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# Functional Genomic Studies of the Intestinal Response to a Foodborne Enteropathogen in a Humanized Gnotobiotic Mouse Model*[S]

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**Members of the genus *Listeria* provide a model for defining host responses to invasive foodborne enteropathogens. Active translocation of *Listeria monocytogenes* across the gut epithelial barrier is mediated by interaction of bacterial internalin (InlA) and its species-specific host receptor, E-cadherin, whereas translocation across Peyer's patches through M-cells is InlA-independent. To define microbial determinants and molecular correlates of the host response to translocation via these two routes, we colonized germ-free transgenic mice expressing the human enterocyte-associated E-cadherin receptor with wild-type (WT) or mutant *L. monocytogenes* strains, or its nonpathogenic noninvasive relative *Listeria innocua*, or with *Bacteroides thetaiotaomicron*, a prominent gut symbiont. Mouse GeneChips, combined with Ingenuity Pathway software, were used to identify canonical signaling pathways that comprise the response to WT *L. monocytogenes versus* the other species. Gain- and loss-of-function experiments with *L. innocua* and *L. monocytogenes*, respectively, demonstrated that the 773-member transcriptional signature of the response to WT *L. monocytogenes* is largely conserved in the Δ*inlA* mutant. Internalin-dependent responses include down-regulation of gene networks involved in various aspects of lipid, amino acid, and energy metabolism and up-regulation of immunoinflammatory responses. The host response is markedly attenuated in a listeriolysin-deficient (Δ*hly*) mutant despite its ability to be translocated to the lamina propria. Together, these studies establish that *hly*, rather than bacterial invasion of the lamina propria mediated by InlA, is a dominant determinant of the intensity of the host response to *L. monocytogenes* infection via the oral route.**

The human gut is inhabited by a complex community of trillions of microorganisms representing all three known domains of life: Bacteria, Archaea, and Eukarya (1–3). Our microbiota is dominated by members of Bacteria, with components of two divisions, the Firmicutes and the Bacteroidetes, comprising >90% of all phylogenetic types in those few individuals where comprehensive 16S rRNA gene sequence-based enumerations have been performed (1, 2). Although most of the estimated 500–1000 bacterial species in the gut microbiota appear to enjoy a mutually beneficial relationship with their host, potential pathogens are also present or may be introduced through the consumption of food or water.

Members of the genus *Listeria* provide a model for comparing host responses to invasive *versus* noninvasive foodborne bacteria. Fully sequenced genomes are available from two species: *Listeria monocytogenes*, an enteroinvasive human pathogen that can cross the intestinal as well as blood-brain and placental barriers; and *Listeria innocua*, a nonpathogenic and noninvasive relative that shares 84% of its genes with *L. monocytogenes* (4). *L. monocytogenes* is estimated to be present in the small intestines of up to 5% of individuals yet only a few, typically those who are immunocompromised, develop invasive symptomatic disease (5).

*L. monocytogenes* and *L. innocua* can cross the follicle-associated epithelium (FAE)[5] that overlies the lymphoid follicles of Peyer's patches with equal efficiency (6). *L. monocytogenes*, unlike *L. innocua*, expresses internalin (InlA), a surface protein that is sufficient to promote bacterial internalization into enterocytes that express its receptor, human E-cadherin (hEcad). Epidemiological (7) and histopathological data (8, 9), as well as experiments using human primary cells and tissue explants (8), indicate that InlA is an important virulence factor in humans, mediating targeting and crossing of both intestinal and placental barriers. A single amino acid difference ($Pro^{16}$ in human *versus* $Glu^{16}$ in mouse) enables human but not mouse E-cadherin to function as a receptor for InlA (10). In conventionally raised, adult transgenic mice expressing hEcad under the control of an enterocyte-specific promoter (*Fabpi-hEcad*), *L. monocytogenes* is able to invade enterocytes that cover small intestinal villi and enter the underlying lamina propria. This

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[5] The abbreviations used are: FAE, follicle-associated epithelium; InlA, internalin; hEcad, human E-cadherin; WT, wild type; LLO, listeriolysin; CFU, colony-forming unit; SAM, significance analysis of microarrays; IPA, Ingenuity pathway analysis; GF, germ-free; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase.

receptor-mediated invasion results in a rate of translocation across the intestinal barrier that far exceeds that of InlA-independent translocation across the FAE (6, 9).

Once internalized, *L. monocytogenes* is able to infect macrophages and other cells that neighbor the epithelium, and ultimately, the bacteria disseminate to organs such as the spleen and liver. The ability of *L. monocytogenes* to survive and multiply in professional phagocytes situated in the lamina propria, or beneath the FAE, is dependent upon another virulence factor that is not present in the genome of *L. innocua*: listeriolysin (LLO). LLO, encoded by the *hly* gene, is a pore-forming, cholesterol-dependent cytolysin that mediates bacterial escape from phagosomes to the cytosol, where the bacterium is able to replicate and spread to neighboring cells (11).

A confounding problem with studies of oral listeriosis in conventionally raised, humanized *Fabpi-hEcad* mice is the extent to which other members of the microbiota contribute to observed host responses. Therefore, in this report, we have generated germ-free (GF) *Fabpi-hEcad* mice to directly assess the host response (*i*) as a function of the route of translocation of *L. monocytogenes* across the epithelial barrier (enterocyte *versus* nonenterocyte) and (*ii*) as a function of residency in the underlying lamina propria in the presence or absence of listeriolysin. To do so, we colonized the animals with (*i*) WT *L. monocytogenes* and isogenic mutant strains with *inlA*, *inlB*, *or hly* deletions; (*ii*) WT *L. innocua* and isogenic strains engineered to express InlA or LLO; or (*iii*) *Bacteroides thetaiotaomicron*, a sequenced, well characterized, human gut symbiont that is an adept, adaptive forager of dietary polysaccharides (12, 13). Our results provide a direct view of the significance of the route of bacterial entry into and residency within the lamina propria on the host response.

## EXPERIMENTAL PROCEDURES

*Bacterial Strains*—*L. monocytogenes* WT reference strain EGD (*L. monocytogenes*, BUG 600) and isogenic mutants in *hly* (*Lm*(Δ*hly*), BUG 1954), *inlA* (*Lm*(Δ*inlA*), BUG 947), and *inlA* plus *inlB* (*Lm*(Δ*inlAB*), BUG 949), *L. innocua* WT reference strain (BUG 499), *L. innocua* expressing InlA (*Li*(*inlA*), BUG 1489), and *L. innocua* expressing LLO (*Li*(*hly*), BUG 226) and the *B. thetaiotaomicron* type stain (VPI-5482) were cultured as described (8, 13).

*Animals*—All experiments involving mice were conducted using protocols approved by the Washington University Animal Studies Committee. GF C57BL/6J transgenic mice were housed in plastic gnotobiotic isolators (14) under a strict 12-h light cycle and fed a standard autoclaved chow diet (B&K Universal) *ad libitum*. $10^9$ colony-forming units (CFU) of bacteria, cultured to mid-log phase, were taken up in 0.5 ml of PBS containing 50 mg of $CaCO_3$ and inoculated by gavage into 12–15-week-old male mice that had been deprived of food, but not water, for 12 h. Animals were sacrificed at 72 h after inoculation; the small intestine was removed and cut into 16 equal-sized segments (numbered 1–16; proximal-to-distal). Spleens were homogenized in PBS (2 ml/spleen), and CFU counts in this material and in luminal contents harvested from intestinal segment 15 were determined by streaking 10-fold serial dilutions onto brain-heart-infusion agar plates and incubating the plates for 2 days at 37 °C under aerobic or anaerobic conditions.

*Immunohistochemical Studies*—Optimal Cutting Temperature TissueTek compound (VWR Scientific)-embedded blocks of small intestinal segment 14 (15) were cryosectioned (7-μm-thick sections) and fixed in ethanol (−20 °C for 5 min). Blocking steps, antibody dilutions, and washes were performed in 1% bovine serum albumin in PBS. Slides were stained with a mouse anti-human E-cadherin IgG (1:100) (9) followed by a goat anti-mouse IgG-fluorescein isothiocyanate conjugate (1:100; Molecular Probes). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (40 ng/ml), and sections were visualized on an Axiovert 200M (Zeiss, Gottingen, Germany) fitted with an Axiocam MRm (Zeiss).

Five-μm-thick sections of formalin-fixed, paraffin-embedded blocks prepared from intestinal segment 13 of mono-associated mice were stained with hematoxylin and eosin. Adjacent sections were stained with *L. monocytogenes* R11 or *L. innocua* R6 primary antibodies (16) [final dilution was 1:500 in blocking buffer (1% bovine serum albumin, 0.3% Triton X-100 in PBS)] followed by horseradish peroxidase-conjugated monkey anti-rabbit Ig (1:400). Sections were counterstained with hematoxylin. Ileal sections were also stained with rat anti-mouse CD45 and CD3 and F4/80 rat monoclonal antibodies (all from Pharmingen) followed by horseradish peroxidase-conjugated rabbit anti-rat Ig (1:400; Invitrogen). Antigen-antibody complexes were visualized by using reagents supplied in the Envision kit (Dakocytomation, Carpinteria, CA). Samples were viewed with an Axioskop 2 (Zeiss) microscope and images captured with an Evolution PM color camera (Mediacybernetics, Silver Spring, MD).

*GeneChip Analysis*—RNA was purified from small intestinal segment 12 of each mouse (Midi RNeasy kit (Qiagen) with on-column DNase digestion). Equivalent amounts of RNA from mice in each treatment group ($n = 4$/experiment) were pooled, and two biotinylated cRNA targets (40 μg/replicate) were independently prepared from each sample (17). cRNAs were hybridized to Affymetrix Mouse Genome U74Av2 GeneChips, and the resulting data sets were analyzed using DNA-Chip and significance analysis of microarrays (SAM) as follows. CEL files[6] were read into dChip, and Present/Absent calls were read in from the accompanying GeneChip operating software (Affymetrix) TXT files. The GeneChip with the median overall intensity served as the base line to which all other GeneChips within that treatment group were normalized. Signals were assigned to each probe set by using model-based expression (perfect match-mismatch model). Unsupervised filtering was performed using the following criteria: (*i*) variation across samples (standard deviation/mean) $>0.40$ and $<10.00$; (*ii*) called Present in $\geq 20\%$ of arrays; and (*iii*) variation between replicates $<0.2$, as assessed by median value of standard deviation/mean. For two-class SAM analysis, expression values were exported from dChip only for probe sets called Present in at least one GeneChip within the group being analyzed. Significance was defined by maintaining a false discovery rate

[6] Gene Expression Omnibus (GEO) accession number GSE7013.

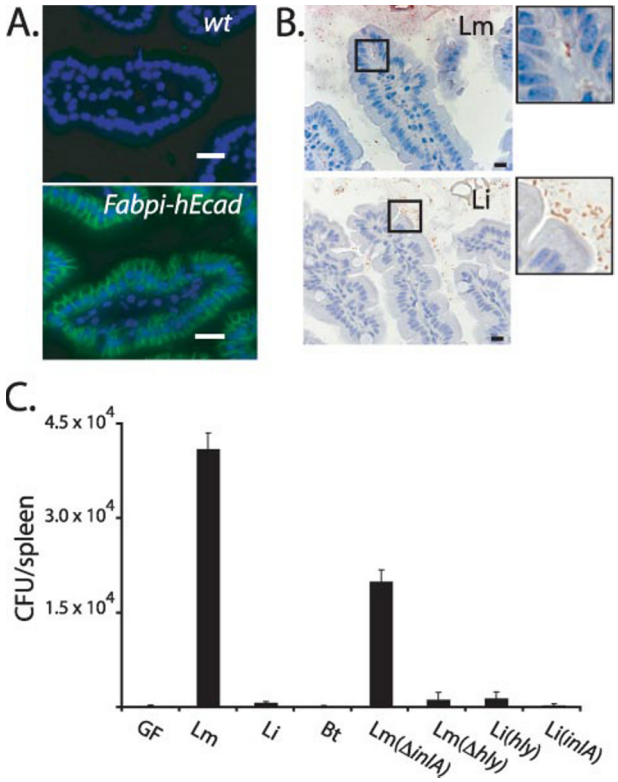


FIGURE 1. **Infection of gnotobiotic *Fabpi-hEcad* mice with *L. monocytogenes.*** *A*, immunofluorescence microscopy showing hEcad expression (*green*) in ileal villus epithelial cells of GF *Fabpi-hEcad* mice. Cell nuclei are stained with 4,6-diamidino-2-phenylindole (*blue*). A GF nontransgenic (*wt*) littermate control is shown. *B*, immunohistochemical detection of bacteria (*red*) using antibodies to *L. monocytogenes* (*Lm*) or *L. innocua* (*Li*) in the ileums of *Fabpi-hEcad* mice. Cell nuclei are counterstained with hematoxylin (*blue*). Stained bacteria are shown at higher magnification in *boxed areas*, either in villus lamina propria (*top right panel*) or in the intestinal lumen (*bottom right panel*). *C*, CFU assays of splenic homogenates prepared from gnotobiotic mice. Mean values ± S.D. are plotted ($n = 4$ animals/group). *Bt, B. thetaiotaomicron. Scale bars*: *A*, 20 $\mu$m; *B*, 10 $\mu$m.

($q$-value) below 1%. Ingenuity pathway analysis (IPA) focus genes were identified as having significantly increased expression in the specified group; 6,351 probe sets were called "Present" in at least one of the GeneChips by GeneChip operating software (Affymetrix).

## RESULTS

*Generation of Germ-free Fabpi-hEcad Transgenic Mice*—*Fabpi-hEcad* C57BL/6J × SJL/J transgenic mice that express hEcad in small intestinal enterocytes under the control of nucleotides −1178 to +28 of the rat *Fabpi* gene (9) were backcrossed to C57BL/6J animals to generation N7 and then rederived as GF (14). The growth rate, adult weight, and fertility of *Fabpi-hEcad* transgenic mice were indistinguishable from that of their GF nontransgenic littermates ($n = 8$ mice/group). Histologic assessment of serial sections, prepared along the cephalocaudal axis of their small intestines, revealed no discernible differences between transgenic and normal littermates. Immunohistochemical studies of 12–15-week-old animals confirmed that the GF transgenic mice expressed hEcad in villus enterocytes (Fig. 1*A*). The FAE in the distal small intestine, but not the colonic epithelium, contained detectable levels of immunoreactive protein (data not shown).

*L. monocytogenes but Not L. innocua Behaves as an Enteroinvasive Microbe in Gnotobiotic Fabpi-hEcad Mice*—Twelve-to-fifteen-week-old GF C57BL/6J *Fabpi-hEcad* mice were inoculated with $10^9$ CFU of WT *L. monocytogenes*, WT *L. innocua*, or *B. thetaiotaomicron*. Animals were sacrificed 72 h after inoculation, a time that corresponds to peak levels of *L. monocytogenes* in intestinal tissue and mesenteric lymph nodes of conventionally raised animals (9, 18). No mortality occurred after 3 days in any group ($n = 8$ animals/group), although *L. monocytogenes*-infected mice exhibited clinical signs of infection (diarrhea, roughcast fur, 5–15% weight loss, and tremor). After 72 h, levels of bacterial colonization in the jejunum (middle third of the small intestine) and ileum (distal third) were not significantly different among mice in each treatment groups ($10^8$-$10^9$ CFU/ml luminal contents; see supplemental Fig. S1). Examination of ileal sections stained with anti-*L. monocytogenes* polyclonal antibodies (16) revealed bacteria invading the tips of intestinal villi and in the lamina propria (Fig. 1*B*), similar to our previously reported observations in infected conventionally raised C57BL/6J × SJL/J *Fabpi-hEcad* animals (9). In contrast, invasion was not detected in the intestines of *L. innocua*-colonized transgenic mice (Fig. 1*B*). Consistent with systemic dissemination, quantitative CFU assays of splenic homogenates disclosed viable bacteria in *L. monocytogenes*-infected animals at levels equivalent to those observed in conventionally raised *Fabpi-hEcad* hosts. In contrast, spleens did not harbor appreciable numbers of viable organisms in *L. innocua*- or *B. thetaiotaomicron*-colonized controls (Fig. 1*C*).

*Intestinal Response to an Enteropathogenic Invasive Bacterial Species*—Histologic studies revealed prominent mononuclear cell infiltrates in the lamina propria of the intestines of *L. monocytogenes*-infected mice (Fig. 2*A*). These mononuclear cells reacted with a pan-leukocyte antibody (anti-CD45) and were predominantly macrophages (F4/80 positive) and T-lymphocytes (CD3 positive) (Fig. 2*B*). In contrast, no lamina propria infiltrates were observed in any of our *L. innocua*- or *B. thetaiotaomicron*-colonized mice (Fig. 2, *A* and *B*, and data not shown).

To further distinguish the host response to *L. monocytogenes* infection *versus* colonization with *L. innocua* or *B. thetaiotaomicron*, we performed GeneChip profiling of ileal RNAs prepared from mice that had been colonized (mono-associated) with each bacterial species, as well as GF transgenic controls ($n = 4$/group). Using the stringent filtering criteria described under "Experimental Procedures," we identified 304 genes with significantly different levels of expression between these four colonization states (Fig. 2*C*). Unsupervised hierarchical clustering of these 304 genes established that WT *L. monocytogenes* elicits a highly reproducible host response, as evidenced by the high degree of similarity in profiles from two independently colonized groups of infected animals ($n = 4$ mice/group) (Fig. 2*C*). The response to *L. monocytogenes* is strikingly different from that elicited by *L. innocua*, despite the high degree of relatedness between these two *Listeria* species (2523 of 2853 *L. monocytogenes* genes have orthologs in *L. innocua*; the 270 genes specific to *L. monocytogenes* include all of its known virulence factors, such as *inlA, inlB, hly*, and *actA*) (4). The clustering also disclosed that the ileal transcriptome of *L. innocua*-

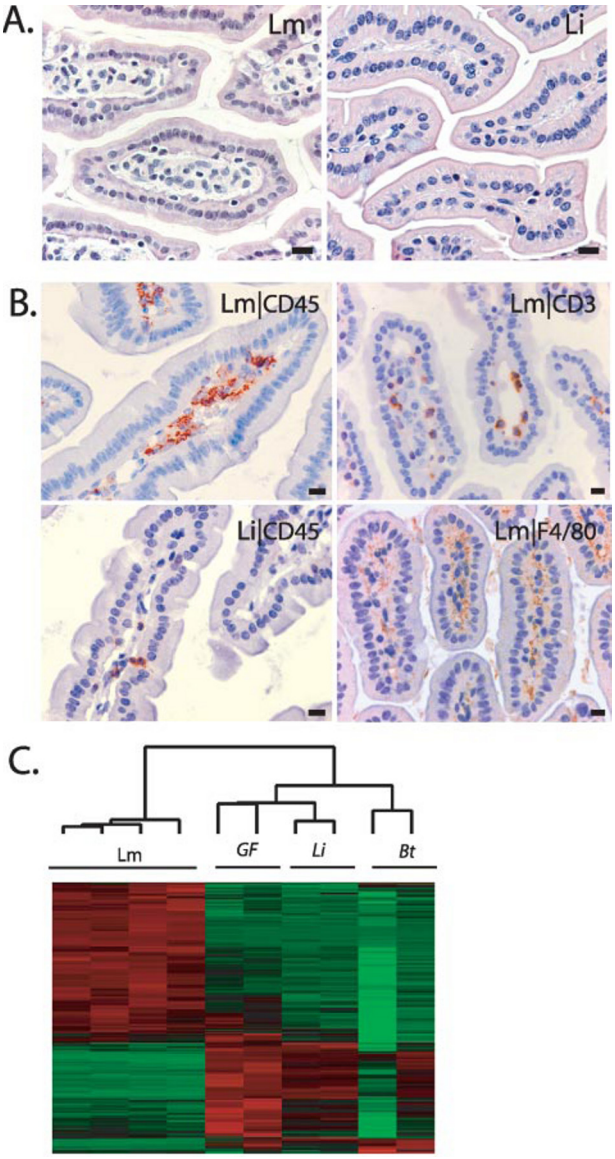


FIGURE 2. **Intestinal response to *L. monocytogenes* infection.** *A*, intestinal villus cross-sections prepared from *Fabpi-hEcad* mice mono-associated with WT *L. monocytogenes* (*Lm*) or *L. innocua* (*Li*) and stained with hematoxylin and eosin, illustrating differences in lamina propria infiltration with mononuclear cells. *B*, immunohistological analysis of lamina propria infiltrates. Intestinal sections were stained with monoclonal antibodies to markers for leukocytes (CD45), T-lymphocytes (CD3), or macrophages (F4/80). *Red*, positive staining; *blue*, cell nuclei. *C*, unsupervised hierarchical clustering of distal small intestinal response in *Fabpi-hEcad* mice to a 72-h colonization with WT *L. monocytogenes*, WT *L. innocua*, or *B. thetaiotaomicron* (*Bt*). GF controls are shown. Using the stringent selection criteria described under "Experimental Procedures," 304 genes were identified as displaying significantly changed expression across the four sample groups. Unsupervised hierarchical clustering, based on this list of 304 genes, established that the host response to *L. monocytogenes* infection was highly distinct from the responses to noninvasive *L. innocua*, or *B. thetaiotaomicron*, both of which cluster with GF controls. See supplemental Table S1 for a list of the 304 genes and their corresponding expression values. *Scale bars* in *A* and *B* are 10 $\mu$m.

colonized animals was more similar to GF controls than the transcriptome expressed in *B. thetaiotaomicron*-colonized mice.

Based on the results obtained with this unsupervised clustering, we used a two-class SAM analysis to compare GeneChip data sets from the ileums of WT *L. monocytogenes*-colonized mice with ileal GeneChip data sets generated from GF, *L. innocua*- and *B. thetaiotaomicron*- mono-associated animals; our goal was to define a set of genes that distinguishes the intestinal response to WT *L. monocytogenes* infection. The comparison yielded 614 genes that exhibited significantly higher expression in *L. monocytogenes*-infected mice and 159 genes with significantly lower expression ($q$-value <1%; total of 773 genes; Fig. 3*A*).

We subsequently used IPA software to identify gene networks that are significantly over-represented among the 614 *L. monocytogenes*-induced genes. This software utilizes a knowledge base of more than 1,000,000 functional and physical interactions for >23,900 mammalian genes (including 8,200 for the mouse). 255 of the 614 genes are functionally annotated in the IPA knowledge base; 83 of these lie within the IPA "immune response" category ($p < 10^{-6}$ by Fisher's exact test; see supplemental Fig. S2). We then utilized 134 genes from immune response and nine other high level IPA functional categories that are over-represented ($p < 10^{-4}$; supplemental Fig. S2) to construct an unsupervised master gene interaction network. Elimination of 51 orphan genes, which lack established interactions with other induced genes, yielded an 83-member "master" network with 237 edges (Fig. 3*D*) that characterizes the response to enteroinvasive WT *L. monocytogenes*.

This analysis shows that many effectors of both the innate and adaptive branches of the immune system are stimulated upon *L. monocytogenes* invasion, including Stat-1 (4-fold difference relative to *L. innocua*-associated, *B. thetaiotaomicon*-associated, and GF controls; $q$-value <1%), i-Nos (Nos2A, 4.8-fold; $q$-value <1%), and lipocalin-2 (Lcn2, 11.2-fold, $q$-value <1%; a component of the innate immune system induced upon Toll-like receptor activation, which limits bacterial growth by binding and sequestering bacterial iron-siderophore complexes) (19). Ccl2 (also known as macrophage chemoattractant protein-1 (MCP-1)), a chemokine secreted by *L. monocytogenes*-infected macrophages that recruits additional monocytes to sites of invasion (20, 21), is up-regulated 6.1-fold. Consistent with the observed leukocytic infiltration of the lamina propria, several additional chemokine and cytokine genes are up-regulated in the ileum by WT *L. monocytogenes*, including interleukin 18 (3.3-fold, $q$-value <1%) and the chemokine ligands Cxcl9, Cxcl10, and Ccl21 (8.3-, 4.2-, 1.8-fold difference, respectively).

By utilizing "focus genes" from the master network and adding the 6,351 probe sets called Present in the ileal RNAs, we were able to show that components of the Jak/Stat, B-cell receptor, interleukin-10, interleukin-6, Toll-like receptor, antigen presentation, NF-kB, granulocyte-macrophage colony-stimulating factor (GM-CSF), ERK/MAPK, PI3K/Akt, G-protein-coupled receptor, integrin, Vegf, Pten, apoptotic, and ephrin receptor signaling pathways are all part of the response of the ileum to *L. monocytogenes* infection (see supplemental Fig. S3). In contrast, relatively few genes showed significant changes compared with GF after colonization with *L. innocua* or *B. thetaiotaomicron* (27 up-regulated by *L. innocua*; 98 by *B. thetaiotaomicron*; see supplemental Fig. S4).

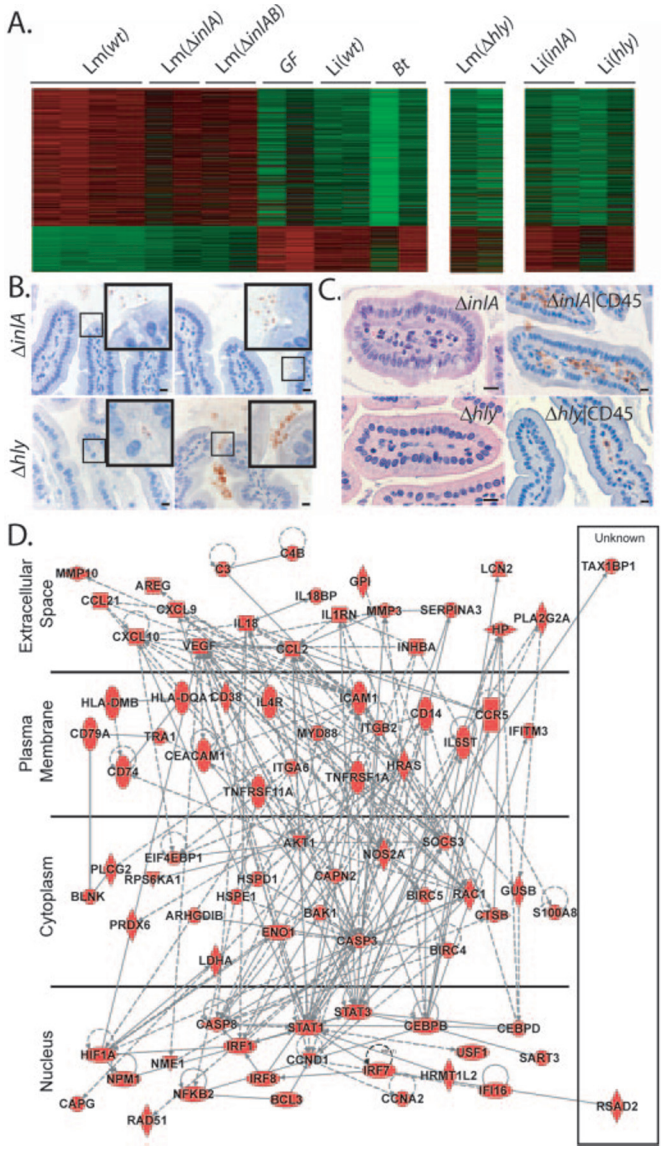


FIGURE 3. **Intestinal response of *Fabpi-hEcad* mice to *L. monocytogenes* depends upon listeriolysin-O.** *A*, results obtained from two-class SAM that identified (*q*-value <1%) a response distinctive for *L. monocytogenes* (*Lm*) compared with that elicited by *B. thetaiotaomicron* (*Bt*)-colonized and *L. innocua*-colonized mice and GF controls. 614 genes show higher expression in the group infected with *L. monocytogenes*, whereas 159 genes exhibit lower expression. The expression pattern for these 773 genes is also shown in gnotobiotic mice colonized with isogenic *Lm*(Δ*hly*) or *Lm*(Δ*inlA*) strains or with mice mono-associated with *L. innocua* expressing InlA (*Li*(*inlA*)) or LLO (*Li*(*hly*)). For a complete list of genes, their associated *q*-values, and -fold differences in expression see supplemental Table S2. *B*, immunohistological localization of *L. monocytogenes* InlA mutant (*Lm*(Δ*inlA*)) or LLO mutant (*Lm*(Δ*hly*)) in the ileums of *Fabpi-hEcad* mice (*red*). Cell nuclei are counterstained with hematoxylin (*blue*). Bacteria are shown at higher magnification in the *insets*. *C*, histochemical (*left panels*) and immunohistochemical (*right panels*) analyses of bacteria populating the lamina propria in *Lm*(Δ*inlA*)- or *Lm*(Δ*hly*)-colonized mice. For immunohistochemical studies, intestinal sections were stained with antibodies to leukocytes (CD45), T-lymphocytes (CD3), or macrophages (F4/80) (*red*, positive staining; *blue*, cell nuclei). *Scale bars* in *B* and *C*, 10 μm. *D*, gene network, organized by cellular location of the gene product, generated using 83 of the genes (*red* nodes) represented in the 10 IPA functional categories that are significantly over-represented ($p < 10^{-4}$, Fisher's exact test) in the list of 614 intestinal genes up-regulated upon oral infection with WT *L. monocytogenes* (see supplemental Fig. S2 for the 10 categories). Established direct interactions are denoted by a *solid line* and indirect interactions by a *dashed line*. Canonical pathways associated with the network are indicated in *blue*.

*InlA Is Not the Major Bacterial Determinant of L. monocytogenes-specific Intestinal Response*—We used this derived 773-member molecular signature of the host response to WT *L. monocytogenes* (Fig. 3) to identify some of its bacterial genetic determinants. Two experiments, one a gain-of-function and the other a loss-of-function, were designed to assess the contributions of the InlA-hEcad interaction. GF *Fabpi-hEcad* mice were colonized with a strain of *L. innocua* engineered for heterologous expression of InlA (*Li(inlA)*) or with mutant strains of *L. monocytogenes* that lack either *inlA* (*Lm*(Δ*inlA*)) or both *inlA* and *inlB* (*Lm*(Δ*inlAB*)). Unlike its isogenic WT *L. innocua* parent, which lacks an InlA homolog, the engineered internalin-expressing *L. innocua* strain is able to invade cultured Caco-2 cells, transfected fibroblasts expressing E-cadherin, guinea pig ileal enterocytes, and *ex vivo* infected *Fabpi-hEcad* mouse intestinal explants (9, 22).

All three bacterial strains colonized the intestines of GF transgenic mice at levels that were not significantly different from their WT parents ($n = 4-8$ mice/group; see supplemental Fig. S1). Expression of *L. monocytogenes inlA* in *L. innocua* (*Li(inlA)*) permits entry into villus enterocytes: however, no bacteria were observed in the lamina propria. Leukocytic infiltrates were not detected and systemic dissemination to the spleen was not observed (Fig. 1*C* plus data not shown). Using the molecular signature defined above as reference, GeneChip analysis of ileal RNAs from *Li (inlA)*-colonized *Fabpi-hEcad* mice revealed a host response that was most similar to that elicited by WT *L. innocua* (Fig. 3*A*).

Strains of *L. monocytogenes* lacking InlA or InlA and InlB (*Lm*(Δ*inlA*); *Lm*(Δ*inlAB*), respectively) were undetectable in villus enterocytes or in the underlying lamina propria ($n = 4-8$ mice/group; *e.g.* Fig. 3*B*). Nonetheless, leukocytic infiltrates in the lamina propria were present in each mouse surveyed (compare Fig. 3*C* with Fig. 2, *A* and *B*). Moreover, like its WT *L. monocytogenes* parent strain, the isogenic *Lm*(Δ*inlA*) mutant disseminated to the spleen (Fig. 1*C*), albeit at a lower level than WT. Together, these findings suggest that internalin-mediated invasion of hEcad-expressing villus enterocytes is not strictly required for systemic dissemination in gnotobiotic mice.

GeneChip analysis indicated that the intestinal response to *Lm*(Δ*inlA*) or *Lm*(Δ*inlAB*) contrasts sharply with that elicited by *L. innocua* or *Li*(*inlA*), and resembles that of WT *L. monocytogenes* (Fig. 3*A*). Direct comparison of ileal expression profiles from *Lm*(Δ*inlA*)- and *Lm*(Δ*inlAB*)-mono-associated mice with WT *L. monocytogenes*-colonized mice revealed 157 genes that are regulated via an internalin-*dependent* mechanism (see supplemental Fig. S5*A*). When the levels of expression of these 157 genes were referenced to the differences noted between WT *L. monocytogenes versus* the GF, *L. innocua* and *B. thetaiotaomicron* base-line groups, it was apparent that the internalin mutants produce attenuated responses for both up- and down-regulated genes (supplemental Fig. S5*B*). Ingenuity-based functional categorization of the 77 genes that were significantly *down-regulated* upon *L. monocytogenes* infection via the hEcad-mediated route (defined as decreased expression in WT *L. monocytogenes* infection compared with *Lm*(Δ*inlA*) and *Lm*(Δ*inlAB*) infection), revealed two broad categories that were enriched: "lipid metabolism" and "small molecule biochemis-

try" (supplemental Fig. S5*A*). The effects of infection via this internalin pathway on intestinal metabolism is evidenced by down-regulation of genes encoding enoyl-coenzyme A hydratase (Ehhadh; −1.6-fold), hydroxysteroid (17-$\beta$) dehydrogenase 4 (Hsd17b4; −2-fold), components of vitamin D signaling pathways [cubilin (Cubn), a transmembrane receptor, −3.8-fold]; the nuclear receptor vitamin D receptor (VDR; −1.5-fold); plus 13 others involved in arginine and proline metabolism ($p < 10^{-8}$), tryptophan metabolism ($p < 10^{-6}$), or histidine metabolism ($p < 10^{-5}$) (supplemental Table S6). Ingenuity-based analysis also revealed a gene interaction network centered around PPAR$\alpha$ signaling further illustrating how internalin-based *Listeria* invasion affects host cell metabolism (supplemental Fig. S5*C* and Table S7). Moreover, genes associated with the mitochondrion are over-represented (GO: 0005739; $p < 0.001$) (23–25).

The 80 genes that exhibited significantly increased expression upon hEcad-mediated infection include four significantly over-represented ($p < 10^{-3}$) functional categories (*e.g.* "cellular movement"; supplemental Fig. S5*A*). In addition, the T-cell and monocyte recruitment chemokines, Cxcl9 and Cxcl10, are significantly more responsive to *L. monocytogenes*-infection if InlA-based enterocytic invasion is intact (2.2- and 2.0-fold increase, respectively, compared with *Lm(*Δ*inlA*) and *Lm(*Δ*inlAB*)).

An Ingenuity-based interaction network, generated using genes showing the largest expression differences that depend upon internalin (up- or down-regulated ≥2-fold compared with *Lm*(Δ*inlA*) and *Lm*(Δ*inlAB*) infection) disclosed that tumor necrosis factor-$\alpha$ is a centrally positioned regulator of the observed host response (supplemental Fig. S5*D* and Table S8). This is consistent with increased expression of the proinflammatory cytokines Cxcl9 and Cxcl10 (2.0- and 2.2-fold, respectively) and down-regulation of catalase (−2.8-fold). Together, our functional genomics studies in gnotobiotic mice demonstrate that whereas InlA-mediated bacterial internalization into enterocytes is not essential for achieving dissemination to spleen, its absence significantly affects the route of invasion and the intestinal transcriptional response to *L. monocytogenes*.

*Critical Role of LLO in Triggering the L. monocytogenes-specific Host Response*—Like *inlA* and *inlB*, the *hly* gene encoding LLO is present in *L. monocytogenes* but not *L. innocua* (4). In cell culture systems, this secreted pore-forming toxin mediates *L. monocytogenes* escape from the internalization vacuole to the cytosolic compartment of host cells, a step critical for intracellular survival and multiplication, particularly in professional phagocytes (11). *hly* expression is also needed to provoke innate and adaptive immune responses in mice infected *intravenously* with *L. monocytogenes* (26); this reflects its important roles in accessing the macrophage cytosol, stimulating key host signaling pathways involved in innate immune responses, and permitting MHC-I presentation of *L. monocytogenes*-derived peptides (20, 27).

As in the case of *L. monocytogenes* internalin, we performed gain-of-function and loss-of-function experiments to examine the role of LLO. GF *Fabpi-hEcad* mice were colonized for 3 days with a strain of *L. innocua* expressing LLO (*Li(hly)*) or with a *L. monocytogenes* mutant with a deletion in *hly*. The efficiency and density of the colonization of the small intestines of these mice were equivalent to that observed with the corresponding WT strains ($n = 4$ mice/group; see Fig. S1). *Li*(*hly*) was not detectable in villus enterocytes, and leukocytic infiltrates in the lamina propria were not evident (data not shown). *Li*(*hly*) was detectable in the spleen but at levels that were 29-fold lower than that observed when mice were colonized with WT *L. monocytogenes* ($p < 0.05$; Fig. 1*C*). GeneChip studies of ileal RNAs revealed that expression of *L. monocytogenes* LLO in *L. innocua* did not produce a host response signature that was significantly different from that triggered by WT *L. innocua* (Fig. 3*A*). These findings are consistent with previous *ex vivo* studies in the J774 macrophage and HEp-2 epithelial cell lines documenting that cytosolic survival and multiplication of *Li*(*hly*) are much reduced compared with WT *L. monocytogenes* (28); they also emphasize that the stepwise progression of *L. monocytogenes* from phagosome to cytosol to invasion of neighboring cells is dependent upon the action of multiple virulence factors not present in *L. innocua* (4).

Histo- and immunochemical analysis of ileums harvested from *Lm*(Δ*hly*)-infected mice showed that these bacteria were able to invade enterocytes positioned at the tips of intestinal villi (Fig. 3*B*) and accumulate at their basal surface within the lamina propria. Despite the ability of *Lm*(Δ*hly*) to invade villi, leukocytic infiltrates were not detected in the lamina propria (Fig. 3*C*). In accord with the inability of *L. monocytogenes* LLO mutants to persist in monocytes/macrophages (29), splenic CFU levels were 34-fold lower in mice mono-associated with the mutant compared with the WT *L. monocytogenes* strain ($p < 0.05$; Fig. 1*C*). Moreover, GeneChip analyses revealed that host response to *L. monocytogenes* Δ*hly* was more similar to that in mice colonized with *L. innocua* and *B. thetaiotaomicron* than to its isogenic WT *L. monocytogenes* parent strain (Fig. 3*A*).

## DISCUSSION

We have used an environmentally and genetically defined, simplified, and "humanized" gnotobiotic transgenic mouse model of oral listeria infection to (*i*) characterize canonical host signaling pathways that distinguish the transcriptional response to this enteropathogen in a specified intestinal habitat from the response evoked by its nonpathogenic relative, *L. innocua*, or the gut symbiont, *B. thetaiotaomicron*; and (*ii*) assess the contributions of *L. monocytogenes* genes to enteroinvasion and this transcriptional response.

In agreement with our previous results in conventionally raised animals (9), WT *L. monocytogenes* is able to invade enterocytes in gnotobiotic *Fabpi-hEcad* mice, gain access to the underlying lamina propria, and disseminate to the spleen. Isogenic *L. monocytogenes* Δ*inlA* and *L. monocytogenes* Δ*inlAB* mutants, although unable to invade enterocytes and the underlying lamina propria, are still able to cross the gut barrier and disseminate to the spleen, most probably reflecting the increased luminal bacterial load in mono-associated gnotobiotic mice compared with infected, conventionally raised animals. However, even though the density of colonization of the ileal lumen by these mutants was identical to WT *L. monocyto-*

*genes* (as defined by CFU/ml ileal contents), the ileal immunoinflammatory response to invasion was significantly attenuated.

Previous work in nontransgenic, conventionally raised mice has shown that Peyer's patches are a site of *L. monocytogenes* translocation after oral administration (30). Translocation of *L. monocytogenes* across the FAE and its underlying lymphoid follicles, or across the colonic mucosal barrier via an as yet undefined InlA-Ecad-independent pathway, provides potential alternate routes for bacterial translocation across the gut barrier (31, 32). Our preliminary experiments in conventionally raised adult *Lt*β*R*(−/−) mice that lack Peyer's patches (33) suggest that these animals are resistant to intestinal invasion by WT *L. monocytogenes*.[7] This finding, together with our observations in gnotobiotic *Fabpi-hEcad* mice, provides direct evidence supporting the notion that InlA-independent translocation through Peyer's patches is a significant route for dissemination of *L. monocytogenes* across the intestinal barrier, in addition to the important InlA-dependent route mediated by enterocyte invasion (9, 34).

The LLO-deficient strain *Lm*(Δ*hly*), although retaining the ability to efficiently colonize the intestine and invade villi via InlA-Ecad interaction, nonetheless failed to evoke a leukocytic infiltrate in the lamina propria, did not produce a signature transcriptional response demonstrably different from that produced by nonpathogenic *L. innocua* or symbiotic *B. thetaiotaomicron*, and failed to disseminate to the spleen. Thus, *Lm*(Δ*hly*) uncouples invasion of enterocytes *and* the lamina propria from an evoked immunoinflammatory response.

The indispensability of LLO likely reflects a number of factors. *First*, LLO is required for *L. monocytogenes* to access the cytosol of professional antigen presenting cells, a necessary step in activating Myd-88-independent secretion of Ccl2 (MCP-1) by infected macrophages; this activation may involve cytosolic pattern recognition systems analogous to Nod-1 and -2 (20). The inability of intraphagosomal *L. monocytogenes* to elicit Ccl2 secretion is also consistent with the lack of monocyte recruitment to the lamina propria noted with our LLO-deficient strain and consistent with our transcriptional profiling results. *Second*, the lack of LLO not only limits the availability of cytosolic *L. monocytogenes* antigens for MHC-I presentation but also eliminates the immunodominant antigen that is derived from LLO (amino acids 91–99) (35). *Third*, LLO-deficient *L. monocytogenes* is unable to replicate and spread to neighboring host cells; the dead-end infection results in a decreased load of invading bacteria. *Fourth*, LLO has been shown to be a potent signaling molecule, able to trigger the NF-kB and MAPK pathways (27, 36).

By using germ-free mice, we were able to directly determine that *L. monocytogenes* LLO is a major determinant of the consequences of the gut barrier invasion by a foodborne pathogen. The power and versatility of the gnotobiotic system, combined with functional genomics, now allows this analysis to be extended to include an assessment of the roles of specified components of the human gut microbiota in determining how *L. monocytogenes* adapts to the gut ecosystem, invades the mucosa, and elicits immunoinflammatory responses.

[7] E. Huillet, P. Cossart, and M. Lecuit, unpublished observations.

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