 1992) into the Listeria monocytogenes EDG-e inLA2 knockout strain (Lingnau et al., 1995). Reversion via homologous recombination was achieved as described (Lingnau et al., 1995). All steps were monitored by PCR analysis. The resulting strain, Lmo-InLA™, carries the intact inIA locus including two point mutations within InLA. The correct reversion was confirmed by PCR sequencing and immunoblotting with monoclonal antibodies against InLA and InLB.

**Invasion Assays**

Uptake of wild-type Listeria monocytogenes EDG serotype 1/2a (ATCC-number BAA-679) and Lmo-InLA™ were analyzed with the human colorectal epithelial cell line Caco2 (ATCC HTB-37) and the murine macrophage-like cell line J774 (ATCC TIB-67). Caco2 cells were cultured in minimal essential medium (MEM) with Earle’s salts (Invitrogen), supplemented with 10% FCS (PAA Laboratories), 2 mM glutamine, 1 mM sodium pyruvate, and 1% nonessential amino acids at 37°C/7% CO2. J774 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% FCS (PAA Laboratories), and 2 mM glutamine at 37°C/7% CO2. Two days prior to infection, 2 × 10⁵ Caco2 cells or 2 × 10⁴ J774 cells were seeded into 24-well plates. An overnight culture of Listeria was diluted 1:50 in BHI medium (Difco) and grown at 37°C till the middle of the log phase. Bacteria were washed twice in medium without FCS, and 8 × 10⁵ (Caco2) or 2 × 10⁵ (J774) bacteria were added to the monolayer (Caco2) or semiconfluent cells (J774)/well, centrifuged 5 min at 500 g, and incubated for 1 hr. Cells were washed with PBS. Medium containing 100 μg/ml gentamicin was added to kill extracellular bacteria. After 1, 2, 3, and 4 hr, cells were washed with PBS and lysed with sterile water containing 0.2% Triton X-100. Serial dilutions of cell lysates were plated onto BHI agar plates and incubated 24 hr at 37°C. Colonies were counted, and the recovery per well was determined. Each data point of one experiment was determined three times, and experiments were independently repeated in triplicate.

Adhesion assays were performed like invasion assays without centrifugation after addition of bacteria to Caco2 cells. Thirty minutes p.i., cells were washed 5-fold with PBS and lysed with 0.2% Triton X-100. Bacterial numbers were determined as described.

**Mouse Infection**

Ten-weeks-old female C57BL/6J mice were purchased from Harlan-Winkelmann (Borchen) and housed for a further week in the specific pathogen-free (SPF) animal facility (Helmholtz Centre for Infection Research) prior to infection. Lmo-EGD and Lmo-InLA™ were grown in brain-heart infusion (BHI) broth (BD-Difco) until the end of the log-growth phase. After washing, bacteria were diluted in PBS. A total of 0.2 ml of the desired inoculum of either strain was mixed with 0.3 ml PBS containing 50 mg CaCO3 (Lecuit et al., 2001). The suspension was inoculated intragastrically into mice (starved overnight, water allowed) with a 21 gauge feeding needle attached to a 1 ml syringe. Animals were then either monitored daily to determine survival rates or they were sacrificed and dissected for histological analysis or for determining bacterial counts in organs at the time points indicated. All animal experiments were reviewed and approved by local authorities. For infection of C57BL/6J mice intravenously, Lmo-EGD or Lmo-InLA™ were prepared as described (Pasche et al., 2005). Survival rates and organ loads were determined as described for oral infections.

**Infection of Pregnant Mice**

BALB/c mice (Harlan-Winkelmann) were infected intragastrically or intravenously on day E13.5 or E14.5 as described above. Animals were examined daily. At indicated time points p.i., mice were sacrificed and the abdominal cavity was opened aseptically. Each placenta and fetus was independently dissected and analyzed with cfu determination or histopathology.
Bacterial Counts in Organs, Placentae, and Embryos
Stomach and small intestine were removed and incubated for 2 hr at 20°C in PBS supplemented with 100 μg/ml gentamicin (Gibco) to kill extracellular bacteria; other organs and embryos were sterilely dissected. Organs and embryos were homogenized, and serial dilutions were plated onto BHI agar, thus allowing a determination of bacterial counts per mg organ/embryo.

Statistical Analysis
Survival curves were statistically evaluated by Kaplan-Meier and Log-rank (Mantel-Cox) analyses. Bacterial loads are listed as median ±95% confidence intervals, statistically evaluated by the Mann-Whitney U nonparametric test. Calculations were done with GraphPadPrism4 (GraphPad Software). Differences were considered significant for $p \leq 0.05$.

Histology and Immunohistochemistry
Organs were fixed in 10% formalin, dehydrated, and embedded in paraffin. Sections of 5 μm were cut and stained with hematoxylin-eosin or used for immunohistochemistry (IHC). For detection of Lmo Listeria, O antiserum (Serotype 1 and 4; BD-Difco) was used. For detection, a secondary, peroxidase-coupled goat-anti-rabbit antibody was used. IHC sections were counterstained with hematoxylin.

Supplemental Data
Supplemental Data include six figures and one table and can be found with this article online at http://www.cell.com/cgi/content/full/129/5/891/DC1/.

ACKNOWLEDGMENTS
We thank our colleagues Daniela Gebauer and Stefanie Edler for excellent technical assistance and Dr. Theresia E.B. Stradal (Division of Cell Biology) for introducing T.W. to cell-biological methods and critical discussions. Funding by the Deutsche Forschungsgemeinschaft as part of the Priority Program 1150 (SCHU 1560/1-1 and 1-2) to W.D.S.; by the European Community (FMRX-CT-1999-01214) to T.W.; and by the Fonds der Chemischen Industrie to D.W.H is gratefully acknowledged. We acknowledge the staffs of beam line BL1, Protein Structure Factory, BESSY (Berlin, Germany), and beam line BW6 (MPG DESY, Hamburg, Germany) for assistance and beam time.

Received: October 20, 2006
Revised: January 30, 2007
Accepted: March 15, 2007
Published: May 31, 2007

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Accession Numbers

Crystallographic data have been deposited with the Protein Data Bank (accession numbers 2OMV, 2OMW, and 2OMY).