The Invasive Phenotype of *Shigella flexneri* Directs a Distinct Gene Expression Pattern in the Human Intestinal Epithelial Cell Line Caco-2*§

Thierry Pédroń‡, Christelle Thibault§, and Philippe J. Sansonetti¶

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Invasion of the human colonic epithelium by *Shigella flexneri* causes inflammation that disrupts the intestinal barrier. Invaded intestinal epithelial cells are the major source of mediators recruiting the inflammatory infiltrate. To better characterize the global response of intestinal epithelial cells to *Shigella* invasion, Caco-2 cells were infected by an invasive isolate of *S. flexneri* 5a, and their transcription was analyzed by Affymetrix (Santa Clara, CA) microarrays (12,000 genes) and compared with those elicited by a non-invasive *Shigella* mutant and tumor necrosis factor (TNF)-α. The invasive and non-invasive strains enhanced transcription of a common pattern of 240 genes, among which genes encoding isoforms of cytochrome P-450 were induced. These genes were not induced by TNF-α. Conversely, both the invasive strain and TNF-α induced a common set of 18 genes, mainly encoding proinflammatory molecules. They also induced specific sets of genes. The transcriptome induced by the invasive strain was characterized by the induction of early genes (*i.e.* expressed within the first 45 min of invasion) and late genes (*i.e.* after 60 min of invasion) whose pattern was strongly biased toward stimulation of granulopoiesis, chemotaxis, activation, and adherence of polymorphonuclear leukocytes. When compared with a non-invasive *Shigella* and TNF-α, invasive *Shigella* induced a narrow transcriptome that seems to program infected epithelial cells to recruit a mucosal polymorphonuclear leukocyte to infiltrate. Dramatic increase in *IL-8* gene transcription points to this chemokine as the major molecule orchestrating mucosal inflammation in shigellosis.

Enteroinvasive bacteria stimulate mucosal inflammation that controls infection at the cost of severe tissue destruction. The acute recto-colitis that follows epithelial invasion by *Shigella* (1), a Gram-negative invasive pathogen for humans and primates, is a paradigm of this process (2). PMN† are massively recruited to the infected epithelial lining through which they translocate, thereby causing rupture of the epithelial barrier that, in turn, facilitates further bacterial invasion (3, 4) and destruction of mucosal tissues. In parallel, PMN achieve degradation of pathogenicity molecules and bacterial killing (5, 6). A central theme in the study of *Shigella* pathogenesis is understanding the cross-talk between bacteria and intestinal epithelial cells that leads to mucosal inflammation, a topic common to the study of inflammatory bowel diseases such as ulcerative colitis and Crohn’s disease. *Shigella* may provide invaluable information by identifying common dysfunctions in signaling pathways.

Recent studies point to infected intestinal epithelial cells (IEC) as major players in the inflammatory process, both as sentinels achieving bacterial sensing and as effectors producing mediators, particularly cytokines and chemokines, which initiate and orchestrate mucosal inflammation (7). Mechanisms of recognition of invasive pathogens by IEC are not yet fully understood. Although the signaling cascades that elicit invasion in response to bacterial invasions may also affect proinflammatory signals, current evidence indicates that recognition of pathogen-associated molecular patterns is likely to dominate (8). Extracellular recognition relies on Toll-like receptors (TLR) (9), particularly TLR5, a receptor for flagellin, (10, 11) which is constitutively expressed by IEC, and possibly TLR2 and TLR4, which recognize bacterial lipoproteins and lipopolysaccharide, respectively, although their expression or actual function in IEC is still a matter of debate, particularly in absence of expression of CD14 (12, 13). In the case of *Shigella*, which is non-flagellated, most recent evidence indicates that bacterial recognition by IEC occurs intracellularly via an “inside-in” signaling pathway involving intracellular pathogen-associated molecular pattern recognition (14) by a cytosolic molecule, Nod1/CARD4 (15, 16). Gram-negative peptidoglycan is the agonist of Nod1 intracellularly.²

Both TLR and Nod lead to activation of the proinflammatory transcriptional factor, NF-κB (14, 15, 17, 18). However, it is likely that the proinflammatory potential of invaded IEC does not simply reflect NF-κB activation. The transcriptional pattern of these cells is likely to encompass a complex combination of activation and repression of several transcriptional systems. NF-κB dominates among these systems, as reflected by the

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† To whom correspondence should be addressed. Tel.: 33-1-40-61-30-93; Fax: 33-1-46-68-59-53; E-mail: psanson@pasteur.fr.

‡ The abbreviations used are: PMN, polymorphonuclear leukocytes; IEC, intestinal epithelial cells; TNF, tumor necrosis factor; EGF, epidermal growth factor; TGF, transforming growth factor; TLR, Toll-like receptors; CYP, cytochromes P-450; VIP, vasoactive intestinal polypeptide; IFN, interferon; GM-CSF, granulocyte-macrophage colony-stimulating factor; CLARP, caspase-like apoptosis-regulating protein.

² Girardin, S. E., Boneca, I. G., Carneiro, L. A., Antignac, A., Jehanno, M., Viala, J., Tedin, K., Taha, M. K., Labigne, A., Zähringer, U., Coyle, A. J., DiStefano, P. S., Bertin, J., Sansonetti, J. J., and Philpott, D. J. (2003) *Science* 300, 1584–1587.
release of IL-8, a common denominator to a variety of invasive microorganisms and epithelial cell types (19). In vitro, the production of IL-8 is largely associated with infected IEC, as demonstrated in the rabbit-ligated loop model of shigellosis. In this model, neutralization of IL-8 causes massive decrease of PMN recruitment, resulting in protection of the epithelium against inflammatory destruction, but also in uncontrolled growth of bacteria in the lamina propria (20). Therefore, in shigellosis, the innate immune response corresponds to a balance between bacterial eradication and destruction of the mucosa. IL-8 produced by IEC plays a major role in this process, although the complexities and subtleties of transcriptional regulation leading to this particular profile are currently unknown and probably vary depending on the nature of the invasive microorganism and timing of the infectious process. In addition, with time, the epithelial response is likely to increasingly reflect a response to the proinflammatory cytokines that are massively released, such as TNF-α, which plays a major role in epithelial destruction in experimental shigellosis (21). In addition, when bacteria reach subepithelial tissues, they encounter other cell populations, particularly resident macrophages and recruited monocytes that will impose their own profile of response, which is dominated by CD14/LR4 recognition of Shigella lipopolysaccharide, as shown by the dramatic changes in mucosal response observed either upon CD14 neutralization (22) or upon infection by a Shigella mutant that was genetically engineered to express a non-endotoxic lipid A (21).

To provide a detailed analysis of the transcriptional response of IEC invaded by S. flexneri (i.e. 45 min to 4 h), we have applied the Affymetrix microarray technology. Thus, the transcriptome of the human colonic Caco-2 cell line was established upon its infection with strain M90T, which expresses an invasive phenotype, due to the presence of the 213-kb virulence plasmid (23). The M90T-induced transcriptome was compared with the transcriptomes obtained by infecting Caco-2 cells with strain BS176, a non-invasive, plasmid-less derivative of M90T, or by exposing cells to TNF-α.

These experiments demonstrate that the transcriptome of Shigella-invaded IEC reflects the invasive phenotype and is significantly different from that induced by TNF-α in that it is almost exclusively devoted to the recruitment, activation, and adherence of PMN. This observation opens the way to studying the extent of transcriptional regulation achieved by the Nod1 cascade, upon stimulation by intracellular Shigella, and suggests that virulence proteins of Shigella, particularly those that are secreted through the type III secretory apparatus, may “remodel” this basic transcriptome.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human IEC line Caco-2 derived from a colonic carcinoma (24) was used in this study. Cells were grown in an incubator at 37 °C, 10% CO₂, in Dulbecco’s modified Eagle’s medium supplemented with 10% decomplemented fetal calf serum, 1% non-essential amino acid, and antibiotics (penicillin-streptomycin, respectively, 100 units/ml and 100 μg/ml). Before infection with bacteria, cells were washed in Dulbecco’s modified Eagle’s medium without serum and incubated at 37 °C for 2 h in the same medium.

**Bacterial Strains and Infection**—Two bacterial strains were used in this study: the wild-type Shigella flexneri 5a (M90T) that possesses the vil and iba virulence plasmid and its plasmid-cured mutant BS176, which is non-invasive (23). One isolated colony on Tris-buffered saline agar containing 0.01% Congo red (25) was seeded in 7 ml of Tris-buffered saline broth for overnight culture. Before cell infection, bacteria were diluted in fresh broth for 2 h to be harvested in exponential phase of growth. Caco-2 cells were grown in 10-cm-diameter Petri dishes. To obtain an efficient cell invasion, non-confluent Caco-2 cell cultures were infected with bacteria with a multiplicity of infection of 100 bacteria/cell. After a 15-min-centrifugation at 2,000 rpm, cells and bacteria were incubated for 45 min and then washed three times in Dulbecco’s modified Eagle’s medium and reincubated for 75 or 195 min with 50 μg/ml gentamicin to kill extracellular bacteria. As confirmed by Giemsa staining, more than 70% of the cells were regularly infected.

**Preparation of mRNA and Hybridization on Affymetrix Chips**—Following washing in cold phosphate-buffered saline, cells were lysed, and the total RNA was extracted by RNeasy Mini kit (Qiagen, Valencia, CA). Integrity and purity of RNA were checked by spectrophotometry and capillary electrophoresis, using the Bioanalyzer 2100 and RNA 3.2 software. The chips kit from Agilent Technologies (Palo Alto, CA) cDNA were synthesized using Superscript Choice system (Invitrogen). Biotin-labeled-cRNA was then synthesized with the Enzo BioArray High Yield RNA transcript labeling kit (Enzo Biochem, New York, NY). After purification with Rneasy columns (Qiagen), 12.5 μg of fragmented cRNA were hybridized to an HG-U95A2 array (Affymetrix), and the slides washed and stained with biotin-labeled streptavidin using a fluids station. Finally, the arrays were scanned at 570 nm with a resolution of 3 μm/pixel, using a GeneArray scanner from Agilent Technologies.

**Analysis of Results**—In the Affymetrix technology (26), 25-mer oligonucleotides are directly synthesized on the glass slides. For each gene sequence, 16–20 different oligonucleotides are present, and for each oligonucleotide, the perfect match is the exact homology of the gene selected, whereas for the mismatch, the nucleotide in position 13 is wrong. For the analysis, two versions of MicroArray Suite from Affymetrix were used, MAS4.0 (27) and MAS5.0 (28). Expression algorithms compute two main metrics for each transcript, the absolute or differential call (present, marginal, or absent) determined from the transcript is reliably detected by the probe array and the average difference or signal reflecting the relative level of expression of the transcript. In the first experiments, the fluorescein obtained was analyzed using MAS4.0 software, based on empirical algorithms. A normalization factor (the scaling factor) was applied, and then after background subtraction and statistical comparison of the hybridizations between perfect matches and mismatches, the presence or absence of the gene was provided, thus constituting the absolute call (present, marginal, or absent). Then the average difference, directly related to the expression of the transcripts, was determined. The comparative analysis, also performed by MAS, allowed the comparison between two samples (in our case, the non-infected and the experiment (infected with invasive or non-invasive bacteria). This analysis indicated whether there was a change in gene expression between the two samples. This was provided as the change call (increase, marginal increase, decrease, marginal decrease, or no change) and also by the -fold change (between the signal of the experimental sample and of the baseline sample). With MAS 5.0, the empirical algorithms are replaced by statistical algorithms; the major difference between the two versions of the software is that in the comparative analysis, the -fold change is shown as a logarithmic scale.

The clustering analysis with Data Mining Tool (DMT version 3.0) of the microarray experiment allowed the identification of gene expression patterns. One of the methods used in this study was the correlation coefficient clustering algorithm, which finds probe set patterns that have similar shapes. Three steps are successively performed for finding clusters of similar probe set patterns: (i) filtering to remove patterns mostly related to noise, (ii) seeding to define the expression patterns of the clusters using a nearest neighbor approach, and (iii) clustering to group patterns that are close to the cluster shape define in the previous step.

**Detection of Protein Expression**—Caco-2 cells were infected with Shigella, as described above, in 12-well culture plates. At different time points (60, 120, 240, and 360 min), culture media were centrifuged (1,500 rpm, 10 min), and enzyme-linked immunosorbent assay tests for immunodetection of IL-8 and CXCL1 chemokines were performed, following the supplier’s recommendations (Quantikine®, R&D Systems, Inc., Minneapolis, MN). Chemokine concentrations were determined from the standard curve provided in the detection kit. The experiments were performed twice in duplicate.

**RESULTS**

**Patterns of Gene Expression in Non-infected and Shigella-infected Human Colonic Caco-2 Cells**—To enable evaluation of the reproducibility among different experiments, 4–5 independent microarray hybridization experiments were performed either on non-infected cells or on cells infected by the wild-type strain M90T or on cells infected by its non-invasive plasmid-less derivative, BS176. Only half of the12,000 human genes displayed on the Affymetrix U95A chip were expressed by Caco-2 cells, based on the absolute calls determined by MAS.
Cluster 1, Genes Up-regulated during a 2-h Infection with either M90T or BS176—We identified a total of 240 genes whose transcription was up-regulated, regardless of the expression of the invasive phenotype by Shigella. Considering that a plasmid-less Shigella is devoid of any specific pathogenic factor (i.e., no adherence-invasive capacity, no protein secretion) and that the species has lost expression of flagella, a major stimulus eliciting a proinflammatory program in IEC via TLR5 recognition (11), the cell response might reflect (i) the recognition of bacteria-associated molecular patterns (i.e., lipopolysaccharide, peptidoglycan, lipoproteins) as well as (ii) the stress response to metabolic products released by infecting bacteria and to the conditions of competition for essential nutrients. Table I shows genes whose ratio of transcriptional up-regulation by Shigella infection, normalized to the background expression in non-infected cells, was over 1.5 with at least one of the two infecting organisms and with a significant absolute call. This ratio was computed from results obtained in four independent samples of cells infected by M90T or BS176 and five independent samples of non-infected cells. Data presentation in Fig. 1 has been organized according to sets of genes reflecting similar functions. The complete list of the cluster, like the other tables shown in this study, may be found as supplemental data. The complete results are available upon request.

Cluster 2, Genes Up-regulated during a 2-h Infection with M90T—Following a 2-h infection, we identified a total of 72 genes whose increased transcription was specifically related to expression of the invasive phenotype by Shigella. Table II reports genes whose transcriptional induction over non-infected cells showed a ratio superior to 1.5.

This transcriptome was clearly dominated by the up-regulation of genes encoding chemokines, cytokines, and adherence molecules, which, altogether, may achieve bone marrow stim-
I

(49). Increased transcription of the gene encoding the MAD-3/aimed at moderating the proinflammatory cascade, particu-
diation-inducible early gene (IAP1) (47). Engagement of a cell protection and proliferation
of transcription of the gene encoding the antiapoptosis protein
BS176, was confirmed by the observation of 6.1
observation that transcription of TNF-
/H9251
(43, 44).

invasion is dominated by massive induction of
in the next paragraph exploring the dynamics of M90T-induced
infection with both the invasive and non-invasive strains of S. flexneri.

These genes belong to cluster 1, generated by analysis of data with the correlation coefficient clustering available in the Data Mining Tool
software from Affymetrix. The genes shown here are those whose transcriptional activation ratio was over a cut-off value of 1.5. As some genes are
represented by more than one probe set, they were indicated two times in this table.

| Category of genes | Accession number | Description of genes | BS176/NI | M90T/NI |
|-------------------|------------------|----------------------|----------|---------|
| Detoxification    | K03191           | Cytochrome P-1-450 (TCDD-inducible) | 8.12     | 7.13    |
|                   | X02612           | Cytochrome P1-450    | 7.30     | 7.36    |
|                   | X02612           | Cytochrome P1-450    | 6.57     | 5.66    |
|                   | U03688           | Dioxin-inducible cytochrome P450 | 3.44     | 3.17    |
|                   | U03688           | Dioxin-inducible cytochrome P450 | 3.30     | 3.04    |
|                   | U07919           | Aldehyde dehydrogenase 6 | 5.27     | 4.70    |
| Apoptosis         | M30704           | Amphiuregulin        | 1.58     | 1.54    |
|                   | AF005775         | Clarp 2              | 1.73     | 1.93    |
|                   | X70340           | Transforming growth factor α | 1.46     | 1.69    |
| Phosphatase       | M25393           | Protein tyrosine phosphatase | 1.59     | 1.21    |
| Receptor          | X77777           | Intestinal VIP receptor related protein | 1.74     | 2.03    |
|                   | U68019           | Mad protein homolog 1 | 1.75     | 2.01    |
|                   | U68019           | Mad protein homolog 2 | 1.74     | 1.82    |
| ATPase-activating protein | U90920 | PTPL1-associated RhoGAP | 1.66     | 1.73    |
| Cellular cycle    | AF01453          | Cyclin E2            | 1.53     | 1.67    |
| DNA               | AF084513         | DNA repair exonuclease (RECL) | 1.51     | 1.41    |
|                   | D83702           | Photolyase            | 1.97     | 1.83    |
| Transcription     | AF078096         | Forkhead/winged helix-like transcription factor 7 (FKH7) | 1.51     | 1.41    |
| Others            | L40904           | Peroxisome proliferator activated receptor γ | 1.62     | 1.41    |
|                   | M26883           | Interferon γ treatment inducible | 2.29     | 2.72    |
|                   | AF099955         | MDC-3.13 isoform 2   | 1.46     | 1.67    |
|                   | AL049399         | DKFZp56600118        | 2.47     | 2.38    |
|                   | AL049953         | DKFZp564K1216        | 1.70     | 1.81    |
|                   | W28850           | Homo sapiens cDNA    | 1.60     | 1.46    |
|                   | AL035447         | Clone 1183211        | 1.57     | 1.50    |

These accession numbers are GenBank™ accession numbers.

The kinetics of transcriptional activation of eukaryotic genes according to the duration of cell invasion by M90T were established following 45, 120, and 240 min of invasion. Because both the experimental results reported in the previous paragraphs and a statistically analysis of variance analysis of var-
ance of the replicate had confirmed high reproducibility of the technique, hybridizations were performed according to a pro-
tocol in which mRNA samples from four different experiments were pooled in a single sample. This process allows us to
minimize the biological variation between samples.

This strong proinflammatory profile was reinforced by the observation that transcription of TNF-α and its regulatory protein A20 (45) were up-regulated by 3.06× and 4.23×, re-
spectively, and that the gene encoding cyclooxygenase-2 was induced by 2.2× (46). In addition, the antiapoptosis profile observed through the common up-regulation of Clarp, amphi-
regulin, and TGF-α, following infection by both M90T and BS176, was confirmed by the observation of 6.1× up-regulation of transcription of the gene encoding the antiapoptosis protein
IAP1 (47). Engagement of a cell protection and proliferation program was confirmed by the additional induction of the rad-
iation-inducible early gene (IEX-1) and the CYR61 gene (49). Increased transcription of the gene encoding the MAD-3/ IκB-1 activity (50) may reflect initiation of a retrocontrol loop aimed at moderating the proinflammatory cascade, parti-
cularly the sustained activation of NF-κB. The induction of genes encoding oncoproteins, such as c-jun and jun-B, will be considered in the next paragraph exploring the dynamics of M90T-induced transcriptional patterns according to duration of cell invasion.

in summary, M90T induces a transcriptome reflecting a proinflammatory program that is strongly biased toward in-
volve ment of PMN, NK, and dendritic cells at the early stage of the innate response to Shigella invasion. Transcriptional activation is dominated by massive induction of IL-8 transcription, thus emphasizing that this chemokine is a key marker of the
epithelial response to bacterial invasion.

Time Course of Transcriptional Activation, Early and Late Genes—The kinetics of transcriptional activation of eukaryotic genes according to the duration of cell invasion by M90T were established following 45, 120, and 240 min of invasion. Because both the experimental results reported in the previous paragraphs and a statistically analysis of variance analysis of var-
ance of the replicate had confirmed high reproducibility of the technique, hybridizations were performed according to a pro-
tocol in which mRNA samples from four different experiments were pooled in a single sample. This process allows us to
minimize the biological variation between samples.

Fig. 2 summarizes the kinetics of transcriptional induction of some of the major genes whose transcription was induced fol-
lowing invasion by M90T. It clearly demonstrates the existence of a set of early genes, exemplified by c-fos, that was strongly
induced as early as 45 min after invasion. Then, despite the persistence of intracellular bacteria, its transcription returned to
background level. Among these early genes, another set was observed whose up-regulation started after 45 min of invasion
and remained high or slowly decreased according to duration of
invasion. The latter comprised the genes encoding cytochromes
P-450 and the gene encoding IEX-1.

Following these early transcriptional programs, up-regula-
tion of the genes encoding proinflammatory molecules, such as IL-8, CXCL-1, -2, -3, ICAM-1, and the chemokine CCL-20,
appeared only around the second hour of cell invasion, thus
their classification as late genes. To demonstrate that increase
in gene transcription was actually followed by translation and release of the relevant proteins in the cell culture medium, we selected IL-8 and CXCL-1 as chemokines whose gene transcription
was most strongly induced, according to Affymetrix microarrays. Analysis was performed by enzyme-linked immu-
nosorbent assay (Quantikine, R&D Systems). Whereas non-
invasive BS176 did not induce significant release of these two
chemokines at any time point, upon infection of Caco-2 cells, invasive M90T did induce their expression and release. Significant amounts could be detected following 240 min of infection (IL-8 = 60 ± 0 pg/ml; CXCL-1 = 16 ± 1 pg/ml). These values then increased according to time, as seen following 360 min of infection (IL-8 = 84 ± 4 pg/ml; CXCL-1 = 27.5 ± 0.5 pg/ml).

Genes Up-regulated in Response to a 2-h Stimulation of Caco-2 Cells by TNF-α—To explore whether IEC uniformly respond to proinflammatory stimulus, whether an invasive microorganism, such as Shigella, or a proinflammatory cytokine, such as TNF-α, the transcriptome of Caco-2 cells exposed to 10 ng/ml TNF-α for 2 h was analyzed, and the ratio of transcriptional up-regulation over untreated cells was recorded. Table III shows only genes whose transcriptional ratio was over a cut-off value of 1.5. As some genes are represented by more than one probe set, they were indicated two times in this table.

| Category of genes | Accession number | Description of genes | Fold increase for M90T/NI |
|-------------------|------------------|----------------------|--------------------------|
| Chemokines and cytokines | M28130 | Interleukin 8 | 304.79 |
| | X54489 | CXCL1 | 133.74 |
| | U64197 | CCL20 | 38.67 |
| | M36820 | CXCL2 | 13.17 |
| | M58465 | TNF-α inducible protein A20 | 4.23 |
| | M36821 | CXCL3 | 3.12 |
| | X02910 | TNF-α | 3.06 |
| | M92357 | B94 protein | 1.66 |
| | M13207 | GM-CSF | 33.24 |
| | M13207 | GM-CSF | 12.64 |
| | S81914 | M24283 | 1.88 |
| | | | |
| Apoptosis | U45878 | Inhibitor of apoptosis protein 1 | 6.1 |
| Adhesion Molecule | M24283 | ICAM-1 | 20 |
| Plasminogen activator | X02419 | uPA gene | 2.38 |
| Transcription and regulation | L65072 | Interferon regulatory factor 1 | 2.34 |
| | M69043 | MAD-3 mRNA (IκB-like) | 4.06 |
| | AF060503 | Zinc finger protein | 1.67 |
| Enzyme | U04636 | Cyclooxygenase-2 | 2.2 |
| EGF family | D30783 | Epiuregulin | 1.97 |
| Elastin specific inhibitor | L10343 | Elafin | 1.94 |
| Heparan proteoglycan | D79206 | Ryudocan core protein | 1.84 |
| Ribonucleoprotein | L22009 | HnRNP | 1.71 |
| GTP-binding protein | M29893 | Low molecular mass GTP-binding protein | 1.5 |
| Oncogene | J04111 | c-jun proto oncoprotein | 2.35 |
| | X13445 | jun-B | 1.64 |
| | AL021977 | Novel MAFF | 1.97 |
| | Y11307 | CYR61 | 2.17 |
| | U66035 | X-linked deafness dystonia protein | 1.79 |
| | AF049140 | MMS2 | 1.95 |
| | AF039945 | Synaptojanin 2B | 1.56 |
| | W28205 | H. sapiens cDNA | 1.9 |
| | AA208187 | H. sapiens cDNA | 1.78 |
| | A1452442 | H. sapiens cDNA | 1.68 |

*These accession numbers are GenBank® accession numbers.

As indicated in the text, the numbers shown are ratios between the average signal computed from 4 different samples of cells infected by invasive strain M90T over the average signal computed from 5 different samples of non-infected (control) cells (NI).

Over a ratio threshold of 1.5, no gene was significantly induced by all three experimental conditions. A common gene expression pattern was observed between cells infected with M90T and cells treated by TNF-α. This pattern clearly encompassed up-regulated transcription of the genes encoding the proinflammatory chemokines IL-8, CXCL-2 and -3, as well as CCL-20, and the adherence molecule ICAM-1. The level of transcriptional activation of IL-8 induced by TNF-α, however, was far from matching the level induced by M90T invasion. Also induced in both conditions were genes encoding IAP1, MAD-3, A20, and IEX-1, v-Maf (51), and Jun-B. The induction of several genes remained specific to one condition, such as CXCL1, GM-CSF, c-jun, CYR61 (49), and AML1 (52) for M90T, and, strikingly, a series of genes encoding NF-κB-related molecules, such as IκB, P-50, I-Rel, NF-κB subunit, and nuclear factor κB DNA binding subunit, as well as the genes encoding receptor protein 4–1BB.

These data indicate that Caco-2 cells were able to differentially regulate transcriptional activation in the presence of different proinflammatory stimuli. This opens the way to further differential analysis of gene transcription with the aim of identifying Shigella-expressed molecules that modulate host cell transcriptional patterns to provide a transcriptome that differs from those induced by other stimuli.

DISCUSSION

First used to annotate the function of microbial genomes, DNA arrays have become a powerful tool to study host-microbe interactions (53–55). Investigations using the DNA microarray technology were performed to analyze the transcriptional pattern of host genes during infection of epithelial cells with different enteric pathogens, such as rotavirus, Salmonella, and enteropathogenic Escherichia coli. Using a microarray covering the entire human genome, Cuadras et al. (56) showed that the expression of 511 genes was modulated when Caco-2 cells were infected with rotavirus. If the up-regulation of GM-CSF and CXCL-1 was slight, transcription of the genes encoding the oncogenes jun and fos increased respectively by 17× and 6.6× during infection, when compared with control cells. Genes belonging to the IFN response family and genes involved in the stress response (i.e. heat shock proteins) were also up-regu-
Gene Expression of Caco-2 Cells during Shigella Infection

Fig. 2. Gene expression during time-course analysis. Caco-2 cells were infected for 45, 120, or 240 min with invasive M90T. After RNA extraction, four replicates were pooled before cRNA synthesis was performed. The resulting biotin-cRNA was then hybridized to Affymetrix U-95A DNA chips. The vertical axis represents the fold increase in gene expression between infected versus non-infected cells. *EGR*, early growth response

The most striking feature of gene activation, however, was the massive induction of IL-8 transcription, followed by CXCL-1, -2, and -3, which was reflected in the actual expression and release of the first two that we checked, due to their dramatic transcriptional increase. These α-chemokines are largely devoted to the recruitment and activation of granulocytes, particularly PMN (40). Regarding PMN, like formylmethionyleucylphenylalanine, C5a, or platelet-activating factor, IL-8 is a potent activator of key microbialic functions. It activates 5-lipooxygenase and the production of LTβ4; it also acts as a priming agent amplifying the stimulating effect of formylmethionyleucylphenylalanine, thus increasing the release of leukotrienes and superoxide anions. It is also a potent degradating agent for both specific and azurophilic granules, thus causing release of antibacterial effectors. Among these effectors, elastase appears active on *Shigella* by achieving proteolytic degradation of its major virulence effectors (Ipa proteins and IcsA), thus disarming its capacity to escape into the cytoplasm and resist bactericidal mechanisms (6). However, IL-8 is also a potent chemoattractant for T lymphocytes and NK cells. We have recently shown that both CD4 T-cells and NK cells are the major sources of IFN-γ, an essential cytokine required to achieve control of *Shigella* infection at the innate stage of the immune response (64). These elements indicate that, as sentinel and signaling cells, IECs are sending soluble signals that seem well adapted to the eradication of *Shigella*, a strategy requiring the counteraction of its particular pathogenic properties, particularly the type III-mediated capacity to kill phagocytes (65, 66), and to hide inside IEC. It is likely that IL-8, owing to its strong properties of chemoattraction and activation of PMN, has been selected to play a key role in the defense against *Shigella*. This is, however, a “double-edged sword” since the massive infiltrate of PMN is deleterious to epithelial tissues, an effect probably enhanced by overexpression of ICAM-1 by epithelial cells, leading to increased binding of PMN (67). The absence of expression of IL-8 in mice (68) may largely account for their inability to develop any symptom of

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\[ ^{3} \text{K. LeBarillic et al., manuscript in preparation.} \]
shigellosis when infected intragastrically or intrarectally, unlike rabbits in which neutralization of IL-8 has been shown to strongly attenuate epithelial destruction in the presence of Shigella infection (20).

Potential recruitment of NK cells, in addition to providing IFN-γ, may also participate in epithelial destruction. In addition, activation of Cox-2 is expected to enhance the proinflammatory capacity of these factors. Conversely, up-regulation of the MAD-3/IAkBα gene, although possibly reflecting initiation of an anti-inflammatory loop, may also correspond to a program of vasoprotection maintaining the integrity of mucosal tissues, due to its alternative activity as growth factor for endothelial cells (50); note that the indigenous flora plays a significant role in the development of the intestinal capillary system (70). In addition to a strong bias toward recruitment of PMN, the Caco-2 cell response to Shigella infection (20), demonstrated in other systems (72).

A second major bias of the transcriptional pattern of the Caco-2 transcriptome also reflected a capacity to recruit dendritic cells, as deduced from the strong induction of the gene encoding CCL-20 (43). A similar observation was made in the case of Salmonella typhimurium infecting the murine intestinal epithelium (44). Although the encounter between dendritic cells and Shigella leads to quick apoptosis of these cells (71), this may still affect the profile of cytokines that will modulate the adaptive response. In addition, apoptotic blebs of infected dendritic cells may act as powerfully immunogenic elements in the adaptive immune response against Shigella, as already demonstrated in other systems (72).

A second major bias of the transcriptional pattern of the Caco-2 cell response to Shigella was toward induction of protective and survival mechanisms. This was observed not only with the wild-type isolate M90T but also with its non-invasive derivative BS176. Up-regulation of genes involved in cell detoxification processes, such as several isoforms of CYP and aldehyde dehydrogenase, was unexpected since BS176 is considered a harmful commensal, whereas it seemed instead perceived as a xenobiotic inducing a program of cell protection. CYP is a superfamily of heme-containing enzymes that play key roles in the metabolism and detoxification of an array of drugs, chemicals, and endogenous substances (73). Several pieces of evidence indicate that infection down-regulates the expression of CYP (74). This is observed in infected animals,

### Table III

**Genes up-regulated during stimulation of Caco-2 cells with 10 ng/ml of rTNF-α**

Caco-2 cells were stimulated during two hours with 10 ng/ml of rTNF-α before RNA extraction. The genes shown here are those whose transcriptional activation ratio was over a cut-off value of 1.5. As some genes are represented by more than one probe set, they were indicated two times in this table.

| Gene category | Accession number | Descriptions | Fold increase |
|---------------|-----------------|--------------|--------------|
| Chemokines, cytokines | X02530 | γ-interferon inducible early response gene | 10.41 |
| | U61497 | CCL20 | 10.18 |
| | M59465 | TNF-α inducible protein A20 | 7.07 |
| | M36820 | CXCL2 | 6.38 |
| | M28130 | Interleukin 8 | 6.25 |
| | M36821 | CXCL3 | 3.91 |
| Apoptosis | U45878 | Inhibitor of apoptosis protein 1 | 7.13 |
| NF-κB family | M60043 | MAD-3 mRNA encoding IAκB-like activity | 5.18 |
| | U91616 | IAκB epsilon | 3.21 |
| | S76638 | p50-NF-κB homolog | 2.26 |
| | S76638 | p50-NF-κB homolog | 2.23 |
| | X61498 | NF-κB subunit | 2.13 |
| | M58603 | Nuclear factor κB DNA binding subunit | 1.94 |
| Oncogene | AL021977 | Novel MAFF (v-maf oncogene family, protein F) | 3.78 |
| | X51345 | jun-B | 1.86 |
| | X56681 | junD | 1.81 |
| Transcription | S81914 | IEX-1 = radiation-inducible immediate-early gene | 4.41 |
| | MS2221 | IκBα | 2.65 |
| | AF001461 | Kruppel-like zinc finger protein Zf9 | 1.78 |
| | U34249 | Putative zinc finger protein | 1.94 |
| Homology to TNF and NGF receptor | U03397 | Receptor protein 4–1BB | 4.66 |
| Adhesion and receptor | M24823 | ICAM-1 | 4.63 |
| | U19247 | Interferon γ receptor α chain | 1.86 |
| | AB000712 | CPE receptor | 1.89 |
| Heparan proteoglycan | D79206 | Ryudocan core protein | 3.53 |
| Enzyme | X53483 | Glutathione peroxidase-like protein | 1.96 |
| | D10495 | Protein kinase C, δ-type | 2.00 |
| Others | AE636885 | IMAGE-1855887 | 2.62 |
| | AL050374 | DKFZp586C1619 | 2.27 |
| | AJ011896 | HIV-1, Nef-associated factor 1 | 2.00 |
| | U42408 | Ladinin | 1.78 |
| | M90657 | Tumor antigen (L6) | 1.78 |

* These accession numbers are GenBank™ accession numbers.
* Numbers indicate the fold increase between stimulated cells and non-stimulated (NI) control cells.

**Fig. 3. Gene expression patterns following infection of Caco-2 cells with invasive M90T or non-invasive BS176 or their stimulation with 10 ng/ml TNF-α.**
most likely in response to inflammatory mediators (75), with the exception of liver infection by Helicobacter hepaticus, which causes a time-related increase in expression of CYP1A2 and 2A5 (76). Moreover, administration of lipopolysaccharide, either systemically to animals and humans, or to cell cultures, almost universally decreases CYP expression (77). These results are a strong incentive to identify xenobiotic substances produced by Shigella, their mechanisms of sensing by IEC, and the signaling cascades and downstream transcriptional complex involved. Up-regulation of the transcription of the gene encoding the dioxin-inducible isozyme of CYP by 2.9× was also observed in the course of an infection of epithelial cells with Pseudomonas aeruginosa (78).

In a similar logic of cell protection against harmful molecules produced by both M90T and BS176, genes encoding apoptosis-regulating proteins, such as the caspase-like apoptosis-regulating protein CLARP (30) and two growth factors, ligands for EGFR receptor, amphiregulin (31) and TGF-α (33), were significantly induced. Amphiregulin is transcriptionally induced by oxidative stress, a possible link with Shigella infection (79). It is an autocrine/paracrine epithelial growth factor that was shown to be the most abundant EGFR ligand produced by Caco-2 cells (32). Amphiregulin secretion appears essentially basolateral in polarized cells and induces strong cell proliferation (32) but no significant differentiating effect (80). Amphiregulin also strongly induces secretion of the matrix metalloproteinases MMP-9 and MMP-2 in cancer cells (81) and is a potent antiapoptotic factor in non-small cell lung cancer cell lines (63). TGF-α is also an autocrine/paracrine growth factor acting primarily in the process of growth initiation by moving cells from the non-cycling state back into the cell cycle (69). Increased transcription of amphiregulin and TGF-α may reflect the neoplastic origin of Caco-2 cells. Similar experiments will be conducted on human epithelial strips obtained from biopsy specimens to confirm these findings. However, these results suggest that commensals and pathogens may influence the survival, proliferation, and attachment of cancer cells, thus enhancing the metastatic capacity of colonic tumors.

Induction of the VIP receptor (34) transcription may follow the same logic of a protective program that would also include increased transcription of DNA repair genes, such as the DNA repair exonuclease Rec1 (35) and the photolyase (36). This program may play a significant role in protecting IEC against apoptotic death, unlike macrophages that rapidly undergo apoptosis upon invasion by Shigella (61).

A last major point to be discussed is differential transcriptional regulation. Beyond the current uncertainties regarding what, in both invasive and non-invasive Shigella, up-regulates transcription of genes aimed at establishing a protective and stent support and encouragement in the course of this work, and Isabel Fernandez, Armelle Phalipon, Duna Philpott, and Régis Tournebize for critical review of the manuscript.

REFERENCES

1. Mathan, M. N., and Mathan, V. I. (1991) Res. Infect. Dis. 13, S314–S3318
2. Sanesnonetti, P. J. (2001) J. Immunol. 165, 410–416
3. Kepp, E. B., and Medzhitov, R. (1999) Curr. Opin. Immunol. 11, 13–18
4. Girardin, S. E., Tournebize, R., Mavris, A. M., Kim, J. M., Truong, F., Eckmann, L., and Kagnoff, M. F. (1999) J. Immunol. 163, 1457–1466
5. Neish, A. S., Gewirtz, A. T., Zeng, H., Young, A. N., Hobert, M. E., Karmali, M., and Mathison, J. C. (2000) J. Immunol. 164, 3214–3221
6. Mandic-Mullec, I., Weiss, J., and Zychlinsky, A. (1997) Infect. Immun. 65, 110–115
7. Jung, H. C., Eckmann, L., Yang, S. K., Panja, A., Fierer, J., Morzycka-Wroblewska, E., and Kagnoff, M. F. (1996) J. Clin. Invest. 98, 55–65
8. Thoma, P., Girardin, S., and Sanseonetti, P. J. (2001) Curr. Opin. Immunol. 13, 410–416
9. Kepp, E. B., and Medzhitov, R. (1999) Curr. Opin. Immunol. 11, 13–18
10. Girardin, S. E., Simon, P. O., Schmitt, C. K., Taylor, L. J., Hagedorn, C. H., O’Neill, A. D., Neish, A. S., and Madara, J. L. (2001) J. Clin. Invest. 110, 99–109
11. Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M., and Adedem, A. (2001) Nature 410, 1099–1103
12. Anderson, K. T., Vora, P., Faure, E., Thomas, L. S., Arnold, E. T., and Arditi, M. (2001) J. Immunol. 167, 1609–1616
13. Carr, B., and Podolsky, D. K. (2000) Infect. Immun. 68, 7010–7017
14. Philpott, D. J., Yamaoka, S., Israel, A., and Sanseonetti, P. J. (2009) J. Immunol. 183, 963–974
