Research

A gene-expression program reflecting the innate immune response of cultured intestinal epithelial cells to infection by *Listeria monocytogenes*

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Abstract

Background: *Listeria monocytogenes* is a Gram-positive, facultative, intracellular bacterial pathogen found in soil, which occasionally causes serious food-borne disease in humans. The outcome of an infection is dependent on the state of the infected individual's immune system, neutrophils being key players in clearing the microorganism from the body. The first line of host defense, however, is the intestinal epithelium.

Results: We have examined the transcriptional response of cultured human intestinal epithelial cells to infection by *L. monocytogenes*, which replicates in the host cell cytoplasm and spreads from cell to cell using a form of actin-based motility. We found that the predominant host response to infection was mediated by NFκB. To determine whether any host responses were due to recognition of specific virulence factors during infection, we also examined the transcriptional response to two bacterial mutants; *actA* which is defective in actin-based motility, and *prfA*, which is defective in the expression of all *L. monocytogenes* virulence genes. Remarkably, we found no detectable difference in the host transcriptional response to the wild-type and mutant bacteria.

Conclusions: These results suggest that cultured intestinal epithelial cells are capable of mounting and recruiting a powerful innate immune response to *L. monocytogenes* infection. Our results imply that *L. monocytogenes* is not specifically detected in the host cytoplasm of Caco-2 cells by intracellular signals. This suggests that entry of bacteria is mediated in the host cell post-translationally, and that these bacteria seek the cytosol not only for the nutrient-rich environment, but also for protection from detection by the immune system.

Background

Listeria monocytogenes is a ubiquitous, Gram-positive, soil bacterium and facultative intracellular pathogen, which on occasion causes severe food-borne disease in newborns, pregnant women or immunocompromised individuals. Although the epithelial cells of the intestinal tract are in constant contact with a diverse population of bacteria, most are not stimulated by these bacteria to produce strong immune responses. However, these cells must be prepared to help fight off infections by bacteria that have evolved mechanisms

to enter and replicate in their cytoplasm. This study examines the basis of host response specificity.

The pathway of infection by *L. monocytogenes* is initiated by binding of bacterial cell surface molecules (including the internalins InlA, and InlB) to specific receptors on the surface of the intestinal epithelia such as E-cadherin in the case of InlA [1-3], and Met or gC1qR for InlB [4,5]. The interaction between InlA and E-cadherin is essential for infection of enterocytes in a mouse model of listeriosis [6]. After being engulfed, the bacterium secretes virulence factors to degrade the phagosome. Listeriolysin O (LLO, *hly*) [7-10], and two phospholipases (PlcA, PlcB) [11-13], work in concert to disrupt the membrane of the phagocytic vacuole, allowing the bacterium to escape into the cytoplasm. Once in the cytoplasm, *L. monocytogenes* recruits the actin cytoskeletal machinery by expressing the protein ActA in a polarized fashion on its surface [14,15]. ActA-dependent actin polymerization generates force and thereby cytoplasmic motility [16]. Moving bacteria that reach the cell plasma membrane form membrane-bound protrusions that are engulfed by neighboring cells, allowing the infection to spread without the bacteria ever leaving the cytoplasmic environment.

Microarrays of cDNAs have become an important tool for analyzing genomic transcriptional programs in diverse biological processes. Recently, microarrays have also become a widely used tool for studying host-pathogen interactions from the perspective of both the host and the pathogen [17-22]. The goals of this work were twofold: to use cDNA microarrays to characterize the primary response of intestinal epithelial cells to a well-studied intracellular pathogen, *L. monocytogenes*, and to try to identify host gene-expression programs which are induced by specific stages of the infection by comparing the wild-type expression profile to that of mutants which are either incapable of intracellular growth and thus avirulent, or which cannot initiate actin polymerization in host cell cytoplasm.

To this end, we have examined the global transcriptional response of a human intestinal epithelial cell line, Caco-2, to infection by *L. monocytogenes* and isogenic mutants which are disrupted in the *actA* or *prfA* gene. Caco-2 cells were chosen because they are a model human cell line for studying the differentiation [23], transport biochemistry [24,25] and immunology of intestinal epithelia [26-29]. The *actA* internal in-frame deletion mutant (DP-L1942) [30], which cannot initiate the polymerization of actin, was used to assess transcriptional responses specific to the motility phase of infection. The *prfA* gene codes for a transcriptional regulator, which controls the virulence genes *hly*, *plcA*, *plcB*, *mpl* and *actA*, as well as negatively regulating itself [31,32]. Mutant bacteria with a transposon insertion in *prfA* (DP-L1075) [33] adhere to the host-cell surface, and induce their own uptake into a phagocytic vacuole, presumably through low-level expression of the internalins, but cannot escape from the phagocytic vacuole into the cytoplasm. Several reports in the literature indicate that LLO, PlcA and PlcB of *L. monocytogenes* induce specific host responses in macrophages and endothelial cells [34-39]. We were therefore particularly interested to see whether these virulence-specific responses would be similar in intestinal epithelial cells, the normal first site of *L. monocytogenes* infection.

Results

Predominance of the NFκB pathway of activation in the transcriptional response of Caco-2 cells to *L. monocytogenes* infection

We used DNA microarrays spotted with 22,594 human cDNAs (representing about half of the transcripts in the human genome) to examine the host transcriptional response of cultured human intestinal epithelial cells (Caco-2) over an 8-hour period following infection by *L. monocytogenes* wild-type strain 10403S. Samples for each time point (0, 30, 60, 120, 240 and 480 minutes post-infection) were infected in parallel. The progress of each infection was monitored using a combination of phase-contrast and fluorescence microscopy (Figure 1). Wild-type bacteria were highly motile within the cytoplasm by 4 hours post-infection, as evidenced by filamentous actin tail structures stained with rhodamine phalloidin (Figure 1b). Overall, the time course of cellular events following *L. monocytogenes* infection in Caco-2 cells closely parallels the time course reported for macrophage-like cell lines and other epithelial cell lines [16].

Messenger RNA was isolated from each experimental sample and used to prepare Cy-5 labeled cDNA ('red') by reverse transcription [40]. To provide an internal standard for each measurement, a pool of reference mRNA (labeled with Cy-3 dUTP, 'green') derived from combining mRNA from 10 different human cell lines was mixed with the Cy-5 labeled experimental samples before hybridization with the cDNA microarray. The reference pool of RNA was designed to provide some signal for as many genes on the array as possible, permitting analysis of changes in gene expression with respect to the ratio of experimental signal (red) to a constant signal for each gene, the reference signal (green) [41]. Temporal changes in gene expression for each gene were evaluated at each time point by subtracting $\log_2$(red/green) of the time zero measurement from the corresponding $\log_2$(red/green) ratio at each time point. The resulting values represent $\log_2$(red T_1/red T_0), or $\log_2$(red T_2/red T_0) and so on, and indicate changes relative to the beginning of the infection. A schematic representation of this zero transformation is shown in Figure 2.

Many genes were strongly induced in response to the infection. Results for corresponding time points from two independent 8-hour time courses performed on different days were averaged, filtered to remove unnamed genes, and ranked by the average change for the entire time course. The

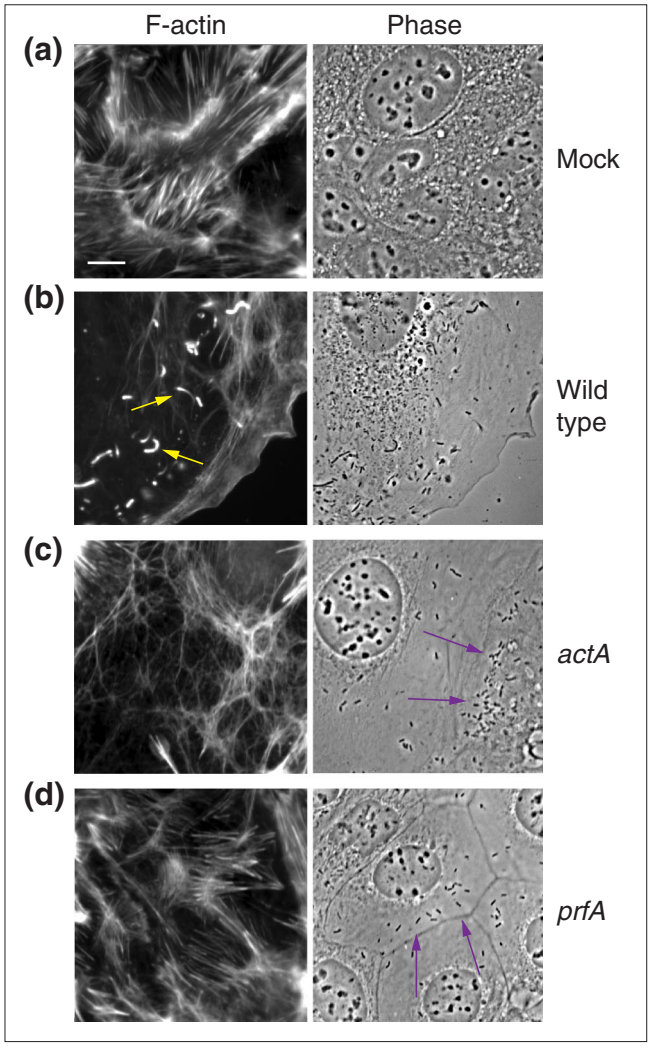

Figure 1
Monitoring of infections. Fluorescence and phase microscopy to monitor infections at 4 h post-infection for **(a)** mock infection, **(b)** wild-type *L. monocytogenes* 10403S, and isogenic mutants deficient in **(c)** *actA* or **(d)** *prfA*. Filamentous actin was stained with rhodamine phalloidin. Yellow arrows in (b) show comet tails behind wild-type *L. monocytogenes* (10403S). Purple arrows in (c) indicate intracellular colonies formed by *actA* (DP-L1942). *prfA* (DP-L1075) bacteria (d) are seen individually as they do not escape the endosome or replicate after being internalized. Scale bar is 10 μm.

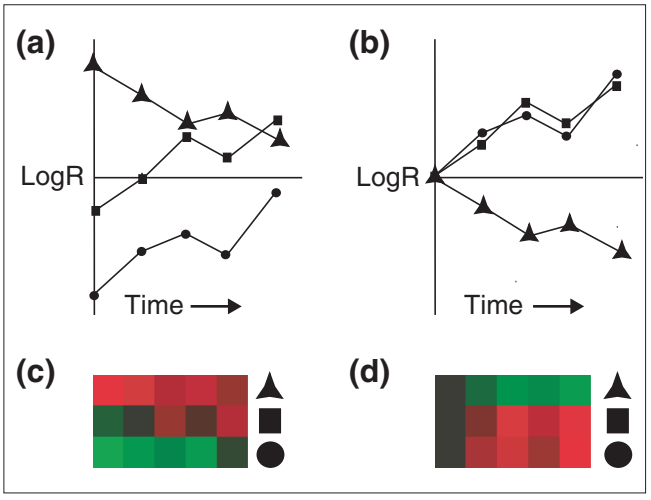

Figure 2
Schematic representation of time-zero transformation. In this process, the logR of the zero time point is subtracted from each time point in the time course. Zero transformation enables visual comparison of trends in gene expression for different genes in the same time course, and also for trends in gene expression from different experiments. **(a)** Time course of three different genes, plotted as the logR relative to the reference pool of mRNA. **(b)** Zero transformation of the time courses in (a). **(c)** Visual representation of trends in gene expression for untransformed data (a) as seen in TreeView. The Color scheme depicts LogR values with gray equalling zero, red>0, and green<0. **(d)** TreeView image of zero-transformed data.

50 most highly induced named genes are shown in Figure 3. Twenty percent of these genes are either involved in NFκB signal transduction or directly activated by NFκB. These include the immediate early response genes Fos, Jun, and Myc, as well as inhibitors of apoptosis (IAP-2), chemokines (Gro-1) and the inhibitor of NFκB, IκB-alpha.

Wild-type and *actA* strains of *L. monocytogenes* infect Caco-2 cells at similar rates and both induce strong NFκB responses

We were interested in determining whether any of the responses we had detected were specific to the actin-based motility phase of the infection. To this end, we carried out similar 8-hour time courses with an isogenic *L. monocytogenes* strain deficient in *actA* (DP-L1942, *ΔactA*), which is unable to nucleate actin polymerization and hence has no capacity for intracellular motility and intercellular spread [30]. As has been described for this mutant, the visible characteristics of the infection were quite different from wild type, and no actin structures are associated with cytoplasmic microcolonies of *actA* mutant bacteria (Figure 1c).

Microarray analysis of the *actA* time courses revealed that the host transcriptional responses were remarkably similar to those observed during infection with the wild-type strain. Genes in the NFκB pathway were strongly induced in both experiments, although the amplitudes of responses for specific genes were a bit different for each experiment. Day-to-day variation for individual time courses as well as overall similarity in the top 500 induced genes can be clearly seen when data from all experiments (including a parallel time course described below) are clustered together (Figure 4, right).

Despite the overall similarity of the gene-expression patterns for the two wild-type infections and the two *actA* infections, there were significant variations between replicants that could compromise our ability to extract what might be subtle differences between wild-type and mutant infections. Careful studies have demonstrated the correlation of

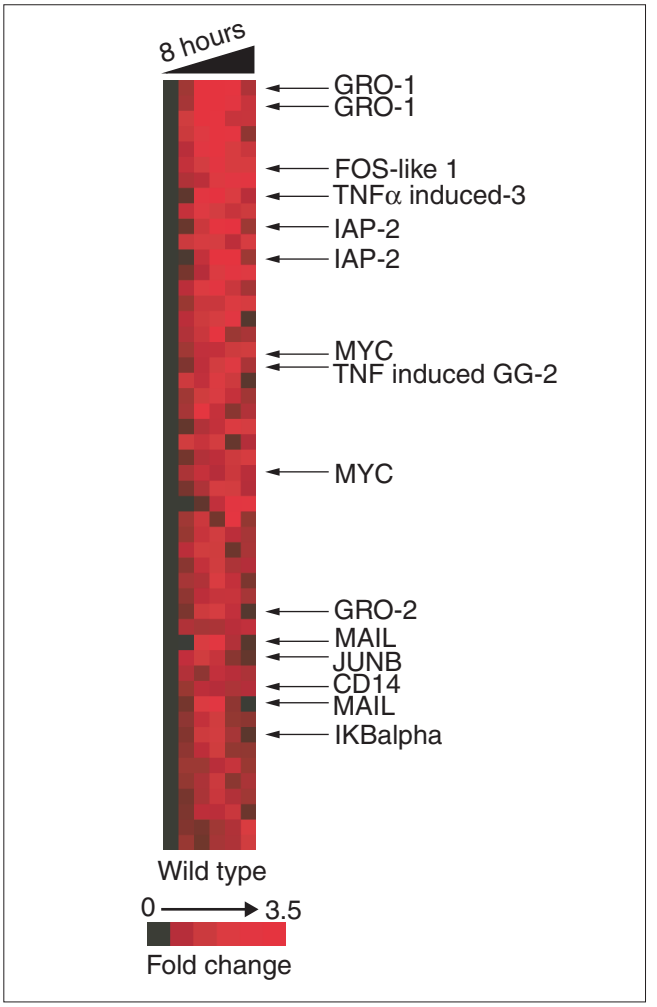

Figure 3
Rank average of two 8-h wild-type time courses. The 50 most strongly induced named genes are shown. Individual time courses were averaged, and transformed to the zero time point measurement (first column: black equals zero). The five time points shown (in min) are 30, 60, 120, 240 and 480. The scale of induction after transformation is shown at the bottom, where the brightest red indicates an induction of at least 12-fold. NFκB-responsive genes are indicated by arrows.

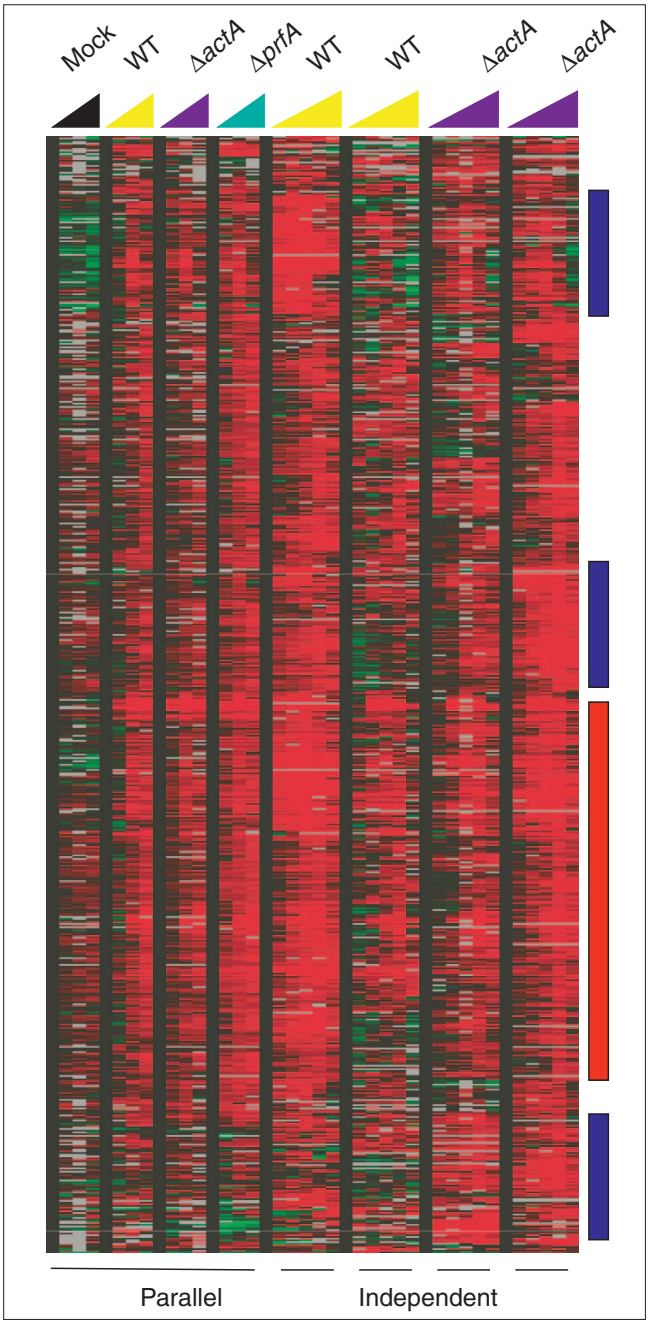

Figure 4
Megacluster of all time-course infections. The data across all time courses was averaged, ranked, and then clustered. All genes that were induced in the mock time course at levels higher than an average $\log_2$(ratio) of 0.5 were omitted. The four parallel time courses are shown on the left, with trends in gene expression appearing very similar for all time courses. Four individual 8-h time courses are on the right, and show the day-to-day variability of the response (which includes dependence on the time zero measurement).

measurements from microarrays with changes in gene expression [42] or genomic DNA content [43], suggesting that the variability we observed was not attributable to the microarray methodology. To determine the source of variability of our measurements, we examined the correlation of measurements from uninfected cells plated at the same relative density and passage number where mRNA was isolated on different days and hybridized at different times, or harvested and hybridized at the same time. We found that the correlations of the global mRNA expression measurements for cultures harvested on different days was only fair (Figure 5a, Pearson correlation coefficient of 0.74), while the correlation between two independent microarray analyses of the same samples gave a correlation of 0.97 (Figure 5b). The correlation of measurements for mRNA samples prepared in parallel on the same day from the same batch of cells was also very high (Figure 5c, average correlation of 0.94). These

results indicate that primary source of variability among the infections carried out on different days is the true day-to-day biological variation of the Caco-2 cell cultures. We therefore decided to repeat the wild-type and mutant infections on cells that had been expanded at the same time and infected in parallel on a single day.

Synchronized infections yielded consistent changes in gene expression dominated by the NFκB response

We repeated the experiments carrying out parallel 4-hour time courses simultaneously for mock infections, wild-type (10403S), *ΔactA* (DP-L1942) and *ΔprfA* (DP-L1075), with time points taken at 0, 60, 120 and 240 minutes after infection. In this parallel experiment, baseline (T_0) mRNA from uninfected cells was independently isolated from three different cultures. The correlation between the expression patterns of these parallel uninfected cultured cell samples was much higher than for samples isolated on different days, albeit not as high as for two independent hybridizations of the same mRNA (Figure 5). The three measurements of $\log_2$(Caco-2 T_0/reference) from the parallel set were averaged to define the pattern of expression in uninfected cells at the beginning of the synchronized parallel time course, and was then used to zero transform the mock, wild-type, *actA* and *prfA* parallel infections.

Genes were ranked by the average of the $\log_2(T_n/T_0)$ for all three infection time courses, then filtered to exclude any genes that were induced in the mock infection (the average $\log_2(T_n/T_0) < 0.5$ and not equal to zero). Of the 50 named genes most highly induced in all infections, 40% are either involved in NFκB signaling, or are responsive to NFκB activation. When these genes were subjected to hierarchical clustering, many also had similar patterns of gene expression as measured by the Pearson correlation of their gene-expression vectors, and the bulk of NFκB-responsive genes formed a single cluster (Figure 6).

Significance analysis of microarrays [44] (see Materials and methods) was used to search for genes whose expression differed significantly between mock-infected samples taken at 2 and 4 hours (class 1), and samples taken at 2 and 4 hours during the bacterial infections (class 2). As missing data for relatively small datasets are not tolerated by the SAM algorithm, the most significantly induced genes identified in this way were not identical to the list generated by ranking the average $\log_2(T_n/T_0)$, but this alternative approach did confirm the observation that the NFκB-responsive genes were significantly induced by all bacterial infections, and that this induction was robust through a 2-4-hour window. Interestingly, far fewer genes were specifically repressed than induced. When we compared the expression patterns at 2 and 4 hours after infection to the corresponding time points of the mock infection, 128 genes were considered significantly induced by SAM, and no genes significantly repressed.

Throughout the time course of all infections, many genes regulated by NFκB were induced at high levels, including some members of the NFκB family itself (Figure 7). Among the most highly induced were the genes encoding NFκB1, RelB, IκB-alpha (the inhibitor of NFκB1), IκKε (IκB kinase-epsilon), and NFκB-inducing kinase or Map-3-kinase-14 (NIK, MAP3K14). Interestingly, CD14 and TLR2, receptors involved in innate immune recognition of microbial

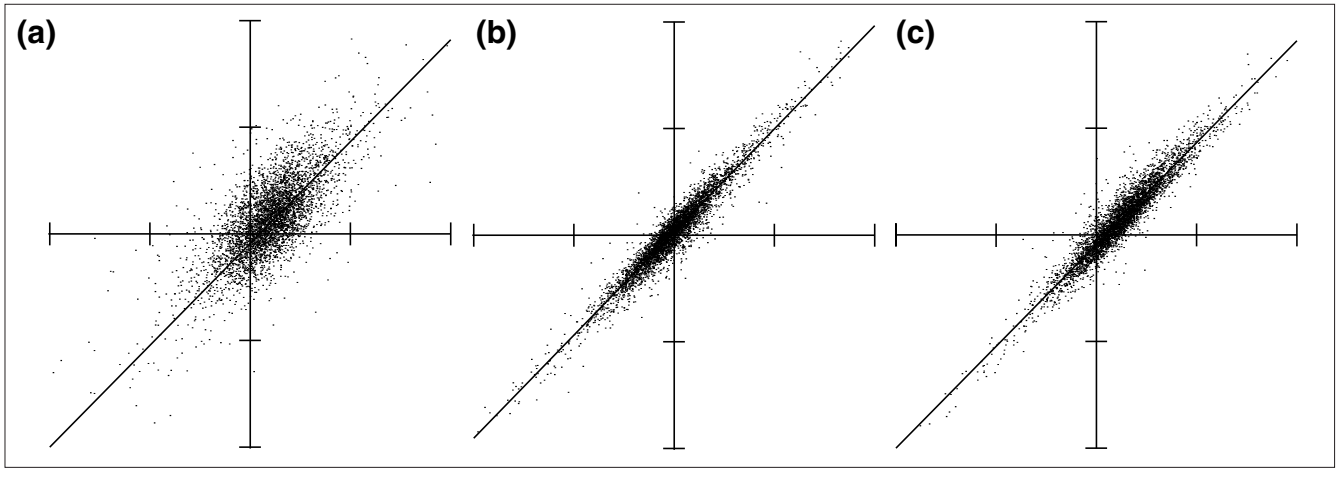

Figure 5
Comparison of gene expression in uninfected Caco-2 cells. Scatterplots were made of log-transformed ratios $\log_2(T_n/T_0)$ of (Caco-2 mRNA/reference pool mRNA). As the reference is relatively constant for each gene, scatter in the data represents biological variability in the Caco-2 cells. The scale for each plot is identical, with the axes ranging from $\log_2(T_n/T_0)$ -4 to 4. **(a)** Comparison of uninfected cells harvested several months apart. Correlation is poor (Pearson correlation coefficient 0.74). **(b)** Comparison of the same uninfected Caco-2 mRNA, hybridized on different days. Correlation is excellent (correlation coefficient 0.97), verifying reproducibility with respect to the reference pool. **(c)** Comparison of uninfected cells harvested from different plates on the same day. Correlation is good (correlation coefficient 0.94).

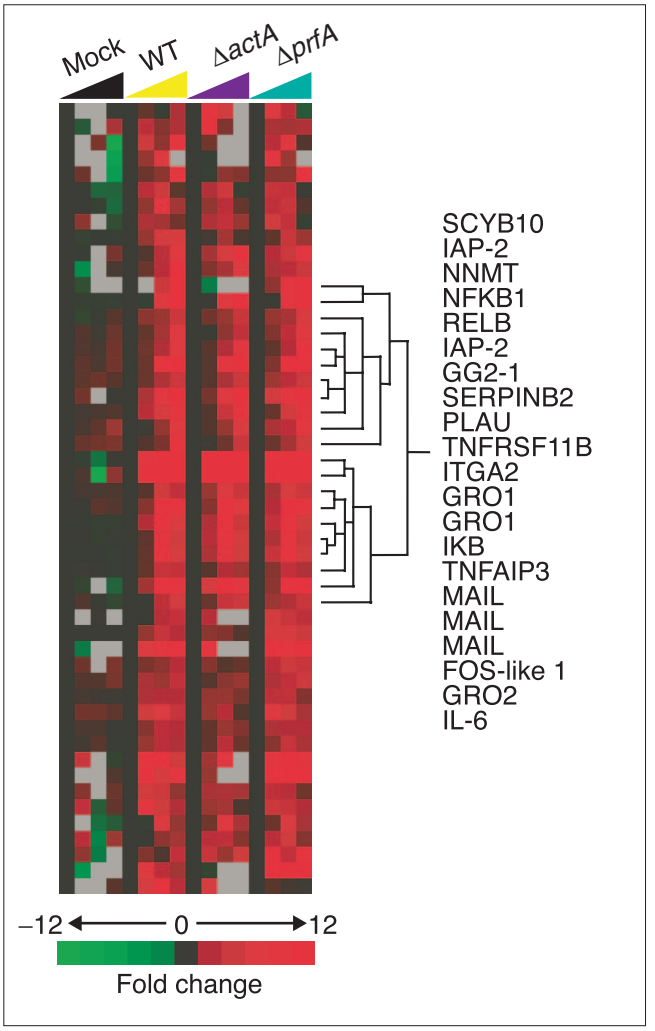

Figure 6
TreeView image of named genes induced during parallel time course. Genes were ranked by average $\log_2$(ratio) over all time courses to look at similarity of induction (the mock time course was filtered for genes induced by the infection protocol), and then clustered. Many NFκB-related and responsive genes form a single cluster, representing 40% (20/50) of the most highly induced genes. Outside the node representing these genes, there are a number of other immune-response-related genes, including small inducible cytokines (SCY) which are also regulated by NFκB. Image contrast is set at $\log_2$(ratio) = 3.5, indicating a minimum of 12-fold induction for the brightest red spots. Gray spots indicate missing data.

antigens, were both induced. A number of chemokines and cytokines that are regulated by NFκB1 were also induced (Figure 7), including IL-6, Gro-1, Gro-2, and a number of small inducible cytokine family members (SCY). Urokinase and urokinase receptors were both specifically induced, as were cell adhesion molecules such as ICAM-1. Both of the gamma interferon (IFNR) receptors were induced, as well as a number of genes induced by tumor necrosis factor alpha (TNFα). Genes not shown in the figure can be seen in the tab-delimited text files of the raw or filtered data.

ActA-dependent cytoplasmic motility of *L. monocytogenes* does not appear to induce *de novo* host transcription

Two primary methods were used to compare differences in gene expression between wild-type and mutant infections; direct subtractive comparisons of mutant from wild type, and SAM analysis of the 2- and 4-hour time points as single classes. All analyses of wild-type and mutant time courses presented here were carried out on the parallel time courses, where the variability of conditions was limited as much as possible. No genes appeared to be significantly induced by any single genotype of *L. monocytogenes*, according to either method of analysis. We also used a variety of additional approaches to search for genes differentially affected by the mutants. Primarily, this included setting filters for experimental time points (with Microsoft Excel), asking for genes that were expressed more highly in one case than another (for example, wild-type T240 greater than mutant T240, or combinations such as wild-type T240 greater than both ActA and PrfA T240, and so on). These methods were used to ensure that the statistical and mathematical criteria were not being influenced by the presence of any 'bad spot' on the microarrays, causing us to miss an important gene. To our surprise, we were unable to identify genes or clusters that could be considered either motility-specific or specific to the presence of cytoplasmic bacteria by any statistical method or filtering criterion.

Many proteins directly involved in the actin-based motility of *L. monocytogenes* have been identified by biochemical and cell biological methods [45]. We carefully examined the transcriptional responses for genes encoding these motility-related proteins to determine whether we might be overlooking interesting but subtle changes in expression levels as a result of the stringency of our statistical criteria (Figure 8, bottom). They are contrasted with genes involved in other aspects of actin-cytoskeletal dynamics, which did change at least threefold at some time point in some infection (Figure 8, top). It is immediately apparent that no genes encoding proteins known to be involved in *L. monocytogenes* actin-based motility are induced by infection.

No detectable transcriptional response to cytoplasmic bacteria or virulence factors expressed during entry

We were surprised to discover that virtually all of the transcriptional response to *L. monocytogenes* infection of these cells results from what is apparently the initial interaction of the bacteria with the cell surface, regardless of virulence phenotype. *prfA* mutant bacteria could still be seen associated with cells by phase-contrast microscopy at 4 hours post-infection (Figure 1d), albeit at much lower levels than wild-type or *actA* bacteria as they were no longer replicating. It is not surprising that these bacteria are adherent, as InlA expression is only partly dependent on PrfA [46]. No actin structures were seen in association with these bacteria, implying that they were indeed deficient in virulence gene expression and failed to escape from the endosomes (Figure 1d).

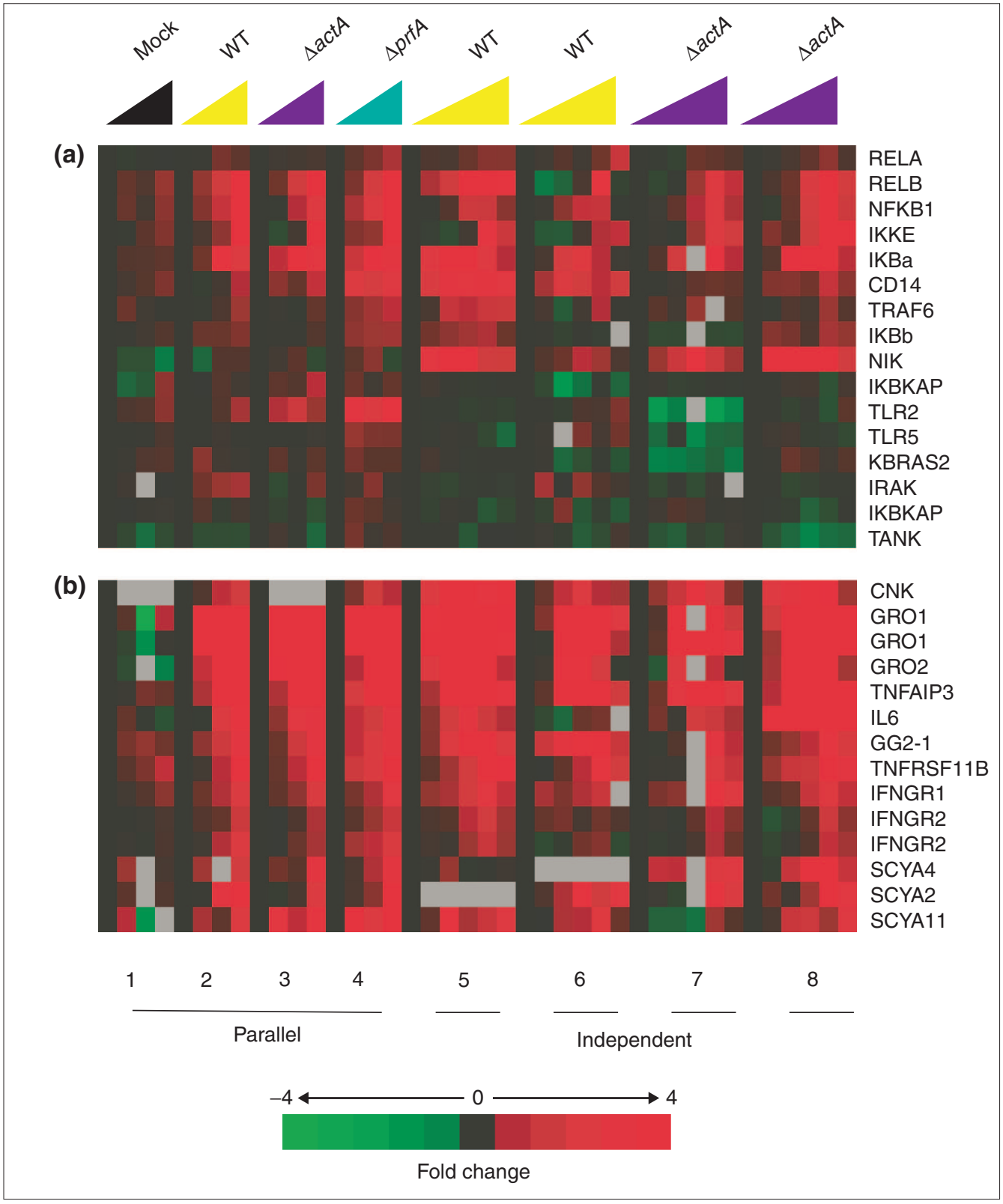

Figure 7
The NFκB and proinflammatory response. **(a)** The response of NFκB family members and related kinases that are present on the 24,000-spot (24K) arrays. **(b)** Cytokines, chemokines and their receptors that are induced during the time courses. Image contrast is set at 2, such that the brightest red is greater than a fourfold change. Gray spots indicate missing data.

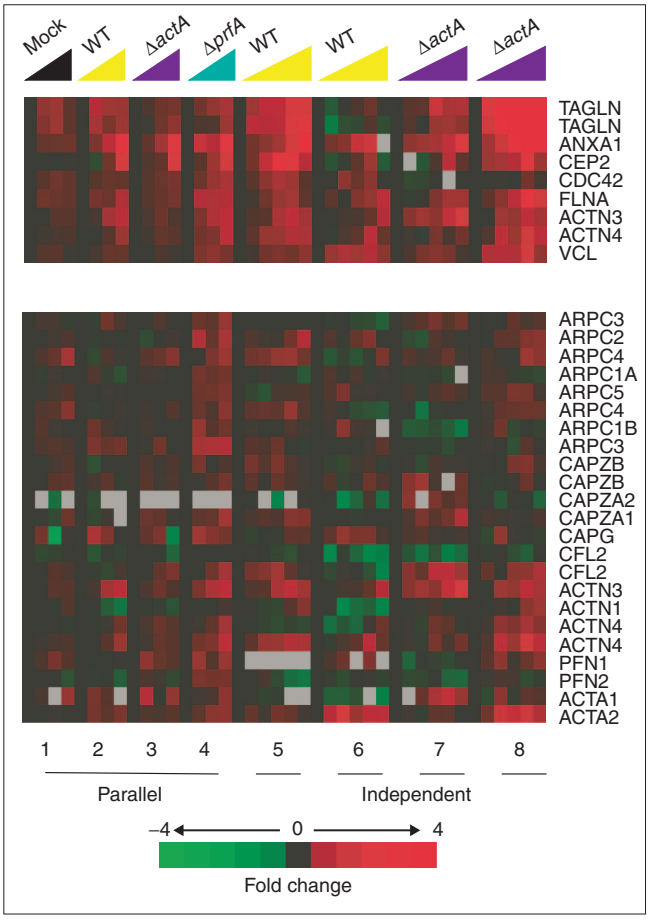

Figure 8
Induced cytoskeletal genes and the genes required for actin-based motility. **(a)** Cytoskeleton-related genes present on 24K arrays which respond to the infection with at least a threefold change. **(b)** Named genes known to be involved in actin-based motility of *L. monocytogenes*. Image contrast is set at 2, such that the brightest red is greater than a fourfold change. Gray spots indicate missing data. Note that genes previously known to be involved in actin-based motility are not specifically induced by wild-type bacteria.

By the same sets of criteria described above for studying the difference between wild-type and *actA* infections, we examined whether there were any genes reproducibly induced by cytoplasmic bacteria (wild-type or *actA*) but not by the avirulent organism (*prfA*). In this dataset, no genes passed these criteria during the first 4 hours of infection. It is likely that differences in the transcriptional program would emerge at later stages of infection, as wild-type and *actA* bacteria replicate and kill the cells, and *prfA* do not.

The innate immune response of Caco-2 cells occurs independently of host-cell differentiation, or of bacterial genotype

We were interested to determine whether changes in gene transcription levels correlated with changes in protein expression. We chose to look at Gro-1 (MSGA) production and secretion as it appeared to be one of the most highly induced chemokines at the RNA level (Figures 2,6), and is known to be a target of NFκB activation [47]. Indeed, at 4 hours post-infection, Gro-1 was induced, as measured by enzyme-linked immunosorbent assay (ELISA), and was detected at similar levels in the supernatants following infections with each of the three genotypes tested, as predicted by the array experiments (Figure 9a). As infections were most efficient in subconfluent Caco-2 cells, which do not have differentiated apical and basolateral surfaces, we chose these conditions for our microarray experiments with the goal of inducing as much actin-based motility as possible. However, given the robust NFκB-dependent response we saw from all three bacterial genotypes, we were interested to know whether the response we detected might depend on the differentiation state of the host cells.

One might expect that polarized intestinal epithelial cells would not react strongly to the presence of bacteria on the apical surface (where bacteria are concentrated in the gut), but that the basolateral surface, which *in vivo* should encounter bacteria only when epithelial integrity is breached, might be more sensitive. We therefore compared the responses of polarized cells to encounters with wild-type bacteria at either the apical or basolateral membranes. Caco-2 cells were grown as confluent monolayers on transwell filters and tight-junction formation was confirmed by the inability of inulin to diffuse from the apical to the basolateral surface. Despite infecting the polarized monolayers at relatively high multiplicity of infection (MOI) with wild-type *L. monocytogenes* strain 10403S, we were not able to detect any intracellular bacteria by either phase-contrast or fluorescence microscopy. We found, however, that the polarized cells secreted Gro-1 in response to exposure to wild-type bacteria at either the apical or basolateral surface, with most of the Gro-1 secreted from the basolateral membrane (Figure 9b). Surprisingly, the apical surface appeared to be most sensitive to the presence of bacteria, but this could be explained by the fact that the membrane supporting the basolateral cell surface reduced the amount of surface area available for receptor expression. It is also possible that a very small amount of lipotechoic acid (LTA) is penetrating the confluent monolayer, thus stimulating a response from the basolateral surface. We reproducibly demonstrated that our monolayers permitted less than 1% inulin diffusion, one standard for polarization of Caco-2 cells; however, this may not have been sufficient to completely block LTA diffusion.

We also found that Gro-1 expression was not affected the presence or absence of serum during these infections, implying that the events stimulated by the bacteria are occurring primarily through interactions with cell-surface moieties on both the bacteria and the host cells. Gro-1 expression was also detected in response to heat-killed bacteria (data not shown), confirming that intracellular infection is not required for activation of the pathway for Gro-1 expression.

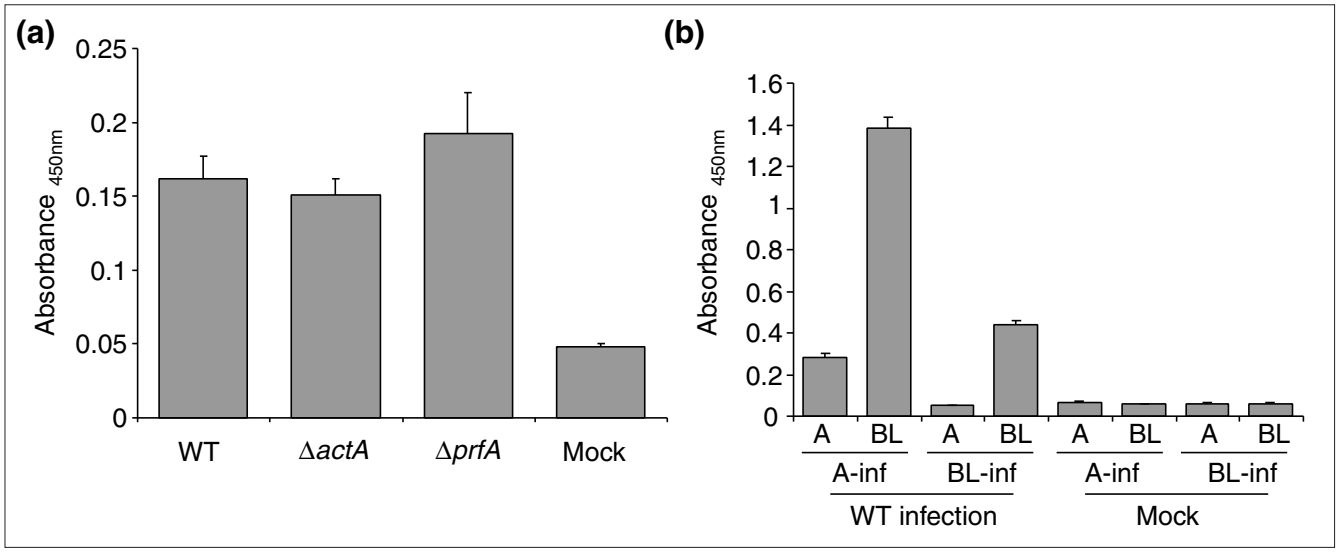

Figure 9
ELISA for the presence of secreted Gro-1 chemokine in supernatants of subconfluent and polarized Caco-2 monolayers. **(a)** Subconfluent monolayers infected with wild-type, *actA*, and *prfA* bacteria under conditions used for microarray analysis. **(b)** ELISA assay for Gro-1 production by polarized Caco-2 cells. Wild-type *L. monocytogenes* (WT) was used to infect either apically (A-inf) or basolaterally (BL-inf). Supernatants from both apical (A) and basolateral (BL) membranes were analyzed for secreted Gro-1.

Discussion

Cultured intestinal epithelial cell lines mount an innate immune response to infection by *L. monocytogenes*

In this report we describe the transcriptional response of human intestinal epithelial cells to infection by both pathogenic and non-pathogenic *Listeria monocytogenes*. Using cDNA microarrays of 22,594 human cDNAs, we have examined transcriptional responses of Caco-2 cells to wild-type and mutant bacteria. The predominant response has pervasive similarities to the transcriptional program mediated by the NFκB system. This response is not specific to intestinal epithelial cells, as a similar suite of genes is induced in promyelocytic THP1 cells infected by wild-type *L. monocytogenes* for 2 hours [19]. Nor is it specific to infection by *L. monocytogenes*; for example, recent experiments using peripheral blood mononuclear cells (PBMCs) have shown a similar response on exposure to a variety of pathogenic and non-pathogenic microbes [48]. The number of activated (and repressed) genes in the PBMC experiments is higher than we have found here, perhaps reflecting the nature of a more 'professional' or specialized immune response. It is also important to note that the microarrays (Lymphochips) used in the PBMC experiments were carefully designed to encompass all of the genes known to be involved in the NFκB pathway and therefore may simply represent a more complete dataset.

In light of recent developments in understanding the biology of Toll-like receptors, the NFκB-dominated responses we observe fit well within a model in which undifferentiated, subconfluent Caco-2 cells can recognize Gram-positive cell-surface moieties and induce what is termed an 'innate immune response'. The Toll-like receptor family [49], which has evolutionary roots throughout invertebrate metazoans for pattern-based recognition of bacterial and fungal cell-surface components [50], activate NFκB through convergent pathways involving the IL-1 receptor-associated kinase (IRAK), the adaptor protein MyD88, TRAF6, NFκB-inducing kinase (NIK), and IκB kinase (IκK) [51]. The Toll-like receptors TLR2 and TLR4 are known to be present on a number of different human intestinal epithelial cells, including primary cells, H4 and Caco-2 cells [52-54]. In addition, TLR5 has recently been shown to signal in response to bacterial flagellin from both Gram-positive and Gram-negative bacteria in a MyD88-dependent manner [55], and TLR5 is constitutively expressed on primary intestinal epithelium [52].

Caco-2 cells respond similarly to infection by pathogenic and non-pathogenic *L. monocytogenes*

A critical property of the intestinal epithelium is the ability to distinguish between pathogenic and non-pathogenic bacteria, and under stress, to flush pathogens from the lumen of the intestine. *In vivo*, intestinal cells are specially adapted to coexist with very high concentrations of commensal bacteria. In the case of newborn mice, there appears to be an innate ability to develop commensal relationships with microbes. A recent report indicates that during the development of host-microbial interactions, essentially no proinflammatory or innate immune responses are detected as the intestines of germ-free mice become colonized [22]. In contrast, infection of the intestinal epithelium by pathogenic bacteria elicits a

strong inflammatory response that includes disruption of the epithelial layer by mechanisms of both cytopathicity and apoptosis [56]. Intraepithelial lymphocytes (IELs), and polymorphonuclear leukocytes (PMNs), which reside in the epithelial mucosa, are recruited to damaged regions. Indeed, neutrophils are critical for controlling infections by *L. monocytogenes* in a mouse model [57,58]. NFκB has an important role in the proinflammatory process in the gut, and chemokines and cytokines involved in establishing an inflammatory or immune response have also been implicated [59,60].

Our results are in broad agreement with these studies with respect to the central role of NFκB in response to infection, but are also puzzling because we would not expect intestinal cells to respond so strongly to the presence of avirulent or heat-killed bacteria, even when presented on the apical surface of a confluent, polarized monolayer, regardless of the presence or absence of serum. The mechanism of intestinal epithelial cell discrimination between pathogens and nonpathogens *in vivo* is unknown. We assume that the response we see in Caco-2 cells is one that intestinal epithelial cells are intrinsically capable of mounting *in vivo* as well as *in vitro*, but that *in vivo* there are other factors which may contribute to the paucity or severity of the response. Other cell types may be able to better distinguish between pathogenic and nonpathogenic *L. monocytogenes*; for example, expression of the virulence factor LLO, the hemolysin responsible in part for invasion of *L. monocytogenes* into the host cell cytoplasm, is necessary for *L. monocytogenes* to activate neutrophils [34], stimulate prolonged TNFα production by macrophages [36,37], and induce NFκB nuclear translocation and cytokine secretion by HUVEC human vascular endothelial cells [35,39]. It is possible that intestinal cells are programmed for a more general response, and that controlling the severity of the response is the critical level of regulation. In the intact intestine, communication between intestinal epithelial cells and resident immune cells may serve to modulate the initial innate immune response mounted by the epithelial cells.

Actin-based motility of *L. monocytogenes* does not induce a specific transcriptional response in infected epithelial cells

Under the conditions tested, we found little difference between responses to wild-type and mutant infections, indicating that nearly all the transcriptional response we observed was due to the initial contact between bacteria and the host-cell surface. Detailed comparisons of the response to wild-type and *actA* mutant infections were designed to uncover gene-expression programs specific to the motility phase of the infection, when bacterially directed actin polymerization is induced to move the bacteria through the cytoplasm. While subtle gene-expression responses were highly sensitive to changes in culture conditions, remarkably we found no convincing evidence for a gene-expression response specifically induced by entry of *L. monocytogenes* into the cytosol, or by bacterial subversion of the cell's actin cytoskeleton. We cannot rule out the possibility that specific alterations in host-cell transcription may have subtle effects by altering the stoichiometry of molecules involved in actin-based motility, but we are not able to detect such subtle changes using the methods described here. It remains possible that specific genes not present on the microarrays used here (representing about half the human genome) may be induced by actin-based motility, but it is nonetheless clear that bacterial subversion of the actin cytoskeleton does not result in any large-scale, coordinated transcriptional response.

This result is exciting from the biochemical perspective of actin-based motility, although disappointing with respect to discovery of novel genes involved in the process. We conclude that any cellular alteration required for this dynamic actin polymerization in an infected cell is supported post-transcriptionally, certainly with respect to known cytoskeletal genes, and that bacterial regulation of cellular systems required for motility is therefore probably mediated by biochemical events. Although studies of global transcriptional changes in both host cell and bacterial pathogens have proved powerful tools in the study of host-pathogen interactions, they cannot fruitfully assist investigation of these types of events.

Materials and methods

Cell culture

The human cells used for all infections were Caco-2, (ATCC: HTB-37). For microarray experiments, cells were maintained as a non-polarized, subconfluent population. All infections were performed on subconfluent cells that were passaged between 7 and 10 times after arrival from ATCC (original stock was p18). Cells were grown in Dulbecco's modified Eagle's medium (DME) containing 10% FBS (Gibco-BRL), antibiotic-antimycotic at 1/100 (Gibco-BRL; 10,000 units/ml penicillin G, 10,000 μg/ml streptomycin sulfate, 25 μg/ml amphotericin B).

Infections

All time courses were subject to identical infection protocols. Subconfluent monolayers of Caco-2 cells were infected with wild-type or mutant bacteria at an MOI of approximately 20. Bacteria were grown overnight at room temperature to promote flagellar assembly and extracellular motility before infection, and the OD_{600} was monitored for MOI normalization. Typically, bacterial cultures were in late log phase after 8-10 h (OD_{600} around 1). Before infection, monolayers were rinsed 3x with DME containing no antibiotics, and left in DME + 10% FBS (P/S-) for at least 1 h. After normalization for MOI, bacteria were pelleted at low speed (IEC centra-7; 1,500 rpm for 15 min), and resuspended in DME + 10% FBS. Concentrated bacteria were diluted into DME + 10% FBS

and distributed to antibiotic-free Caco-2 monolayers. Infections were initiated for 30 min, after which cell-free bacteria were rinsed away by washing 3x, and then continued in DME + 10% FBS. After 1 h, gentamycin was added at 100 μg/ml to kill any remaining extracellular bacteria. Mock infections were carried out in parallel, omitting bacteria at the time of inoculation.

It should be noted that on a very small scale, infections may be more precisely synchronized by spinning the bacteria down onto the target cells, and washing soon after. Under these conditions, differences in response to the mutants might be teased out with more time point measurements. Although the sensitivity and reliability of both mRNA amplification and detection have improved immensely in the last few years, these methods were not possible at the time of our microarray analysis, making centrifugation-mediated infections impossible.

Time courses

Infections were carried out under the guidelines of a single protocol, and several different experiments were performed. Four individual 8-h time courses were carried out at different times. Two 8-h wild-type time courses, and two 8-h *actA* time courses were initially examined. The time points for each of these were 0, 30, 60, 120, 240 and 480 min. In addition, a single experiment was performed where a mock infection, wild-type, *actA* and *prfA* mutants were all compared under the same conditions. For this parallel experiment, three independent mRNA samples were prepared before infection for the zero time point for the purpose of averaging during time-zero transformation of the data (see Data extraction and analysis). For the parallel time course, 0, 60, 120 and 240 min time points were measured. At each time point, cells were lysed directly in the culture dishes for mRNA isolation using oligo(dT) binding buffer (Invitrogen FastTrack 2.0) containing proteinases for ribonuclease degradation (see below).

Bacterial strains and infections

Three strains of *L. monocytogenes* were used; wild type (10403S), a mutant for the transcriptional regulation of virulence genes, *prfA* (DP-L1075) [33], and a mutant deficient for intracellular motility, *actA* (DP-L1942) [30]. Bacteria were maintained on brain-heart infusion (BHI) agar plates, and grown overnight in liquid BHI.

Cell staining and microscopy

Cells used for infections and mRNA isolations were seeded into dishes containing 20 x 20 mm coverslips. At each time point during a time course, coverslips were removed and fixed with 3.5% formaldehyde in PBS for 15 min. Coverslips were washed 3x with PBS and stored at room temperature before staining. Rhodamine phalloidin was used to stain and visualize actin structures in the cell [61]. Cells on coverslips were permeabilized and blocked with PBS containing 100 mg/ml BSA (Sigma), and 0.1% Triton X-100 (Sigma) for 1 h. Cells were stained with the above blocking buffer containing 1 μg/ml rhodamine phalloidin for 1 h, and rinsed 3x with PBS. Coverslips were inverted, and placed on 5 μl sterile glycerol on a glass microscope slide, and sealed with nail polish. Images were acquired with a Zeiss Axioplan 2 fluorescence microscope equipped with a Princeton Instruments CCD camera, and Metamorph imaging software. All images were initially acquired with a 40x objective and multiplied 2x by the CCD camera for 80x final magnification. Phase and epifluorescence images were acquired simultaneously.

cDNA microarrays

The microarrays used in these experiments were generated in the Brown and Botstein laboratories at Stanford University using standard techniques [62]. All experiments were performed with 24K-SH arrays containing spots representing 22,594 human transcripts and ESTs, from a number of different print runs. The print list for our data can be seen at [63] and print run cDNA quality-control information is archived at [64]. The raw data from these experiments, including information about genes present on these arrays, and the array images, can be viewed, searched, or downloaded from the supplementary website [63] which includes limited public access to the Stanford Microarray Database (SMD; Oracle) [65,66].

mRNA isolation, labeling and hybridization

mRNA was isolated from monolayers of infected cells by direct lysis in FastTrack 2.0 binding buffer (Invitrogen), and subsequent oligo(dT) purification as per the manufacturer's instructions. Protocols for labeling and hybridization can be found at the Brown laboratory website [62]. cDNA from experimental samples was generated by reverse transcription in the presence of Cy-5 dUTP, and was mixed with a reference pool of cDNA derived from 10 different cell lines (Brown laboratory control reference 'E'), labeled with Cy-3 dUTP. The mixture of Cy-5- and Cy-3-labeled cDNA was hybridized to PCR-based cDNA microarrays containing 22,594 genes. Microarrays were scanned using an Axon Instruments two-channel scanner (635 nm and 532 nm), and spots were defined with GenePix 3.0 Pro software (Axon Instruments).

Data extraction and analysis

Termed the 'type II' experimental approach, measurements for each gene during each condition were made relative to a constant reference pool. All subsequent analysis was performed on $\log_2$ of the ratio (Cy-5/Cy-3). For all arrays, red and green channels were normalized by the medians of the distribution of spot intensities, and transformed to the zero time point of each individual time course. Time zero transformation was carried out by subtracting the $\log_2$ (ratio) of the zero time point for each gene from each time point in the time course. By subtracting the $\log_2$ (ratio) of the zero time point from each point in the time course, one effectively eliminates the reference from the analysis: for example, for the 1-h time point: $\{\log_2(T_{60}\text{red}/\text{REFgreen}) - \log_2(T_0\text{red}/\text{REFgreen}) = \log_2(T_{60}\text{red}/T_0\text{red})\}$. For the parallel time course, three

zero time-point measurements were averaged before subtraction. The behavior of each gene over the time course of the experiment is presented as the relative change from its own zero time point.

Before statistical or simple mathematical comparison of wild-type and mutant infections, the dataset from the parallel time course was filtered such that only genes changing at least threefold at some point during the time courses ($\log_2(T_n/T_0)$ of 1.75) were included for further consideration.

Significance analysis of microarrays (SAM) was applied to both the parallel and individual datasets. Both a web-based version and an Excel macro containing the algorithm for SAM are available for download to academic users at [67]. For wild-type and mutant comparisons of the parallel time course, the 2- and 4-h time points were grouped as single classes. This analysis tests only for genes induced specifically by wild type but not by mutant infections at both 2- and 4-h time points. For the *actA* analysis, the wild-type and *prfA* were grouped as a single class. For the *prfA* analysis, wild-type and *actA* were a single class. For individual time-course analysis, which later included the parallel time course, individual time points remained as single classes.

Data were further analyzed by filtering with Microsoft Excel, thresholding many combinations of time points, and subsequently clustering (for example, the $\log_2(T_n/T_0)$ for wild-type time points 2 and 4 h must both be greater than 1.75, and all other 2- and 4-h time points must have a log(ratio) less than 1.8).

All data described in the text of this report are available for download. They are provided as tab-delimited text files, as they were generated during analysis. It should be noted that many of the original printed spots on the arrays were ESTs, which belong to a new UniGene cluster. It is possible that spots on these arrays have been renamed since the data were analyzed for this manuscript and we therefore recommend that if the reader has a real interest in any particular gene that they go to the Stanford Microarray Database [65,66] and carefully examine the data as it stands today. The raw and filtered data are available in tab-delimited text format, which can be opened by Microsoft Excel (including SAM), Cluster [68] or TreeView [68]. For convenience, all data have been normalized by the medians of the intensity distribution for each array, and converted to the $\log_2$(ratio of red/green raw intensity). Normalized data are available as original log ratios where the experimental samples are measured in the red channel, and reference RNA is measured in the green channel. Time-zero transformed data are also available for download.

Polarization of Caco-2 cells

Caco-2 cells were plated at high density (1×10^6 per well) on 3 μm pore size transwell filters (2.4 cm diameter), and allowed to polarize for 2 weeks. To test for tight-junction formation, [^{3}H]inulin was added to the medium on the apical surface, with cell-free 0.45 μm filters as a control for rapid diffusion. Samples of 10 μl from both apical and basolateral supernatants were counted in a scintillation counter 30 min after addition. 1% diffusion or less indicates tight-junction formation.

ELISA for Gro-1 production

Supernatants from infected cultures were harvested at each time point and frozen before ELISA analysis. Gro-1 Quantikine ELISA kit was purchased from R&D Systems and protocol was followed according to the manufacturer's instructions. In each case, reactions were stopped at 30 min, and A_{450} was measured on a fluorescence plate reader. The data shown are in absorbance units, not absolute quantities of Gro-1, and have been normalized to total volumes from the experiment, but not cell number, used during the infections (the cell number/culture volume ratio for the polarized-cell experiments was much higher than for the comparisons of wild type to mutant on subconfluent Caco-2 cells).

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