Host microarray analysis reveals a role for the *Salmonella* response regulator *phoP* in human macrophage cell death

Corrella S. Detweiler*, Dolores B. Cunanan, and Stanley Falkow

Department of Microbiology and Immunology, Stanford University Medical Center, Stanford, CA 94305

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Bacterial pathogens manipulate host cells to promote pathogen survival and dissemination. We used a 22,571 human cDNA microarray to identify host pathways that are affected by the *Salmonella enterica* subspecies *typhimurium phoP* gene, a transcription factor required for virulence, by comparing the expression profiles of human monocytic tissue culture cells infected with either the wild-type bacteria or a *phoP*::*Tn10* mutant strain. Both wild-type and *phoP*::*Tn10* bacteria induced a common set of genes, many of which are proinflammatory. Differentially expressed genes included those that affect host cell death, suggesting that the *phoP* regulatory system controls bacterial genes that alter macrophage survival. Subsequent experiments showed that the *phoP*::*Tn10* mutant strain is defective for killing both cultured and primary human macrophages but is able to replicate intracellularly. These experiments indicate that *phoP* plays a role in *Salmonella*-induced human macrophage cell death.

Salmonellosis is caused by ingestion of contaminated food or water. The bacteria are resistant to the acidic environment of the stomach and usually invade or are phagocytosed by cells lining Peyer's patches in the small intestine (1). *Salmonella enterica* subspecies *typhimurium* replicates in macrophages; in mice, it is thought that either macrophages or dendritic cells carry the bacteria from the Peyer's patches to the adjacent lymph nodes, spleen, and liver (2). *S. typhimurium* can kill macrophages by a caspase-1-dependent mechanism that also releases proinflammatory cytokines (3–6). Caspase-1 activation correlates with the ability to colonize the lymph nodes, spleen, and liver in mice (7). In immunocompetent humans, *S. typhimurium* does not usually cause systemic disease, and bacterial replication is instead limited to the intestine, with resulting gastroenteritis (1). It is unclear why *S. typhimurium* causes different diseases in mice and humans. Whereas *S. typhimurium* can kill human macrophages, the role of macrophage cell death in human disease is not yet known.

S. typhimurium phoP::*Tn10* mutants are avirulent in mice (8). It is unknown whether *phoP* is required for human gastroenteritis, but *S. typhi phoP*::*Tn10* mutants fail to cause typhoid fever in humans (9). *phoP* is the DNA-binding partner of a two-component response regulatory system that is activated after the bacteria enter host cells and that regulates transcription of diverse bacterial genes. For instance, *phoP* activates transcription of genes such as *pmrA*/*B, pmrE, pmrF,* and *pagP*, whose products play a role in bacterial resistance to anti-microbial peptides by chemically modifying lipopolysaccharide (LPS; ref. 10). *phoP* also induces genes involved in magnesium transport (*mgtA, mgtCB*), and has been shown to play a role in bacterial resistance to bile (11, 12). In addition, *phoP* represses genes that are important for bacterial entry into epithelial cells, namely those in the *Salmonella* pathogenicity island 1 (SPI1) (13).

What is the host molecular response to the activities of *phoP*-regulated genes? It has been demonstrated that *phoP* expression affects host cell antigen processing and presentation, but the downstream mediators, in both the bacteria and the host, are unknown (14). Microarrays provide us with a new tool for identifying host molecular pathways that a virulence determinant affects by enabling comparative analysis of the host transcriptional response to infection with virulent and avirulent mutant bacteria. Here, we identify differentially expressed genes from a human monocytic cell line infected with either wild-type *S. typhimurium* or an isogenic *phoP*::*Tn10* mutant under conditions in which bacterial growth was comparable. Based on these data, we hypothesized that *phoP* plays a role in macrophage cell death. Subsequent cell biological experiments showed that, indeed, *phoP*::*Tn10* mutants are defective for killing human U-937 monocytes and peripheral blood mononuclear cells (PBMCs). Thus, the *phoP* regulatory system controls gene product(s) that promote the death of human macrophages.

Materials and Methods

Bacterial Strains and Growth Conditions. *Salmonella* strains are derived from SL1344 (15). The *phoP* mutant (*phoP*::*Tn10*Tet^R) has an insertion at the *phoP* locus (8). Strain P7G2 (*spiA*::*mTn5*K_m) has an insertion in the *spiA* gene, a putative outer membrane component of the SPI2 type III secretion system (16). BJ66 (*orgA*::*Tn10*Tet) has an insertion in *orgA*, a gene within SPI1, and is unable to cause mouse macrophage cell death within 2 hr of infection (6). Bacteria were grown overnight in LB without agitation at 37°C, diluted 1:50 in LB and grown 3 hr without agitation at 37°C (17). Before inoculation, bacteria were harvested by centrifugation and resuspended in PBS (for the microarray experiments) or diluted into PBS (all other experiments).

We confirmed the phenotype of the *phoP*::*Tn10* mutant strain by showing that it is unable to induce a known *phoP*-dependent promoter (*pmrE*/*pagA*) in N-minimal media with 8 μM magnesium, conditions under which the wild-type strain induced a *phoP*-regulated *pagA* promoter fused to a green fluorescent protein (GFP) reporter 5-fold relative to expression in 2 mM magnesium (data not shown) (11). Secondly, the *phoP*::*Tn10* mutant strain fails to replicate in the mouse macrophage RAW264.7 cell line, as previously reported (8, 18, 19).

Tissue Culture Infections and Bacterial Replication Assays. For the microarray experiments, 5.4×10^7 U-937 cells per T175 flask were treated with phorbol 12-myristate 13-acetate (PMA, 10 ng/ml) to promote differentiation and adherence. After 46 hr, there were $\approx 1.6 \times 10^8$ cells per flask, and the medium (RPMI 1640, 10% FCS) was replaced with fresh medium without PMA. At 48 hr, *S. typhimurium* was added at a multiplicity of infection

Abbreviations: PBMC, peripheral blood mononuclear cell; PMA, phorbol 12-myristate 13-acetate; moi, multiplicity of infection; cfu, colony-forming unit; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; LDH, lactate-dehydrogenase; DAPI, 4′,6-diamidino-2-phenylindole; LPS, lipopolysaccharide; STAT, signal transducers and activators of transcription; SSI-3, STAT-induced STAT inhibitor 3.

*To whom reprint requests should be addressed. E-mail:corried@leland.stanford.edu.

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(moi) of 4–12 colony-forming units (cfu) per cell. An equivalent volume of PBS was added to uninfected controls. Thirty minutes after inoculation, gentamicin (100 μg/ml) was added to kill extracellular bacteria. Two hours after inoculation, the medium was replaced with medium containing 10 μg/ml gentamicin. Adherent cells were harvested at 0.5, 1, 2, 3, and/or 4 hr (time 0 is the time at which bacteria were added) after infection by scraping in 2 ml PBS and centrifugating for 2 min at 200 $\times g$. The cell pellet was frozen in liquid nitrogen.

For the bacterial replication assays, 1×10^5 cells were seeded into 24-well dishes and infected in parallel with the T175 flasks at an moi of 10. Adherent cells were collected at 2 and 24 hr, lysed with 1% Triton X-100 for 5 min with vigorous pipetting, and plated for cfu (6, 17). The replication assay was repeated on four independent occasions, each in triplicate.

In all experiments, the bacteria were grown under conditions that allow for activity of the SPI1 type III secretion system, which secretes a cytotoxic protein(s) into host cells. However, by 4 hr, only a quarter of the wild-type-infected U-937 cells were dead as determined by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining (Fig. 3). Additionally, 4 hr after infection with wild-type *Salmonella*, we could consistently isolate reasonable amounts of high quality (as determined by distribution on an agarose gel) U-937 mRNA (data not shown).

RNA Isolation, Labeling, and Hybridization. Cell pellets were thawed and homogenized in 2 ml Trizol (GIBCO/BRL, Germany) for 30 s with a Polytron PT 1200C (Kinematica, Littau-Lucerne, Switzerland), and total RNA was purified according to the manufacturer's instructions. Labeled cDNA was synthesized from 20 μg of RNA with an oligo(dT) primer and hybridized to a sequence-verified 22,571 spotted cDNA microarray on glass slides (20). The reference RNA we used was poly(A)$^+$ RNA derived from a mixture of U-937 cells and the AGS human gastric tissue culture cell line (American Type Tissue Culture Collection). This reference RNA was labeled as previously described and hybridized to each array to allow for comparisons between different experiments (20). The hybridized slides were scanned and analyzed with a Gene Pix Scanner 4000A and the GENEPIX program (Axon Instruments, Foster City, CA).

Data Analysis. Data were analyzed with the Stanford University Microarray Database, Microsoft EXCEL and ACCESS, and the SIGNIFICANCE ANALYSIS FOR MICROARRAYS (SAM) program (http://www-stat-class.stanford.edu/SAM/SAMServlet; ref. 21). mRNAs for which at least 75% of the spots had a regression correlation greater than 0.6 were used in analysis (22). Missing data points were estimated with a *K*-Nearest-Neighbor imputator, where *K* equaled 10 (21).

To determine which mRNAs differed in the wild-type vs. uninfected samples, SAM was run on the mean of the Log_2 red/green normalized ratio where the time points from all experiments were grouped together. Arrays were paired according to experiment and time point. For example, the wild-type strain 2-hr time point from the third experiment was paired with the uninfected 2-hr time point from the third experiment. The differences in transcript levels between wild-type strain and *phoP*::*Tn10* mutant strain-infected samples, and the *phoP*::*Tn10* mutant strain vs. uninfected samples were calculated similarly. SAM calculates a list of significant mRNAs and a false discovery rate (FDR), which is an estimate of the percentage of false positives. The relative levels of gene expression and standard errors reported in Tables 1 and 2 are the SAM-generated r_i and $(s_i + s_o)$ values, respectively, where s_i is the standard deviation and s_o is an estimate of error (21). Data from all of the arrays used in this paper are available at http://genome-www4.stanford.edu/MicroArray/SMD/.

Table 1. mRNAs that are more abundant in wild-type-infected and *phoP*::*Tn10* mutant-infected than in uninfected macrophages*

	Ratio†	SE	Ratio‡	SE
Inflammatory				
IL-8, C-X-C chemokine	3.1	1.2	2.3	1.4
GRO1, C-X-C chemokine§	7.0	1.2	8.6	1.3
GRO1, C-X-C chemokine§	7.9	1.3	5.5	1.6
GRO2, C-X-C chemokine	7.2	1.3	6.5	1.6
Mip-1-α, C-C chemokine	4.1	1.3	2.3	1.9
Mip-1-β, C-C chemokine	7.8	1.5	3.9	2.2
IL-1-β, cytokine§	4.8	1.3	6.4	1.5
IL-1-β, cytokine§	4.3	1.3	3.5	2.0
IL-23 p19 subunit, IL-6 family	2.4	1.2	2.6	1.6
Inhibin, beta A (activin A)	3.5	1.2	4.4	1.4
Prostaglandin-endoperoxide synthase 2	7.4	1.3	6.7	1.5
Plasminogen activator inhibitor, type II	3.4	1.2	3.2	1.5
Transcription factor AP-1 subunit (Jun)	3.0	1.2	2.1	1.7
Transcription factor Spi-B	3.1	1.2	2.1	1.4
Cell death/survival				
NF-κB inhibitor (IKBA) (NKBI)	3.3	1.3	2.6	1.7
TNF receptor-associated factor 1 (TRAF1)	2.4	1.2	2.5	1.4
PRG1 (GLY96, DIF2, IEX1)	3.7	1.3	3.2	1.6
Other				
B-cell derived transcription factor	2.0	1.3	2.0	1.6
Connexin 26, gap junction protein	6.0	1.3	4.0	1.5
Connexin 26, gap junction protein	3.5	1.2	2.2	1.9
Superoxide dismutase 2, mitochondrial§	6.4	1.2	6.2	1.5
Superoxide dismutase 2, mitochondrial§	6.7	1.2	4.9	1.6
Transcription factor AP-2 alpha	4.3	1.3	3.4	1.6
Trinucleotide repeat containing 3§	2.6	1.2	2.8	1.4
Trinucleotide repeat containing 3§	2.7	1.2	3.1	1.5
Phorbol myristate acetate-induced protein	5.6	1.3	4.5	1.5
Similar to HMG-box transcription factor	2.6	1.2	2.0	1.4

*The false positive rate was 0.0% for the wild-type-infected vs. uninfected, and 9.3% for the *phoP*::*Tn10* mutant-infected versus uninfected.

†Relative expression levels of wild-type-infected macrophages and uninfected macrophages.

‡Relative expression levels of *phoP*::*Tn10* mutant strain-infected macrophages and uninfected macrophages.

§Two different cDNAs on the array represent the same gene.

Northern. Northerns were performed on the same RNA samples used in the array experiments following standard protocols (23). Twenty micrograms of RNA were loaded per well. Blots were analyzed on a Molecular Dynamics PhosphorImager. GPD-1 (glycerol-3-phosphate dehydrogenase 1) was chosen as a loading control because the array data indicated that levels of this mRNA varied little between samples (data not shown). The Northern probes were identical to the cDNAs spotted onto the array.

Cell Death Assays. For the lactate-dehydrogenase (LDH) release assays, 1×10^5 macrophages were seeded in 24-well dishes and infected with bacteria (moi 10) as described above. Twenty-four hours after infection, LDH release was measured with the Promega CytoTox 96 kit according to the instructions by using a Bio-Tek Instruments (Burlington, VT) plate reader at 490 nm. The LDH experiments were performed in triplicate on three separate occasions.

For the TUNEL-fluorescence-activated cell sorter (FACS) assays, 1×10^6 macrophages were seeded in 6-well dishes and infected with bacteria (moi 10) as described above. Twenty hours after infection, the media and two washes were collected and combined with cells lifted with 0.5 mM EDTA in PBS. Cells were harvested by centrifugation, fixed in 1% formaldehyde for 1 hr

Table 2. mRNAs that are more abundant in wild-type-infected than in *phoP*::*Tn10* mutant-infected macrophages*

	Ratio†	SE
Inflammatory		
CD9 antigen (p24)	2.1	1.7
E74-like factor 1 (ets domain transcription factor)	2.5	1.5
Leukocyte immunoglobulin-like receptor (ILT3)	1.9	1.4
Cathepsin D (lysosomal aspartyl protease)	2.1	1.4
Cell death/survival		
STAT-induced STAT inhibitor 3 (SSI-3)	2.2	1.5
PIM-1 serine/threonine kinase	2.0	1.5
Tumor necrosis factor-α-induced protein 3 (A20)	1.9	1.7
Cell cycle		
Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	2.0	1.5
v-myb viral oncogene homolog-like 2	1.9	1.4
Dihydrofolate reductase	1.9	1.4
Other		
Thrombospondin 1; ECM, secreted, TGFB activator	1.9	1.5
Contactin 1, cell adhesion	2.0	1.6
Solute carrier family 15 (H+/peptide transporter)	3.0	1.7
Human rhotekin mRNA, Rho GTPase inhibitor	2.2	1.6
Carbonic anhydrase-related protein 10	2.6	1.5
Ceruloplasmin (ferroxidase) copper scavanger	1.9	1.4
UDP glycosyltransferase 2 family, polypeptide B4	1.9	1.6
Fatty acid binding protein 4, adipocyte	2.6	2.2
Fibroblast activation protein-α	2.0	1.7
α-methylacyl-CoA racemase	2.3	1.6
Suppression of tumorigenicity 14	2.1	1.7

*False positive rate = 5.9%.
†Relative expression levels of wild-type-infected macrophages and *phoP*::*Tn10* mutant-infected macrophages.

at room temperature, washed 3× with PBS, permeabilized in 0.25% Triton X-100 and 0.25% sodium citrate for 1–2 min at 4°C, washed 3× in PBS, and stained for TUNEL, according to kit instructions (Roche *In Situ* Cell Death Detection Kit, Germany). Cells were analyzed on a Becton Dickinson FACSCalibur and gated according to size. Samples were done in triplicate on three separate occasions, and a minimum of 800 events were counted per sample. TUNEL-positive cells were defined by comparing the amount of TUNEL staining to cells incubated in the reaction buffer without the enzyme.

For the fluorescence microscopy assay, 1×10^5 U-937s or PBMCs were harvested, seeded onto cover slips in 24-well dishes, and infected with bacteria (moi 10) as described above (24). Four hours after infection, cells were fixed in 1% formaldehyde, permeabilized in 1% T Triton X-100 and 1% sodium citrate for 1–2 min at 4°C, and stained with the TUNEL kit. Cover slips were mounted in media containing 1.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Samples were prepared in triplicate on three separate occasions, and a minimum of 300 cells (as determined by DAPI staining and light microscopy) were counted per coverslip.

Results

***phoP*::*Tn10* Mutant *Salmonella* Replicate in U-937s.** There are disparate reports on the ability of wild-type *S. typhimurium* to survive in PMA-treated U-937s (25, 26). We began our study by asking whether wild-type and *phoP*::*Tn10* mutant *S. typhimurium* strains replicate intracellularly under the experimental conditions used in our laboratory. We infected PMA-treated U-937 cells with wild-type and *phoP*::*Tn10* mutant *Salmonella* and measured bacterial cfu 2 and 24 hr after infection. Fig. 1 shows that, in U-937s, both the wild-type and *phoP*::*Tn10* mutant strains invaded and replicated, consistent with the results of Baker *et al.* (26). The U-937 cell line was not permissive for all *Salmonella* mutants; a *spiA*::*mTn5* mutant strain, which lacks part of the SPI2 type III secretion machinery required for intracellular replication in all known cell types, failed to replicate in U-937 cells by 24 h (Fig. 1; ref. 27).

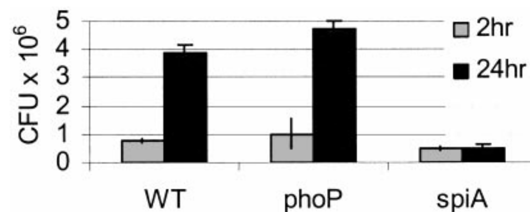

Fig. 1. Wild-type and *phoP*::*Tn10* mutant *Salmonella* replicate in PMA-treated U-937s. At 2 (gray bars) and 24 (black bars) hr after infection, cells were lysed, and bacterial cfu were plated. WT, wild-type; phoP, *phoP*::*Tn10* mutant; spiA, *spiA*::*Tn5* mutant.

To confirm that the *phoP*::*Tn10* mutant bacteria maintained the tetracycline-marked transposon over the course of our experiments, performed in the absence of tetracycline, we assayed several individual colonies recovered after 24 hr of intracellular growth: 36 colonies of 36 tested were tetracycline resistant (data not shown). We conclude that the recovered bacteria retained the transposon and that the *phoP*::*Tn10* mutant can replicate at wild-type levels in PMA-treated U-937s.

Expression Profiles of *Salmonella*-Infected Vs. Uninfected Tissue Culture Cells. We first profiled the transcriptional response of human tissue-culture macrophages to infection with wild-type *Salmonella* by comparing the expression profiles of wild-type-infected and uninfected U-937 cells by using a 22,571 human spotted cDNA microarray. PMA-activated U-937 macrophages were incubated with wild-type *S. typhimurium* or PBS for 30 min, and cells were harvested at 0.5, 1, 2, 3, and 4 hr after infection. Four independent experiments were performed. RNA was isolated, converted to labeled cDNA, and hybridized to the human arrays. Twenty-two of the RNA samples were independently labeled and hybridized to two different arrays to test reproducibility; the median correlation coefficient of the $\log_2$ red/green normalized ratio was 0.86 ± 0.18.

The data from wild-type-infected cells were compared with the uninfected control by using the SAM program (see *Methods*). Time points were pooled to improve statistical significance. Sixty-eight mRNAs had a 2-fold or more difference in level. Fifty-five of the mRNAs had known or ascribable functions. Table 1 shows a subset of the mRNAs expressed at higher levels in wild-type *Salmonella*-infected cells that have known or putative functions. A complete version of Table 1 can be viewed in Table 3, which is published as supplemental data on the PNAS web site, www.pnas.org. An interesting set of genes in Table 1 includes those induced by the transcription factor NF-κB, such as the cytokine IL-1, the chemokine IL-8, an NF-κB inhibitor, and the growth regulator PRG1 (28, 29). The NF-κB pathway is induced in response to bacterial LPS, and its downstream mediators promote an acute inflammatory response and affect cell survival (28). Other inflammatory mediators induced by the infected macrophages included Jun, prostaglandin synthase, and inhibin A. These results agree well with previously published reports, in which individual gene products were assayed or smaller arrays were used, and reflect the ability of Gram-negative bacteria to stimulate innate immunity (30–32).

A *phoP*::*Tn10* Mutant Strain Elicits Expression of Many of the Same Macrophage mRNAs as Wild-Type *Salmonella*. To identify host pathways that the *phoP* gene may affect, we examined the

expression profiles of tissue culture macrophages infected with either a wild-type *Salmonella* strain or the *phoP*::*Tn10* mutant strain. RNA was harvested from U-937 cells infected with a wild-type *Salmonella* strain, a *phoP*::*Tn10* strain, or no bacteria, over replicate 4-hr time courses. A comparison between *phoP*::*Tn10* mutant *Salmonella*-infected and uninfected macrophages with the SAM program revealed that the *phoP*::*Tn10* mutant strain elicited the expression of 36 mRNAs by 2-fold or more. The *phoP*::*Tn10* mutant strain elicited many of the same mRNAs as the wild-type bacteria, as 33 of the 36 mRNAs (91.7%) that were more highly expressed in *phoP*::*Tn10*-infected macrophages were also more highly expressed in the wild-type-infected macrophages, relative to uninfected cells (Table 1). Also, all but one [macrophage inflammatory protein-1β (Mip-1β)] of the 33 mRNAs were induced to within 1 SD of the wild-type-infected macrophage expression levels. This analysis indicates that, overall, the inflammatory response of U-937 macrophages to wild-type *Salmonella* and the *phoP*::*Tn10* mutant strain is similar.

A *phoP*::*Tn10* Mutant *Salmonella* Fails to Elicit Host Cell Induction of Some Cell Death and Cell Cycle Genes. On direct comparison of the wild-type strain and the *phoP*::*Tn10* mutant-infected macrophage data, we did not find any mRNAs whose abundance was lower in wild-type *Salmonella*-infected cells than in *phoP*::*Tn10* mutant-infected cells. However, we identified 34 mRNAs with expression levels that were 1.9-fold lower in *phoP*::*Tn10*-infected macrophages than in wild-type-infected macrophages. Twenty-one of these mRNAs had known or putative functions (Table 2). Two of the known mRNAs affect antigen presentation, a process that *phoP* is known to interfere with (14). Cathepsin D is a lysosomal protease important for MHC peptide presentation (33). ILT3 is a cell surface molecule of the Ig superfamily that can both bind antigens and interfere with intracellular signaling to block cell activation by other surface molecules (34).

Nearly one-third of the twenty-one known genes that were differentially expressed in wild-type vs. *phoP*::*Tn10*-infected monocytes are known to play roles in cell death, the cell cycle, or both (Table 2). SSI-3 (STAT-induced STAT inhibitor 3) encodes one of a family of proteins that suppress apoptosis via the signal transducers and activators of transcription (STAT) proteins, and SSI-3 itself has been shown to block cytokine-induced apoptosis (35, 36). PIM-1 is a kinase that protects against apoptosis and functions downstream of STAT family members (37–39). A20 is a zinc-finger protein that regulates cell death and limits inflammation by inhibiting NF-κB activity (40). The *p27/Kip1* gene product promotes G_1 cell cycle arrest in certain situations, such as upon *Helicobacter pylori* infection, that are followed by cell death (41, 42). The *v-myb* gene product is a member of the myb family of proteins, which are transcription factors that affect both cell cycle progression and cell death (43).

Northern Analysis of mRNA Samples. To confirm our microarray expression data by an independent method, we assayed by Northern analysis the relative abundance of two mRNAs that, according to the SAM analysis, had different expression profiles in wild-type and *phoP*::*Tn10*-infected macrophages. Fig. 2 shows the mRNA levels of SSI-3 and PIM-1, as quantified by array (*Left*) or Northern (*Right*) hybridization at the 2- through 4-hr time points. Whereas the Northern and array data show quantitative differences in relative expression levels, they agree that SSI-3 and PIM-1 are expressed at higher levels in wild-type-infected cells than in either *phoP*::*Tn10* mutant-infected or uninfected cells.

The *phoP*::*Tn10* Mutant *Salmonella* Kills Fewer Tissue Culture Macrophages than Wild-Type *Salmonella*. The array data showed that U-937 macrophages infected with the *phoP*::*Tn10* mutant do not express genes associated with cell death relative to that seen during wild-type infection. We therefore asked whether the *phoP*::*Tn10* mutation affects U-937 survival during *Salmonella* infection. We monitored cell death 4 hr after infection, by which time wild-type *Salmonella* infection is known to cause some macrophage cell death (data not shown; refs. 3 and 6). A TUNEL assay, which detects double-stranded breaks in chromosomal DNA, was used to quantify the percentage of dying cells. Fig. 3 shows the results of TUNEL staining monitored by FACS (Fig. 3*A*) or fluorescence microscopy (Fig. 3*B*). The results of both assays showed that about half as many macrophages were killed by infection with *phoP*::*Tn10* mutant *Salmonella* as with wild-type by 4 hr. Furthermore, the *phoP*::*Tn10* mutant strain promoted as little cell death as an *orgA*::*Tn10* mutant strain, which is defective for SPI1-mediated host cell killing in mouse macrophages (6).

Macrophage cell death was also monitored at later times after inoculation to see whether the difference in the relative levels of

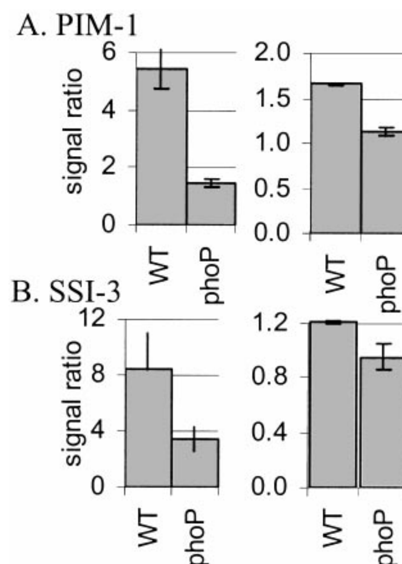

Fig. 2. Comparison of array and Northern data depicted as levels of mRNA in infected relative to uninfected macrophages. RNA from one of the array experiments was processed for Northern and probed with labeled PIM-1 or SSI-3 cDNA. (*A*) PIM-1 array (*Left*) and Northern (*Right*) data. (*B*) SSI-3 array (*Left*) and Northern (*Right*) data. The Northern data were normalized to a glycerol-3-phosphate dehydrogenase 1 (GPD-1) Northern. Data are expressed as the geometric mean of the bacterial-infected vs. uninfected macrophage ratios from the 2- to 4-hr time points. WT, wild-type-infected macrophages; phoP, *phoP*::*Tn10*-infected macrophages.

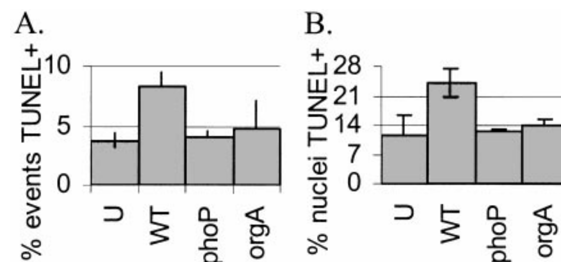

Fig. 3. A *phoP*::*Tn10* mutant killed fewer U-937 macrophages than wild-type *S. typhimurium* by 4 hr after infection. (*A*) Suspended and adherent cells were harvested and processed for FACS. The percentage of TUNEL-positive events is shown. (*B*) Adherent U-937s were TUNEL and DAPI stained for fluorescence microscopy. U, uninfected; WT, wild-type; phoP, *phoP*::*Tn10* mutant; orgA, *orgA*::*Tn10* mutant.

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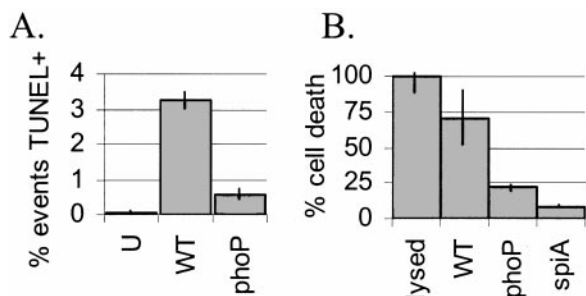

Fig. 4. A *phoP*::*Tn10* mutant killed fewer U-937 macrophages than wild-type *S. typhimurium* by 20 or 24 hr after infection. (*A*) Suspended and adherent cells were harvested and processed for FACS 20 hr after infection. The percentage of TUNEL-positive events is shown. (*B*) LDH release was measured 24 hr after infection. Results are presented as the percent cell death, where 100% is the amount of LDH in uninfected lysed cells. lysed, uninfected lysed; WT, wild-type; phoP, *phoP*::*Tn10* mutant; spiA, *spiA*::*Tn5* mutant.

wild-type and *phoP*::*Tn10* mutant *Salmonella*-mediated killing increased over the course of infection. We quantified the number of TUNEL-positive cells by FACS 20 hr after infection and found that wild-type-infected macrophages had approximately four times more TUNEL-positive events than *phoP*::*Tn10* mutant *Salmonella* (Fig. 4*A*). The number of TUNEL-positive events in wild-type-infected macrophages was lower at 20 hr than at 4 hr (Fig. 3*A*), likely because cells die and disintegrate throughout the 20-hr incubation. We used a different assay to measure cell death 24 hr after infection. The amount of the eukaryotic cytoplasmic enzyme LDH released into the media reflects the fraction of cells with damaged plasma membranes. Wild-type *Salmonella*-infected macrophages released approximately three times more LDH than *phoP*::*Tn10*-infected macrophages (Fig. 4*B*). Thus, *phoP* is an important factor in *Salmonella*-induced U-937 cell death.

***phoP* Is Important for Killing Human PBMCs.** To determine whether *phoP* plays a role in the death of primary human macrophages, we isolated PBMCs from healthy donors and counted the number of TUNEL-positive cells 4 hr after infection with wild-type and *phoP*::*Tn10* mutant *Salmonella*. We found that PBMCs, like the U-937 tissue culture cells, were less likely to die upon infection with the *phoP*::*Tn10* mutant bacteria than with wild-type *Salmonella* (Fig. 5).

Discussion

We used a spotted cDNA microarray to analyze *Salmonella* infection of the human monocytic U-937 cell line. It was not clear at the outset of these experiments whether it would be possible to distinguish between the response of tissue culture cells to wild-type *S. typhimurium* and avirulent *phoP*::*Tn10* mutant strains. Indeed, a recent study using a smaller array to compare the responses of macrophages incubated with live *Salmonella* vs. purified LPS did not detect any differences (31).

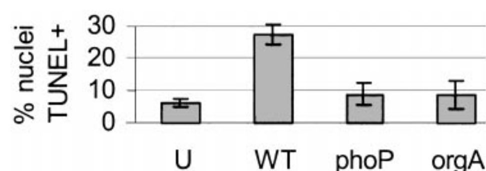

Fig. 5. A *phoP*:: *Tn10* mutant strain is defective for killing human PBMCs. Adherent PBMCs were TUNEL and DAPI stained for fluorescence microscopy 4 hr after infection. U, uninfected; WT, wild-type; phoP, *phoP*::*Tn10* mutant; orgA, *orgA*::*Tn10* mutant.

We first analyzed the expression profile of macrophages infected with wild-type bacteria relative to uninfected macrophages. Only 68 mRNAs of the 22,571 mRNAs on the array had significantly different levels of expression (Table 1). Most of the mRNAs elicited by wild-type *Salmonella* encode genes whose products function in the innate immune response. In addition, inflammatory-response mRNAs tended to be more abundant than genes that function in other processes. These results agree well with a recent study that examined expression of 588 mouse macrophage cDNAs by using a membrane-based array. However, our experiments identified a much lower percentage of differentially abundant mRNAs (0.3% vs. 13%), perhaps because our array was not specifically enriched for genes involved in immunity, a feature that enabled us to survey noninflammatory as well as inflammatory macrophage responses (31).

We then examined the molecular phenotype of *phoP*::*Tn10* mutant-infected macrophages. Over 90% of the macrophage mRNAs induced by the *phoP*::*Tn10* mutant *Salmonella* were also induced by the wild-type strain, indicating that, overall, both strains elicit similar inflammatory responses. These results are consistent with experiments showing that LPS isolated from a *phoP*::*Tn10* mutant strain stimulates monocyte innate immune responses *in vitro* (44).

Direct comparison of array data from *phoP*::*Tn10* mutant *Salmonella*-infected cells to wild-type-infected cells revealed 21 mRNAs of known or ascribable function that were expressed at higher levels in wild-type *Salmonella*-infected macrophages than in *phoP*::*Tn10* mutant-infected macrophages (Table 2). Two of these mRNA gene products affect antigen presentation, a process in which *phoP* has been implicated (14). Five of the mRNAs can play roles in cell death. Whereas the differences we observed in mRNA levels between wild-type- and *phoP*::*Tn10*-infected macrophages were only 2-fold, it should be noted that relatively small changes in mRNA levels can significantly affect cell physiology. For example, small differences in either *bcl-xL* or *survivin* mRNA levels resulted in 4- to 5-fold increases in the number of dead tissue-culture breast carcinomal- or lung adenocarcinomal-derived cells, respectively (45, 46). Furthermore, we were most interested not in individual mRNAs that changed, but in groups of mRNAs that function in the same physiological process and were differentially expressed. Thus, based on the observation that five mRNAs that can play roles in cell death were differentially expressed between wild-type-infected macrophages and *phoP*::*Tn10*-infected macrophages, we pursued the hypothesis that *phoP* affects cell death and demonstrated that *phoP* is required for *Salmonella*-induced human macrophage cell death (Figs. 3–5).

How does *phoP* mediate human macrophage cell death? During or shortly after *S. typhimurium* entry into host cells, the protein SipB is secreted into the host cell cytosol via the SPI1 type III secretion system (3, 4). SipB promotes macrophage cell death by cleaving and thus activating caspase-1, which triggers a cell death proteolytic cascade (4). A second type III secretion system, located in SPI2, has also been implicated in cell death. The SPI2 type III secretion system is activated after *Salmonella* have entered the phagosome and has been recently shown to promote mouse macrophage cell death by 12 h after infection (47). It is unknown which SPI2-secreted effecter(s) is involved. There are at least two ways by which *phoP* could mediate macrophage cell death via a type III secretion system. First, it is possible that *phoP*-regulated genes affect both SPI1 and SPI2 macrophage cell death, as a *phoP*-regulated outer membrane protein or LPS modification could be important for the translocation of effecter molecules by the type III secretion systems. Second, it has recently been shown that *phoP* positively regulates expression of *srfJ*. This gene encodes a protein homologous to eukaryotic lysosomal glucosyl ceramidases, which can play a role in host cell death. It has been proposed that the SrfJ protein is

secreted into the cytosol of host cells, either by SPI2 or an alternative export system, where it could possibly play a role in host cell death (48). Finally, the *phoP* role in cell killing could also be independent of SPI1 and SPI2. For example, human macrophages may detect a *phoP*-regulated outer membrane protein or an LPS modification and respond by activating a cell death pathway.

What is the role of macrophage death in infection? In mice, cell death is correlated with establishing deep tissue infection, as caspase-1$^{-/-}$ mice are resistant to oral infection with *S. typhimurium*. The bacteria can colonize the Peyer's patches, but are impaired in disseminating to the mesenteric lymph nodes, liver, and spleen (7). Caspase-1-mediated cell death is accompanied by local inflammation, which results in the recruitment of inflammatory cells, and *Salmonella* may use macrophages and dendritic cells as a conduit to deeper tissue (2). It is unknown whether SPI2- or *phoP*-mediated cell death utilizes caspase-1 or promotes inflammation.

The experiments described in this report used microarrays to profile the transcriptional response of macrophages infected with *Salmonella.* It is not surprising that inflammatory genes constitute the major class of mRNAs that macrophages express in response to *Salmonella*. Indeed, the host reaction to most infectious agents is a generalized response mediated by the innate immune system and causing fever, malaise, and anorexia. In contrast, by directly comparing the expression profiles of wild-type and avirulent mutant *Salmonella*-infected macrophages, we dissected out a host pathway that a bacterial virulence determinant acts on. We then confirmed the relevance of this pathway with conventional cell biological methods. By using this approach, where two nearly identical pathogen strains are compared in a defined model system, we should be able to learn considerably more about the host pathways that individual virulence determinants affect. Thus, host microarrays will become particularly powerful tools for pathogenesis.

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