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Salmonella typhimurium Infection and Lipopolysaccharide Stimulation Induce Similar Changes in Macrophage Gene Expression

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Changes in macrophage phenotype induced during infection result from the recognition of bacterial products as well as the action of bacterial virulence factors. We used the unprecedented opportunity provided by gene arrays to simultaneously study the expression of hundreds of genes during Salmonella typhimurium infection of macrophages and to assess the contribution of the bacterial virulence factor, LPS, in initiating the host responses to Salmonella. We found that S. typhimurium infection caused significant changes in the expression of numerous genes encoding chemokines, cell surface receptors, signaling molecules, and transcriptional activators at 4 h postinfection of the RAW 264.7 murine macrophage cell line. Our results revealed changes in the expression of several genes that had not been previously implicated in the host responses to S. typhimurium infection, as well as changes in the expression of several genes previously shown to be regulated by S. typhimurium infection. An overlapping spectrum of genes was expressed in response to virulent S. typhimurium and purified S. typhimurium LPS, reinforcing the major role of this surface molecule in stimulating the early response of macrophages to bacterial infection. The macrophage gene expression profile was further altered by activation with IFN-γ, indicating that host cell responses depend on the activation state of the cell. The Journal of Immunology, 2000, 164: 5894–5904.

In the in vivo mouse model of human typhoid fever, IFN-γ is released by NK and T cells 2–3 days following S. typhimurium infection. IFN-γ is a potent stimulator of macrophage gene expression and is necessary for clearance of S. typhimurium and other intracellular bacteria (6–8). A variety of studies, including the use of gene arrays, have supplied a wealth of data regarding differential gene expression in response to IFN-γ stimulation (9, 10). These pleiotropic effects on gene expression translate into alterations of receptor expression, Ag presentation, phagocytosis, cell proliferation, metabolism, and the antimicrobial oxidative and NO burst (11, 12). While IFN-γ is thought to prime the macrophage to respond more rapidly and effectively against invading pathogens, the spectrum of genes whose expression is altered during bacterial infection in unprimed vs IFN-γ-primed cells has not been extensively analyzed. Investigating how IFN-γ activation alters the ability of S. typhimurium to affect macrophage gene expression may lead to the identification of genes that contribute to IFN-γ’s critical role during S. typhimurium infection.

Macrophages have evolved the ability to recognize bacterial products and initiate an immune response to clear the microbe. An innate pattern of macrophage response is triggered by conserved bacterial products such as LPS, porins and other outer membrane proteins, fimbrial proteins, flagella, lipoproteins, glycoproteins, and peptidoglycan (13). These bacterial components, termed modulins, signal through CD14 or other pattern recognition receptors to modulate overlapping as well as unique host cell gene expression. These signals help to initiate the innate and specific immune responses to clear the bacterial infection (14, 15). The bacterial surface component LPS is a potent immunostimulatory molecule that initiates both rapid changes in macrophage signaling pathways and adaptive changes in macrophage gene expression. LPS alters the expression of a variety of genes including transcription factors, cytokines, chemokines, receptors, and cationic antimicrobial peptides (16–19). Other structural components of
Salmonella such as porins and flagella induce cytokine gene expression independently of LPS (20–22). To promote their survival, bacterial pathogens such as S. typhimurium secrete specialized protein effectors that induce alterations in host cells responses (23). These effectors specifically affect host cell functions such as cytoskeletal architecture, vesicle trafficking, cell signaling, and apoptosis to create a more hospitable intracellular niche (24–28). Most studies to date have shown how bacterial effectors modify existing host proteins rather than examining how host gene transcription is affected.

One way to analyze both the complex interactions between host and pathogen as well as the priming effects of IFN-γ is with a general approach such as gene arrays. Gene array technology has recently been used for a more global view of differential gene expression in such fields as inflammatory diseases (29), tumor biology (30), human cytomegalovirus infection (31), superantigen stimulation of T cells (32), S. cerevisiae metabolism (33), and genetic variability of Mycobacterium tuberculosis (34, 35). One proven strength of this experimental approach has been the ability to study the expression of hundreds of genes simultaneously without biasing conclusions drawn from a subset of genes presumed to be involved in a particular process. We capitalized on gene array technology to obtain, for the first time, a more comprehensive picture of how host gene expression is altered during infection by a pathogenic bacterium. Differential host cell gene expression was examined in an in vitro model of S. typhimurium infection using the RAW 264.7 murine macrophage cell line, a common model for the intracellular growth of S. typhimurium. Gene arrays were used to test two hypotheses: 1) that most of the gene expression changes in macrophages infected by S. typhimurium can be induced by LPS, the major constituent of S. typhimurium outer membranes, and 2) that the priming of macrophages by IFN-γ alters the spectrum of genes induced by S. typhimurium infection. We found that S. typhimurium infection altered the expression of a large number of macrophage genes and that an individual virulence factor, LPS, could itself cause many of the same changes in host gene expression. The macrophage gene expression profile following infection was altered by priming with IFN-γ, revealing how host cell activation state alters macrophage responses to bacterial infection at the molecular level.

Materials and Methods

Bacterial and cell culture strains and growth conditions

The S. typhimurium strain SL1344 was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and grown in Luria-Bertani broth. For macrophage infections, highly invasive bacterial cultures were prepared by diluting an overnight culture 1:34 in Luria-Bertani broth and subculturing aerobically with shaking for 3 h at 37°C. The murine macrophage cell line RAW 264.7 (ATCC) was maintained in DMEM (Life Technologies, Burlington, ON) supplemented with 10% FBS (Life Technologies, Burlington, ON) and enriched with antibiotics at a final concentration of 100 μg/ml gentamicin (Sigma, St. Louis, MO) for 24 h before infection.

Infection conditions

For immunofluorescence studies and bacterial colony counts, 24-well plates were seeded with 2.5 × 10^5 RAW 264.7 cells per well. Bacteria were diluted in culture medium to give a nominal multiplicity of infection (MOI) of ~20. Invasion was allowed to proceed for 10 min in a 37°C, CO2 incubator. Cells were washed twice with PBS to remove extracellular bacteria and then incubated in DMEM plus 10% FBS containing 50 μg/ml gentamicin (Sigma, St. Louis, MO) to kill any remaining extracellular bacteria and prevent reinfection. After 2 h, the gentamicin concentration was lowered to 5 μg/ml. Colony counts and immunofluorescence were subsequently performed in parallel to compare the variability in the actual number of intracellular bacteria per cell with the average number per cell for the population, as determined by colony counts. To determine invasion efficiency, samples of cells were washed twice with PBS to remove gentamicin and lysed with 1% Triton X-100/0.1% SDS in PBS at 2 h postinfection. Numbers of intracellular bacteria were calculated by colony counts. At various times postinfection, immunofluorescence was performed as described previously (36) using a rabbit polyclonal anti-LPS Ab diluted 1:200 (S. typhimurium O Ag group B factors 1, 4, 5, and 12; Difco, Detroit, MI) and Alexa 488-conjugated mouse anti-rabbit secondary Ab diluted 1:400 (Molecular Probes, Eugene OR). Cells were counted within randomly selected fields. Consistently, macrophages were infected by an average of one to three bacteria per cell as assessed by standard plate counts and immunofluorescence studies.

RNA isolation

RAW 264.7 macrophage cells were seeded at 5.6 × 10^5 cells in 20 ml media per 150 mm diameter tissue culture dishes and cultured overnight. RAW 264.7 macrophages were infected with S. typhimurium at an MOI of 20 or stimulated with 100 ng/ml S. typhimurium LPS (Sigma) for 4 h. After stimulation, the culture medium was removed for measurement of cytokine production. The cells were washed once with distilled pyrogen-free treated PBS and scraped to detach the cells from the dish. RNA was then isolated using TRIzol (Ambion, Austin, TX) according to the manufacturer’s directions. The RNA pellet was resuspended in RNase-free water containing RNase inhibitor (Ambion, Austin, TX). Contaminating genomic DNA was removed using DNaseI (Clontech, Palo Alto, CA) in the presence of 50 U RNase inhibitor for 1 h at 37°C. The reaction was stopped by adding 1/10 volume 10× termination mix (0.1 M EDTA, pH 8.0, 1 mg/ml glycerol) and extracted twice with phenol/chloroform/isomyl alcohol (25:24:1) and once with chloroform. The RNA was then precipitated with 2.5 volumes 100% ethanol and 1/10 volume sodium acetate, pH 5.2, resuspended in RNase-free water with RNase inhibitor, and stored at −70°C in aliquots to minimize freeze-thaw cycles. Thirty micrograms of total RNA, as determined by OD260 reading, was routinely isolated from one 150-mm dish of cells. The quality of the RNA was assessed by gel electrophoresis and ethidium bromide staining. The RNA purity was confirmed by using the isolated RNA as a template for PCR amplification using β-actin-specific primers (5'-GTCCCTGTATGCCTCTGGTC-3' and 5'-GATGTCAACGCCAGATTTC-3') in the absence of reverse transcriptase. The absence of an amplicon after 35 cycles was checked by agarose gel electrophoresis and ethidium bromide staining.

Mouse cDNA expression arrays

Atlantic mouse cDNA expression arrays I (no. 7741-1; Clontech) consist of a matched set of positively charged membranes containing duplicate spots of 588 mouse partial cDNAs. Information on the genes represented on these arrays and hybridization protocols can be found on the manufacturer’s website: www.clontech.com. Briefly, 32P-labeled radiolabeled first-strand cDNA probes were prepared from 2–5 μg of total RNA from each cell population using Moloney murine leukemia virus reverse transcriptase and pooled primers specific for the 588 genes. 32P-labeled cDNA probe was separated from unincorporated nucleotides using the provided ChromaSpin columns, and probe activity was measured using a scintillation counter. The arrays were prehybridized for 1 h with ExpressHyb containing 100 μg/ml heat-denatured herring sperm DNA (Sigma) to block nonspecific hybridization. The filters were then incubated with 1.5 × 10^6 cpm of denatured cDNA probes in 5 ml of hybridization solution in hybridization bottles. Hybridization was performed overnight at 71°C in a hybridization oven, and bottles were rotated at 5 rpm. The filters were then extensively washed at low and high-stringency conditions in hybridization bottles at a rotation speed of 15 rpm, exposed to a phosphoimager screen (Molecular Dynamics, Sunnyvale, CA) for 3–5 days at 4°C, and the resulting hybridization signals measured using a PSI Phosphoimager (Molecular Dynamics).

Image analysis

Atlas Image 1.0 (Clontech) and Excel 5.0 (Microsoft, Redmond, WA) software were used to quantify and compare the hybridization signals. The intensities for each spot were corrected for background levels and normalized for differences in probe labeling using the average values for genes observed to vary little between our stimulation conditions: β-actin, ubiquitin, GAPDH, calcium binding protein CAB45, and ribosomal protein S29 (37). Spots were included with an intensity ≥300 units, as calculated by Atlas Image, exhibited higher variability and a low signal-to-noise ratio and were therefore not included in the analysis. Genes included in all tables were selected by the following criteria: the mean hybridization intensity

3 Abbreviations used in this paper: MOI, multiplicity of infection; MIP, macrophage inflammatory protein; Hox, homeobox transcription factor; DP-1, DRTF polypeptide-1; I-BEB, inhibitory xB; iNOS, inducible NO synthase.
values for macrophage genes were altered by >2-fold upon S. typhimurium infection; the averaged data was representative of the individual data sets; duplicate spots on the array gave similar hybridization signals; and the specific hybridization signal was not confounded by background hybridization. Intensity values of zero were replaced by the value of 20 to permit ratio calculation.

**Northern blots**
cDNA was prepared from total RNA purified from RAW 264.7 cells using oligo(dT) and SuperScript II reverse transcriptase (Life Technologies). The following primer pairs were designed to amplify portions of the indicated macrophage cDNAs to produce templates for probe synthesis: DRFT polypeptide-1 (DP-1), 5'-TCCAATGGGCTCTAGTACGC-3', 5'-TGTCACTGTGCTCTGCTTGTGAGG-3', 5'-CTTGTGCTCTGCTTGTGAGG-3', 5'-CTGCTCTGCTTGTGAGG-3'; cyclin D1, 5'-CAGCTTAATGTGCCCTCTGCTTGTGAGG-3'; tristetraprolin, 5'-GGACTCGTCATTGCTGTGG-3'; 5'-GAATTCATGGCCTTGTGAGG-3'; CD14, 5'-CTGCTACGCGCCCTGCTCC-3'; 5'-CAGGAGGTGGAAATTGTGAGG-3'; 5'-CTGGGATGGAATTGTGAGG-3'. Anti-sense cDNA probes were prepared by PCR using 50 ng of the appropriate PCR product template, the 3' oligo, and modified nucleotides to facilitate repeated stripping of blots (Strip-EZ PCR; Ambion). These single-stranded PCR products were column purified (Qiagen, Mississauga, ON) and labeled with biotin using psoralen-biotin (Ambion) and cross-linking with 365 nm UV light. Northern blots were performed with the NorthernMax-Gly kit (Ambion) which uses glyoxal/DMSO to denature the RNA as an alternative to formaldehyde. RNA was transferred to a positively charged membrane (Ambion) and cross-linked with long-wave UV light and baked at 80°C for 30 min. Labeled probe (3 ng in 10 ml UltraHyb or ZipHyb; Ambion) was used for hybridization at 45°C. The BrightStar nonisotopic detection kit (Ambion) was used for probe detection according to the manufacturer’s protocols. Northern blots were analyzed using an Alphalmager system (Alpha Innotech, San Leandro, CA).

**Cytokine assays**
The concentration of TNF-α, IL-1β, and macrophage inflammatory protein (MIP)-1α in culture supernatants from RAW 264.7 cells was determined by ELISA (R&D Systems, Minneapolis, MN).

**Results**

**Establishment of array hybridization conditions**
Gene array technology was used to examine differential gene expression in the RAW 264.7 murine macrophage cell line following S. typhimurium infection. The arrays chosen for this study contained 588 murine cDNAs encoding proteins with a wide range of functions and included several gene families whose role during macrophage responses to infection have not been characterized. Macrophages were infected with S. typhimurium SL1344 for 10 min, after which cells were washed and treated with gentamicin to kill any remaining extracellular bacteria and prevent reinfection. The short invasion time permitted a synchronous wave of bacterial invasion to induce a coordinated change in gene expression that could be measured 4 h postinfection. Total RNA was isolated from RAW 264.7 cells that were unstimulated or stimulated with virulent S. typhimurium or 100 ng/ml purified S. typhimurium LPS. Fig. 1 shows images of identical arrays hybridized with 32P-labeled cDNA probes prepared from RAW 264.7 macrophages that were either left unstimulated, infected with S. typhimurium, or stimulated with purified S. typhimurium LPS.

To permit comparison between multiple array experiments, the data sets were normalized to each other, and the unsupervised average expression level of five genes. Table I compares the hybridization intensities of these five genes and shows that their expression levels under different experimental conditions deviated by not more than 0.7- to 1.4-fold, indicating valid data normalization. To determine the reproducibility of the gene arrays, we compared the hybridization intensities of two identical array membranes hybridized with probes synthesized from two separate RNA preparations of un-

stimulated RAW 264.7 cells. Less than 5% of genes expressed by unstimulated cells varied by >2-fold between the two hybridization experiments (data not shown).

**Effect of S. typhimurium infection on RAW 264.7 macrophage gene expression**
Our application of gene array technology provided a cross-section of the diversity of genes whose expression is altered at a given time point after S. typhimurium infection. Due to the extensive amount of data accumulated from the gene array experiments, we have made the data sets for all 588 genes available on our web pages (http://www.cmdr.ubc.ca/salmonellaarray). At 4 h postinfection...
with *S. typhimurium*, the expression levels for 40 of the 588 genes represented on the array were altered in RAW 264.7 macrophages by 4-fold or greater from their uninfected level (Fig. 2). When a cut-off of 2-fold induction or inhibition was applied to the data, 77 genes showed changes in expression. Fig. 2 shows the mean hybridization intensity values for macrophage genes, encoding a broad spectrum of proteins, that were induced by >4-fold upon *S. typhimurium* infection. Many of these up-regulated genes encode effectors with well-characterized proinflammatory or direct antimicrobial properties. For example, inducible NO synthase (iNOS), which encodes the enzyme responsible for producing the potent antibacterial molecule NO, was strongly induced upon *S. typhimurium* infection (38). Highly elevated expression levels were also observed for the chemokines MIP-1α, MIP-1β, and MIP-2α (40), which selectively recruit other effector cells to infection sites (41). The expression of IL-1β, which contributes to the proinflammatory and acute-phase responses, was also up-regulated (42).

### Table I. Hybridization intensities of genes used for data normalization

<table>
<thead>
<tr>
<th>Gene</th>
<th>Average Intensity for Duplicate Array Spots</th>
<th>Ratio Relative to Unstimulated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin</td>
<td>15698</td>
<td>1.2</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5102</td>
<td>0.9</td>
</tr>
<tr>
<td>β-actin</td>
<td>9782</td>
<td>1.2</td>
</tr>
<tr>
<td>Cab45</td>
<td>1446</td>
<td>1.2</td>
</tr>
<tr>
<td>Ribosomal S29</td>
<td>12582</td>
<td>0.9</td>
</tr>
</tbody>
</table>

The average hybridization signals for duplicate cDNA spots of ubiquitin, GAPDH, β-actin, Cab45 calcium binding protein, and ribosomal protein S29 were calculated and corrected for background. The values for these five genes were normalized to each other to account for differences in probe labeling efficiency between experiments. Table 1 contains the mean hybridization intensities of these five genes after normalization for the eight hybridization experiments summarized in Figs. 2 and 3. The appropriate normalization coefficient was then used to normalize the entire data sets to make possible direct comparison between them.

**FIGURE 2.** A. RAW 264.7 macrophage gene expression induced by *S. typhimurium* and LPS. Gene arrays were hybridized using cDNA probes prepared from unstimulated RAW 264.7 cells or from cells 4 h after *S. typhimurium* infection or *S. typhimurium* LPS stimulation. Listed are genes whose mRNA levels were raised by at least 4-fold. The mean hybridization signal for each gene was calculated from two to three data sets obtained from hybridization experiments using probes prepared from independent batches of RNA. The fold increase in hybridization signal upon *S. typhimurium* infection or LPS stimulation was calculated relative to the value for unstimulated cells. B. Graphical representation of selected induced genes. A graphical representation of the mean hybridization signals for a subset of induced genes in unstimulated cells (■) and following bacterial infection (□) or LPS stimulation (□) is shown. Error bars represent the SD from the mean of three intensity values for infected and LPS-stimulated cells and the range of two intensity values for unstimulated cells. Due to the extensive amount of data accumulated from the gene array experiments, we have made the complete data sets for all 588 genes available on our web pages (http://www.cmdr.ubc.ca/salmonellaarray).
the proinflammatory cytokine TNF-α was up-regulated, as was CD40. CD40 binds to a ligand on T lymphocytes, and this interaction induces the production of many inflammatory mediators, primes T cells (43), and augments survival of mice infected with Salmonella dublin (44). A subset of the induced genes shown in Fig. 2 may serve to control or inhibit the inflammatory response. Tristetraprolin was highly induced upon S. typhimurium infection and can decrease TNF-α synthesis by decreasing mRNA stability (45). Elevated transcription of the inhibitory κB (I-κB) and inhibitory subunits of NF-κB was also observed, and these proteins are known to down-regulate the transcriptional program initiated by the translocation of NF-κB to the nucleus (46). Elevated expression of the antiinflammatory cytokines TGF-β1 and -β2 was also observed. TGF-β can have potent effects on macrophage activities, and administration of recombinant TGF-β has been shown to protect mice from a lethal dose of S. typhimurium (47). Elevated mRNA levels for signaling molecules that are involved in cell death or the response to IFN-γ were also observed. These include the apoptosis-associated genes ICE protease (caspase 1), TNF receptor 1, Fas, TDAG51, and TRAIL, and the IFN-γ-induced IFN regulatory factor 1 (IRF-1). Some of the S. typhimurium-up-regulated genes also encode proteins involved in macrophage migration. For example, ICAM-1 is required for vascular extravasation during migration to sites of infection, and urokinase plasminogen activator receptor participates in extracellular matrix remodeling (48). Dystroglycan 1 promotes extracellular matrix formation, and its transcriptional down-regulation (Fig. 3) may cooperate with the up-regulated genes encoding various proteases to remodel the extracellular matrix and promote tissue infiltration by macrophages (49, 50).

The pattern of altered gene expression caused by S. typhimurium infection is reminiscent of the antiproliferative and prodifferentiating transcriptional program that occurs during myeloid development. A number of genes with well-characterized roles in macrophage differentiation were up-regulated by S. typhimurium infection (Fig. 2). For example, leukemia inhibitory factor (LIF) was up-regulated. LIF is secreted by macrophages in response to LPS and promotes myeloid differentiation (51). A number of transcription factors were also regulated by S. typhimurium infection. Expression of Egr-1, NF-E2, IRF-1, and c-Rel was up-regulated, while expression of Ski, B-myb, Fli-1, and c-Fes was down-regulated by 2-fold (Fig. 3). Egr-1 controls both monocyte development and appears necessary for maintenance of macrophage differentiation, as the expression of many cytokines and receptors important during infection are regulated by Egr-1 activity (52). B-myb is a negative regulator of macrophage terminal differentiation, and its down-regulation by bacterial products promotes macrophage development. These transcription factors all regulate macrophage differentiation, and their coordinated expression in response to bacterial products may serve to promote development of the macrophage’s antibacterial abilities (53, 54). Expression of these transcription factors during macrophage maturation is usually coupled with an inhibition of cell proliferation. The expression level of many genes controlling cell cycle G1 to S phase transition

FIGURE 3. A, RAW 264.7 macrophage gene expression inhibited by S. typhimurium and LPS. The mean hybridization intensity values for RAW 264.7 macrophage mRNA levels that were repressed by at least 2-fold after S. typhimurium infection or LPS stimulation for 4 h are shown. The average hybridization intensities were calculated from two to three independent data sets obtained from hybridization experiments using probes prepared from independent batches of RNA. B, Graphical representation of selected repressed genes. A graphical representation of the mean hybridization signals and SD for a subset of repressed genes in unstimulated cells (■) and following bacterial infection (□) or LPS stimulation (□) is shown. Error bars represent the SD from the mean of three intensity values for infected and LPS-stimulated cells and the range of two intensity values for unstimulated cells. Due to the extensive amount of data accumulated from the gene array experiments, we have made the data sets for all 588 genes available on our web pages (http://www.cmrdr.ubc.ca/salmonellaarray).
were down-regulated. Modest decreases in the mRNA levels of cyclin D1 and its partner cyclin-dependent kinase (cdk) 4 were measured. This kinase complex phosphorlates the retinoblastoma gene product, causing it to dissociate from the DP-1:E2F heterodimer, which then translocates to the nucleus and initiates cell cycle progression. The array results also revealed down-regulation of DP-1 and cyclin E, as well as up-regulated expression of various retinoblastoma-related genes (data not shown), all known to block entry into S phase (55).

Effect of IFN-γ activation on gene expression by infected macrophages

IFN-γ primes macrophages for enhanced microbicidal responses to bacterial infection. The established importance of IFN-γ production during S. typhimurium infection invites a molecular examination of how the macrophage’s gene expression profile following S. typhimurium infection is affected by prior IFN-γ activation. To this end, gene arrays were hybridized with cDNA probes prepared from uninfected and S. typhimurium-infected RAW 264.7 macrophages, with or without prior IFN-γ activation. Table II presents genes that were differentially expressed by IFN-γ-activated and unactivated macrophages 4 h after S. typhimurium infection.

We found that IFN-γ treatment altered the expression of a number of genes and, importantly, that it modulated the ability of S. typhimurium to alter macrophage gene expression. IFN-γ often up-regulated gene expression in infected cells, such as BST-1, MIG monokine, and MIP-1α. For some genes, this expression level was further enhanced by S. typhimurium infection. Examples include iNOS, 1-κBα, NF-κB p65, JunB, JunD, TDAG51, tristetraprolin, and TNF-α. For other genes, such as MIG, IFN-γ up-regulated their expression but bacterial products did not significantly increase expression levels above the IFN-γ-stimulated level. Prior IFN-γ stimulation resulted in gene expression upon infection, such as the transcription factors Cdx2 and Bm3.2, which was not observed at the same time point in infected cells not primed by IFN-γ. For other genes, IFN-γ treatment up-regulated mRNA levels in infected cells, which was repressed following S. typhimurium infection. The IFN-inducible protein 1 is an example of this pattern of gene expression that may provide negative feedback.

The most striking trend was an increase in the steady-state mRNA levels encoding transcription factors such as tristetraprolin, three members of the jun family, Fos B, I-κBα, C-EBP, Stat 5a, and elk-1. Of note was an induction in the expression of MIG, NF-κB p65, Egr-1, Hox-4.2, caudal-type homeobox 2, and Brn 3.2 POU transcription factors. Expression of the Hox-4.2, caudal-type homebox 2, and Bm 3.2 POU transcription factors was up-regulated by S. typhimurium infection of IFN-γ-treated macrophages to a much greater extent than in unprimed macrophages. Homeobox gene expression provides critical roles during development, and the homeobox genes Hox-B3, Hox-B4, and Hox-B7...
have been implicated in orchestrating various stages of myeloid differentiation (53, 56). This is the first data, to our knowledge, suggesting that other homeobox genes may play a role in macrophage responses stimulated by bacterial products.

Contribution of LPS signaling to S. typhimurium-induced changes in gene expression

LPS is a potent inducer of macrophage inflammatory functions (13, 17). Because S. typhimurium is a Gram-negative bacteria with an outer membrane rich in LPS, our hypothesis was that many of the effects of S. typhimurium on macrophage gene expression are due to its LPS. Gene arrays were used to identify the relative contribution of the bacterial component, LPS, to the overall pattern of macrophage gene expression observed during S. typhimurium infection. This analysis revealed that the gene expression profiles overlapped considerably (Figs. 2 and 3). In most cases, 100 ng/ml LPS caused equivalent or greater increases in steady-state mRNA levels than S. typhimurium infection. The 100 ng/ml dose of purified LPS used was probably greater than the amount of LPS that the macrophages encountered during a 10-min invasion by S. typhimurium. Therefore, of special interest are genes, such as tristetraprolin, that this semiquantitative technique suggests are preferentially induced or repressed by Salmonella invasion in comparison to LPS stimulation.

Confirmation of array data using Northern blots and ELISAs

Despite its reproducibility, gene array analysis is only semiquantitative. Therefore, Northern blots were used to confirm and more accurately measure the regulation of genes identified in our gene array analysis to be regulated by S. typhimurium infection or LPS stimulation. mRNA levels for both CD14, a receptor for LPS, and IL-1β, a proinflammatory cytokine, were up-regulated, while cyclin D1 levels were decreased in macrophages by S. typhimurium and purified LPS from Northern blot analysis, confirming previously published data (data not shown). Northern blots were also used to confirm the induction or repression of candidate genes identified using array technology where there was little precedence in the literature. We analyzed mRNA levels of DP-1 and tristetraprolin relative to GAPDH in RAW 264.7 macrophages at 1, 4, and 6 h following S. typhimurium infection or LPS stimulation. DP-1 binds to members of the E2F gene family to form a heterodimeric transcription factor that can regulate cell cycle progression (57, 58). Expression of DP-1 is necessary for progression from G1 to S phase, as shown by studies with dominant negative mutants (59). To date, two DP genes and five E2F genes have been identified, and heterodimer subunit composition determines specificity for different E2F DNA binding sites (60). Therefore, regulated expression of DP-1 may coordinate expression of a subset of genes involved in entry into S phase. According to the two array hybridization results, both S. typhimurium and LPS stimulation decreased DP-1 expression by 40% in unprimed macrophages. We confirmed this data by Northern blot analysis, in that DP-1 expression decreased at 6 h following infection or LPS stimulation (Fig. 4A). To our knowledge, this is the first report of repressed DP-1 mRNA levels in macrophages during bacterial infection. An important finding from this Northern blot analyses is that a decrease

FIGURE 4. Confirmation and quantification of genes differentially expressed upon S. typhimurium infection and LPS stimulation using Northern blots. RAW 264.7 cells were infected with S. typhimurium at an MOI of 20:1 or stimulated with 100 ng/ml S. typhimurium LPS and compared with unstimulated cells. Total RNA was isolated from macrophages after 4 and 6 h. RNA was separated by denaturing gel electrophoresis, immobilized on a positively charged membrane, and probed sequentially with biotinylated single-stranded cDNA probes specific for (A) DP-1, (B) tristetraprolin, and (C) GAPDH. The hybridization intensities were quantified using a densitometer and normalized to GAPDH expression. Graphs depict the fold change subsequent to bacterial infection ( ■) or LPS stimulation ( □) relative to unstimulated cells ( ▪) at each time point for the Northern blot shown. These data confirm results from three separate array hybridizations and are representative of at least two Northern blot experiments.
in macrophage gene expression as small as 40% can be detected by array hybridization and confirmed and quantified by Northern blot analysis.

The expression of tristetraprolin was greatly up-regulated by both *Salmonella* infection and LPS, according to the array data sets. Tristetraprolin, encoded by the gene zfp-36, has been hypothesized to be a transcription factor due to its zinc finger motif and its ability to translocate to the nucleus (61). Tristetraprolin regulates mRNA stability as studies with knockout mice show that tristetraprolin lowers TNF-α protein levels by binding to the AU-rich elements in TNF-α mRNA and destabilizing it (62). Tristetraprolin is encoded by an early response gene that is rapidly induced by mitogens (63) and LPS (45). In Northern blot experiments, we found that expression of tristetraprolin was increased as early as 1 h poststimulation by virulent *S. typhimurium* or by LPS (data not shown) and then decreased to a lower level at 4 and 6 h (Fig. 4B). The apparent increase in tristetraprolin mRNA was smaller when quantified by Northern blot analysis compared with the array data, suggesting that the array technique accurately detects trends in altered gene expression but can overestimate ratios. This could be explained by the inability of the semiquantitative array technique to accurately quantify low levels of gene expression, for example in unstimulated cells. Quantitation of the Northern blotting results revealed that macrophages infected by *S. typhimurium* exhibited a higher level of tristetraprolin mRNA compared with macrophages stimulated by 100 ng/ml LPS. This confirmed the array data, which suggested that infection by one to three bacteria per macrophage induced a 30% higher level of tristetraprolin mRNA than following stimulation by LPS.

To confirm that changes in mRNA levels detected by the array hybridizations translated into similar changes in protein abundance for a subset of genes, growth media was collected from the cells used for RNA isolation and tested for proinflammatory cytokine levels by ELISA. In culture supernatants from cells infected with *S. typhimurium* or stimulated with LPS, levels of MIP-1α (both conditions resulted in 6.3–8.8 ng/ml) and TNF-α (infection, 1.3–2 ng/ml; LPS, 2.8–3.4 ng/ml) were elevated at 4 h, while levels of IL-1β were elevated at 24 h (0.3–0.5 ng/ml) when compared with unstimulated cells. For each ELISA, proinflammatory cytokine concentrations in culture supernatants of cells stimulated by *S. typhimurium* or LPS were similar, supporting our array data. By array analysis, iNOS expression was induced by *S. typhimurium* infection and LPS stimulation. Elevated levels of nitrate in the culture supernatants were detected at 24 h (data not shown), indicating increased iNOS activity and confirming that elevated iNOS expression translated into increased NO production.

**Discussion**

Significant progress has been made toward understanding how pathogenic bacteria promote their survival within the host through the regulated expression of bacterial virulence genes. Much less is known about how the host responds to these pathogens to shape the outcome of a potentially fatal liaison with pathogenic microbes. This is the first report to capitalize on gene array technology to profile how the expression of hundreds of host genes are altered by a virulent bacterium.

Gene array technology is a powerful tool that can be used to expand our current understanding of this relationship for a number of reasons. First, this technique permits one to study simultaneous changes in expression of a large number of genes under uniform experimental conditions, including infectious dose and cell passage number. While the selection of genes for inclusion on the array introduces some bias, the wide range of gene families allows rapid identification of genes previously not known to be involved in the host response to pathogens. In this study, we identified genes that have never been directly implicated in macrophage responses to *S. typhimurium* infection and identified novel gene targets of LPS signaling. These include dystroglycan, which is involved in extracellular matrix formation, and DP-1, which regulates cell cycle progression. Second, gene arrays permit comparison of expression profiles obtained from multiple stages of infection, from stimulation with purified microbial products, or from infection with bacterial virulence factor mutants. Our comparison of macrophage gene expression altered by bacterial infection to stimulation with purified LPS suggests that LPS serves a principal role in altering host gene expression during *S. typhimurium* infection. Third, gene arrays measure changes in individual genes in the context of how the expression of other members of the gene family, their receptors, ligands, or transcriptional activators are altered. This allows a more comprehensive understanding of host responses to bacterial infection by identifying patterns of gene expression that would not be evident from studying each gene in isolation. Indeed, this approach enabled us to detect the induction of families of transcription factors in IFN-γ-activated macrophages following *S. typhimurium* infection.

We were able to identify novel macrophage gene targets of IFN-γ activation or *S. typhimurium* infection by looking at <600 genes. The genes presented in this study likely underestimate the total number of affected genes due to limitations of accurately quantifying very low levels of gene expression. This suggests that gene array filters, used in this study, can complement the use of gene chip technology, which can analyze the expression of thousands of genes, because different cross-sections of the genome can be studied in each case. The use of commercially available filter-based gene arrays is an accessible approach to generate testable hypotheses of how hosts respond to pathogens. These arrays have the advantage of containing characterized genes for which reagents such as Abs, mutant cell lines, and knockout mice may be available for hypothesis testing. An even more comprehensive view of host response could be obtained by extending this approach to using gene microarrays incorporating thousands of genes. For this to be successful, improved bioinformatics resources are needed as well as a conceptual shift in the way we analyze and publish large amounts of data. The findings of many studies similarly rest on our assumption that changes in steady-state mRNA levels often correlate with meaningful changes in protein levels. While increased protein levels have been measured for many of the genes found to be differentially expressed in this study, others are bound to be regulated at the level of protein synthesis, posttranslational modification, or intracellular localization. This also highlights the need for high-throughput strategies to confirm changes in genes of interest at the level of transcription, translation, and protein localization to pursue the biological relevance of array data.

Our gene array results suggest that the macrophage’s transcriptional program undergoes a massive overhauling during bacterial infection and highlight the myriad of ways in which macrophages attempt to control and clear *Salmonella* infection. The majority of differentially expressed genes were up-regulated upon *S. typhimurium* infection, and several of these are known to play well-characterized roles during bacterial infection. In general, we observed a strong proinflammatory response that may be tempered by up-regulated expression of TGF-β, IL-10, and tristetraprolin, all of which have demonstrated antiinflammatory properties. This suggests that there may be a balance between proinflammatory responses and negative feedback regulation during *S. typhimurium* infection (42). Stimulation by LPS enhances the macrophage’s
ability to interact with other cells through the coordinated expression of various receptors, such as CD40 and ICAM-1 (44). Extracellular matrix remodeling, through alterations in the expression of various proteases, protease inhibitors, and dystroglycan may promote macrophage entry into infected tissues (48). Differentially expressed genes identified using the arrays were not limited to genes with characterized proinflammatory or antibacterial properties, because *S. typhimurium* had numerous effects on the cell cycle regulator and transcription factor gene families within macrophages. With myeloid cells, LPS has anti-mitotic effects by down-regulating the expression of cyclins and cyclin-dependent kinases and by influencing levels of positive and negative transcriptional activators (55). Northern blots for cyclin D1 and the transcription factor DP-1 revealed that the expression of both are decreased to an equivalent extent by LPS and *S. typhimurium*. This suggests that *Salmonella* infection may affect the cell cycle via LPS signaling. Our data supports a reprioritizing of host gene expression away from normal physiology toward establishing an antibacterial state.

While *S. typhimurium* initially invade naïve unactivated murine macrophages in vivo, macrophages are more likely to be stimulated by IFN-γ during later stages of *S. typhimurium* infection (7). IFN-γ-activated macrophages display enhanced microbialic activities upon bacterial infection, due to changes in the expression of genes such as iNOS and MIP chemokines (64). However, the spectrum of host responses affected by IFN-γ priming is not fully understood at the molecular level. We analyzed the expression patterns of hundreds of genes to gain a more comprehensive understanding of how priming by IFN-γ alters macrophage gene expression, and hence responses, to *S. typhimurium* infection. We identified a variety of gene expression patterns in IFN-γ-primed RAW 264.7 macrophages, which included up-regulated gene expression in uninfected cells, synergistic effects between IFN-γ and *S. typhimurium* infection, and elevated expression of genes following infection of IFN-γ-primed cells that was not seen following infection of unprimed cells. IFN-γ signaling has been shown to increase the amount of NF-κB in the macrophage cytoplasm that, upon LPS stimulation, translocates to the nucleus more rapidly and effectively than without prior priming by IFN-γ (64, 65). This model of priming by IFN-γ may explain the differential response to *S. typhimurium* mediated by IFN-γ, by altering the kinetics of gene activation, so that genes are elevated at our 4 h window. Alternatively, IFN-γ may supply a necessary first signal so that a second stimulus provided by the bacteria triggers gene expression, which is not possible in unactivated cells. Either mechanism could make IFN-γ-primed macrophages more sensitive to stimulation by bacterial products and permit a more rapid and effective antimicrobial response against invading *S. typhimurium*.

To our knowledge, this is the first report of the application of gene arrays to the study of macrophage biology by profiling how RAW 264.7 macrophages respond to various stimuli, such as IFN-γ and LPS. Maturation of myeloid cells into terminally differentiated macrophages involves an arrest in proliferation and the differential expression of many transcription factors (54), some of which were identified using the arrays. Both LPS and IFN-γ exert anti-mitotic effects while promoting development of the antimicrobial properties of myeloid cells. Many of the cell cycle regulatory and transcription factor genes expressed by RAW 264.7 cells in response to LPS stimulation have previously been reported using primary macrophages (18, 45, 46, 53, 66–70). This suggests that RAW 264.7 cells may provide an adequate model for identifying genes involved in macrophage responses to infection, which can then be further characterized using primary macrophages. The most striking class of gene induction in IFN-γ-activated cells 4 h after *S. typhimurium* infection was a group of >15 transcription factors. In infected unactivated cells, many of these transcriptional activators, namely of the homeodomain class, were not induced above our detection level. Hox transcription factors play crucial roles during developmental patterning (71). A previous report has connected the processes of developmental patterning and macrophage differentiation by implicating the expression of the Hox transcription factor Hox-2.4 (Hox-B8) in the terminal differentiation of a hemopoietic cell line along the macrophage lineage (56). This differentiation required expression of Egr-1, which was up-regulated upon infection of IFN-γ-activated RAW 264.7 macrophages in this study. Because IFN-γ activation of macrophages results in differentiation of monocytes into macrophages, it is possible that expression of Hox transcription factors upon infection of RAW 264.7 macrophages, identified in this study, may promote further maturation of the cell’s antibacterial phenotype. Alternatively, these transcription factors may serve as an yet uncharacterized role during macrophage response to *S. typhimurium* infection.

We hypothesized that LPS, a structural component of all Gram-negative bacteria and the most well-characterized modulin, should play a principal role in stimulating the early innate response of macrophages to bacterial infection. To test this hypothesis, we compared changes in host gene expression caused by virulent *S. typhimurium* and purified *S. typhimurium* LPS to investigate the relative contribution of this virulence factor. LPS exerts its effects through its lipid A moiety, which is buried in the cell wall of live bacteria. During our infection model, cells would be stimulated by the lipid A of LPS shed by live bacteria, extracellular bacteria killed by antibiotics, or intracellular bacteria killed by macrophages. There was a remarkable degree of overlap between genes induced by virulent *S. typhimurium* and purified *S. typhimurium* LPS. The 100-ng/ml dose of LPS was likely much higher than the amount of free LPS that stimulated the cells during infection and caused equivalent or higher alterations in gene expression when compared with bacterial infection. The overlap in the macrophage expression data following stimulation with virulent *S. typhimurium* or purified *S. typhimurium* LPS suggests that there is redundancy in host response to bacteria. Gene expression regulated by LPS stimulation has also been shown to be altered by other bacterial components. The ability of both *S. typhimurium* LPS and flagellar proteins to trigger TNF-α and IL-1β release by macrophages (20, 22) supports the concept that different bacterial inputs can initiate a conserved program of macrophage responses.

The remarkable overlap in macrophage gene expression induced by *S. typhimurium* or purified *S. typhimurium* LPS suggests that *Salmonella* specifically affects a relatively small subset of macrophage processes to secure their survival rather than completely dampening the inflammatory response. A number of host proteins and signaling cascades have been identified that are modified by specific bacterial virulence effectors. For example, the *S. typhimurium* virulence factor SopE up-regulates IL-8 production by epithelial cells (25), and SipB binds and activates caspase 1 (ICE) protease to promote macrophage apoptosis (24). The majority of these studies have used epithelial cells and have measured how *S. typhimurium* invasion and virulence factor expression specifically alter host protein abundance or activity. Our results in macrophages, at the level of altered gene expression, invites a comparative study in epithelial cells to identify similarities and differences in gene expression profiles between these two infection models. Because *S. typhimurium* resides within macrophages to cause systemic disease, bacterial factors independent of LPS likely specifically modulate macrophage phenotype at the levels of gene expression, protein abundance, and protein activity to secure this intracellular niche. We identified some genes induced to a higher...
extent by S. typhimurium infection compared with LPS stimulation and have confirmed this higher level of expression for tristetraprolin. While the differential increase in expression was small, it may be significant that another bacterial factor can produce a higher induction in gene expression compared with a relatively large dose of LPS. This raises the intriguing possibility that another virulence factor up-regulates tristetraprolin mRNA levels in macrophages. Our ability to confirm array data for differential tristetraprolin expression suggests that other differentially expressed genes identified by array hybridization may be altered by additional bacterial virulence factors acting synergistically or antagonistically with the effects of LPS. Experiments using killed bacteria or macrophages from LPS-hyperresponsive mice will more accurately quantify the contribution of LPS-independent factors in altering host gene expression. We are presently employing more quantitative techniques to determine whether macrophage genes shown in this study as being differentially expressed to a greater extent upon S. typhimurium infection than by 100 ng/ml LPS, such as tristetraprolin, are specifically responding to an active bacterial process. Array technology is also ideally suited to the study of host gene expression in response to characterized Salmonella mutants to address the contribution of other specific bacterial virulence factors in modulating host gene expression.

This application of array technology will provide insight into how pathogenic bacteria use some of their many virulence effectors to specifically alter host cell biology and secure their niche. Array technology is highly applicable to studying numerous host-pathogen interactions. Comparison of array data from host cells infected with a variety of pathogenic bacteria will likely reveal how specific virulence factors trigger a unique pattern of host gene expression in response to the particular pathogen. Comparison of these data sets with those obtained from LPS and other structural components will likely reveal an overall conserved host gene expression profile that serves as a common signature of infection. Gene array technology promises to provide much-needed insight into host cell gene expression during infection and to broaden our understanding of host-pathogen interactions.

Note added in proof. Cohen et al. (72) recently profiled gene expression changes in human THP-1 cells following Listeria monocytogenes infection using gene arrays.

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References


