Mycobacterium tuberculosis Sigma Factor E Regulon Modulates the Host Inflammatory Response

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Mycobacterium tuberculosis **survives in macrophages and usually subverts the bactericidal mechanisms of these phagocytes. The understanding of this host-pathogen interaction is relevant for the development of new treatments for tuberculosis. The adaptation of *M. tuberculosis* to intracellular life depends on its ability to regulate the expression of its genes. Sigma factors are important bacterial transcription activators that bind to the RNA polymerase and give it promoter specificity. Sigma factor E (SigE) controls the expression of genes that are essential for virulence. We have identified the SigE regulon during infection of macrophages, and we analyzed the impact of this regulon on the transcriptional response of phagocytes. Our results indicate that SigE regulates the expression of genes involved in the maintenance of *M. tuberculosis* cell envelope integrity and function during macrophage infection. Analysis of the phagocytes' transcriptional response indicates that the SigE regulon is involved in the modulation of the inflammatory response.**

Tuberculosis remains one of the major public health challenges in the world, despite more than a century of effort to combat the disease. The ability of *Mycobacterium tuberculosis* to overcome the bactericidal action of macrophages is a characteristic that contributes to the success of this microorganism as a human pathogen. For this reason, studies leading to a better understanding of this intriguing macrophage-pathogen interaction can help to develop improved intervention strategies to prevent or cure tuberculosis.

Among the most important bacterial transcription activators are the sigma factors. By binding to the RNA polymerase, sigma factors provide the specificity for particular promoters. The interaction between enzyme and promoter region provides a means of regulating gene expression in response to various environmental conditions, because different sigma factors bound to RNA polymerase allow different groups of genes to be expressed [1]. A subgroup of sigma factors in the extracytoplasmic function (ECF) family regulates the expression of genes associated with the ability of the microorganism to respond to environmental changes, and in some cases these genes are also involved in bacterial virulence [2]. The presence of 13 genes coding for sigma factors in *M. tuberculosis* is an indication of the ability of this microorganism to adapt to different stress conditions. The expression of these genes has been characterized [3], and the role played by many of these sigma factors in *M. tuberculosis* virulence has been extensively analyzed [4]. Interestingly, although mutants have been created for different ECF sigma factors of *M. tuberculosis,* only the *sigE* mutant is attenuated in resting and activated macrophages, suggesting that this regulator controls genes directly related to the intracellular survival of the bacterium. In many bacterial species, sigma factors similar to SigE have been identified as master regulators responding to extracellular stress and controlling the expression of genes relevant for pathogenesis [5]. Expression of *sigE* in *M. tuberculosis* is up-regulated under SDS,

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oxidative, and heat shock stress, and an *M. tuberculosis sigE* mutant is more sensitive to these conditions [3]. The in vitro treatment of *M. tuberculosis* with SDS has been used to mimic the cell envelope stress that *M. tuberculosis* presumably encounters inside macrophages, and the SigE regulon under SDS stress has been analyzed [6]. However, these experiments are a limited and probably not totally accurate representation of the conditions that *M. tuberculosis* faces during infection.

The analysis of the global transcriptional profiles of macrophages infected with *M. tuberculosis* and of intracellular bacteria has provided fundamental information regarding the macrophage environment and how *M. tuberculosis* responds to this milieu [7, 8]. However, Rengarajan et al. [9] demonstrated a poor correlation between the genes that *M. tuberculosis* upregulated inside macrophages and those that are essential for the intracellular survival of the bacterium. Hopefully, the analysis of the global transcription of an *M. tuberculosis* strain attenuated for survival in macrophages, such as the *sigE* mutant, can lead to the identification of bacterial pathogenic factors that are essential for virulence. In the present study, the global gene expression of the parent H37Rv strain and a *sigE* mutant growing in human macrophage–like THP-1 cells were each compared with the profiles of the respective broth-grown bacteria. The transcriptional response of human and murine macrophages infected with the *M. tuberculosis* wild-type strain or a *sigE* isogenic mutant was also analyzed. These experiments have allowed us to identify genes that are specifically controlled by SigE during infection of human macrophages and that could include potential virulence factors. We expect that this study will increase the understanding of the *M. tuberculosis*–macrophage interaction and will contribute to the search for potential drug targets.

METHODS

Bacterial cultures. The *M. tuberculosis* strain H37Rv (American Type Culture Collection [ATCC]) and the isogenic *sigE* mutant (strain ST28) [6] were used for all experiments. Liquid cultures were prepared in Middlebrook 7H9 medium (Difco) supplemented with albumin-dextrose complex, with bacteria growing in 7H10 agar plates [8].

Macrophage cultures. The human monocytic cell line THP-1 was obtained from the ATCC, and cultures were grown as described elsewhere [8]. Mouse bone marrow cells were extracted from the femurs and tibiae of C57B/6 mice. Red cells were removed with lysis solution (0.15 mol/L NH_4Cl, 1 mmol/L $KHCO_3$, and 0.1 mmol/L EDTA). Cells were cultured for 7 days in Dulbecco's modified Eagle medium supplemented with 20% fetal calf serum and 25% supernatant derived from confluent L929 cell cultures as a source of macrophage colony-stimulating factor (M-CSF). This procedure yields a pure population of adherent, M-CSF–dependent bone marrow macrophages (BMMs). Animal experiments were performed in accordance with the guidelines of the Ethical Review Committee of the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of the Public Health Research Institute (PHRI).

Macrophage infections for bacterial RNA preparation. Ten 175-cm^2 flasks were seeded with 100 mL of 1×10^6 THP-1 cells/mL, and infections of cells with *M. tuberculosis* were performed as described elsewhere [8]. At 24 h after infection, cells were processed for bacterial RNA extraction.

Bacterial and macrophage RNA extraction and purification. *M. tuberculosis* RNA was extracted and purified from in vitro cultures and from macrophage cultures as described elsewhere [8]. Macrophage RNA was obtained from cultures of 4×10^6 THP-1 cells or BMMs seeded in 75-cm^2 flasks. Forty-eight hours before infection of BMMs, cells from some flasks were treated with 100 U/mL interferon (IFN)–γ. Cultures were infected with *M. tuberculosis* strains at a MOI of 5. Uninfected cells were processed as controls. After 24 h of infection, monolayers were disrupted with 1 mL of TRI reagent, and extraction of RNA proceeded as described elsewhere [8].

DNA microarrays. *M. tuberculosis* DNA microarrays were printed at the Center for Applied Genomics (CAG; http://www.cag.icph.org/) at the PHRI. The *M. tuberculosis* microarray consists of 4295 oligonucleotides of 70mer. The complete gene list and array layout can be found at http://www.cag.icph.org/downloads_page.htm. Arrays were prepared by spotting oligonucleotides (Tuberculosis Genome Set; version 1.0; Operon Biotechnologies) onto poly-L-lysine–coated glass microscope slides, using a GeneMachines Omnigrid 100 arrayer (Genomic Solutions) and SMP3 pins (TeleChem). *M. tuberculosis* RNAs were analyzed using 0.5–1 μg of total RNA from each sample for each microarray. RNA samples from the same strain under both conditions were hybridized in 1 chip (i.e., H37Rv from macrophages vs. H37Rv from broth culture). The detailed labeling and hybridization protocol can be obtained at http://www.cag.icph.org/downloads_page.htm. Slides were scanned using an Axon GenePix 4000B scanner (Molecular Devices). Images were processed using GenePix (version 5.1). Data were filtered by removing all spots that were below the background noise. Chips were normalized by the print-tip Lowess method [10].

The human 19K microarray and the mouse 22K microarray were used to hybridize RNA from human THP-1 cells and BMMs, respectively (http://www.cag.icph.org/). Arrays were printed at the CAG using the Human OligoLibrary (HUMLIB96) and the Mouse OligoLibrary (MOULIB96T) (Compugen/Sigma Genosys). cDNAs were generated from 5 μg of total RNA per sample by means of the 3DNA Submicro Oligo Expression Array detection kit (Genisphere) and Superscript II (Invitrogen). Slides were scanned using an Axon GenePix 4000B scanner (Molecular Devices). Images were processed and the chips normalized as explained above for the bacterial arrays.

Table 1. Genes under sigma factor E regulation during macrophage infection.

Gene ID	Gene symbol	Description	Function	H37Rv[a]	*sigE* mutant[b]	Fold change[c]
Rv0479c	*Rv0479c*	Conserved membrane protein	Cell wall	2.6 ± 0.7	1.2 ± 0.4	2.3
Rv0896	*gltA2*	Citrate synthase glta2	IM	3.0 ± 0.8	1.3 ± 0.2	2.3
Rv1630	*rpsA*	Ribosomal protein s1 rpsA	IP	5.3 ± 1.4	2.3 ± 0.8	2.3
Rv2094c	*tatA*	Sec-independent protein translocase membrane-bound	Cell wall	4.5 ± 1.5	1.6 ± 0.1	2.9
Rv2271	*Rv2271*	CHP	CHP	3.3 ± 0.6	1.5 ± 0.2	2.3
Rv2272	*Rv2272*	Transmembrane protein	Cell wall	5.1 ± 2.2	1.7 ± 0.2	3.1
Rv2276	*Rv2276*	Cytochrome p450 (cyp121)	IM	2.0 ± 0.3	1.0 ± 0.2	2.0
Rv2519	*PE26*	PE family protein	PE/PPE	3.0 ± 0.7	1.2 ± 0.2	2.5
Rv2684	*arsA*	Arsenic-transport integral membrane protein arsA	Cell wall	3.96 ± 1.3	1.9 ± 0.2	2.0
Rv2971	*Rv2971*	Oxidoreductase	IM	5.50 ± 1.7	2.6 ± 0.5	2.1
Rv3050c	*Rv3050c*	Transcriptional regulatory protein	RP	2.09 ± 0.5	1.0 ± 0.1	2.0
Rv3140	*fadE23*	Acyl-CoA dehydrogenase	Lipid metabolism	2.55 ± 0.9	0.9 ± 0.2	2.9
Rv3592	*Rv3592*	CHP	CHP	2.48 ± 0.4	1.0 ± 0.1	2.5
Rv3634c	*rmlB2*	UDP-glucose 4-epimerase (galactowaldenase)	IM	2.25 ± 0.4	1.1 ± 0.1	2.1
Rv3767c	*Rv3767c*	CHP	CHP	2.52 ± 0.5	1.2 ± 0.3	2.0
Rv3826	*fadD23*	Probable fatty-acid-coA ligase	Lipid metabolism	2.00 ± 0.4	1.1 ± 0.1	1.9

NOTE. A false-discovery rate of <2% and a regulation of at least 1.8-fold were used as criteria to consider a gene to be differentially regulated. CHP, conserved hypothetical protein; IM, intermediary metabolism; IP, information pathways; PE, proline–glutamic acid; PPE, proline–proline–glutamic acid; RP, regulatory proteins.

[a] Average ± SD of expression ratios for H37Rv (macrophages vs. Middlebrook 7H9 medium).
[b] Average ± SD of expression ratios for the *sigE* mutant (macrophages vs. Middlebrook 7H9 medium).
[c] Fold change in gene expression for the H37Rv strain, compared with that for the *sigE* mutant.

Statistical analysis. For *M. tuberculosis,* 4 *sigE* mutant microarrays (macrophages vs. broth culture) and 5 H37Rv microarrays were compared by significance analysis of microarrays (SAM; unpaired test) [11]. A false-discovery rate of <2%, a regulation of at least 1.8-fold, and $P < .05$ (t test) were used as criteria to consider a gene to be differentially regulated between *sigE* and H37Rv. Macrophage microarray data analysis was performed using the Institute for Genomic Research MultiExperiment Viewer (MeV; version 3.1). We evaluated 5 biological replicates of THP-1 cells infected with H37Rv or the *sigE* mutant versus uninfected cells, 5 biological replicates of resting BMMs infected with H37Rv or the *sigE* mutant versus uninfected cells, and 8 biological replicates of IFN-γ–treated BMMs infected with H37Rv or the *sigE* mutant versus uninfected cells. One-class SAM was performed with the MeV software comparing macrophages infected with H37Rv or the *sigE* mutant to the uninfected cells. A median false-discovery rate of 0 was chosen for all of the comparisons in order to have the same percentage of false-positive results in all of the lists obtained. The lists were then filtered to retain only the average fold changes of at least 1.8-fold. Those lists were then compared to find transcripts that changed differentially between infection with H37Rv and the *sigE* mutant ($P < .05$ by the t test and at least 1.8-fold difference). Data were deposited in the Gene Expression Omnibus repository under the accession numbers GSE7963 *(M. tuberculosis),* GSE7870 (THP-1 cells), and GSE7871 (BMMs).

Quantitative reverse-transcription polymerase chain reaction (RT-PCR) with SYBR green. RT-PCR primers were designed using OLIGO (version 6.6; Molecular Biology Insights) and were purchased from Integrated DNA Technologies. RT-

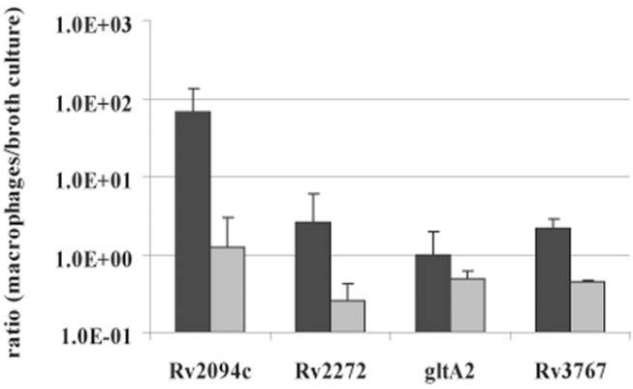

Figure 1. Expression of *Mycobacterium tuberculosis* genes under the control of sigma factor E (SigE). The expression of *M. tuberculosis* genes found to be differentially regulated by SigE in the DNA microarray analyses were confirmed by quantitative reverse-transcription polymerase chain reaction with SYBR green. The figure shows the arithmetic mean ± SD for 3 biological replicates. Results are expressed in logarithmic scale. The expression of each gene was normalized to the expression of *sigA* for the H37Rv strain *(dark gray columns)* or the *sigE* mutant *(light gray columns).* Gene expression for each strain inside macrophages was compared with gene expression for the same strain in broth culture.

Table 2. Genes differentially regulated after infection of THP-1 cells with H37Rv or the *sigE* mutant.

Gene ID	Description	H37Rv[a]	*sigE* mutant[b]	*sigE* mutant/ H37Rv[c,d]
Genes up-regulated after infection with the *sigE* mutant				
NM_007264	G-protein coupled receptor	1.13	2.83	2.51
AK000422	cDNA FLJ20415 fis, clone KAT02189	1.36	3.65	2.68
NM_013351	T-box 21 (TBX21), mRNA	1.43	3.50	2.44
NM_004900	Phorbolin	1.25	2.44	1.96
NM_013340	Protocadherin beta 1 (PCDHB1), mRNA	1.51	3.49	2.32
AJ133115	mRNA for TSC-22–like protein	0.79	2.32	2.94
NM_003523	H2B histone family, member H (H2BFH)	1.35	2.82	2.08
NM_012447	Stromal antigen 3 (STAG3), mRNA	1.42	3.87	2.73
NM_018381	Hypothetical protein FLJ11286 (FLJ11286)	1.18	3.26	2.77
NM_001252	TNF ligand superfamily, member 7 (TNFSF7)	1.63	4.62	2.84
AB014532	mRNA for KIAA0632 protein, partial cds	1.56	2.98	1.91
NM_003548	H4 histone family, member N (H4FN) mRNA	1.10	2.96	2.68
Genes up-regulated after infection with H37Rv or the *sigE* mutant				
NM_017414	Ubiquitin specific protease 18 (USP18), mRNA	3.75	14.31	3.81
NM_018171	Hypothetical protein FLJ10659 (FLJ10659)	5.63	18.57	3.30
S68954	Metallothionein MT-1g isoform	3.95	11.03	2.79
AF035035	Myosin-reactive IgG light chain variable region	5.80	13.60	2.35
NM_002751	Mitogen-activated protein kinase 11 (MAPK11)	2.83	6.00	2.12
NM_005218	Defensin, beta 1 (DEFB1)	2.81	5.81	2.07
X72308	mRNA for monocyte chemotactic protein–3 (MCP3)	4.47	9.07	2.03
AB040912	mRNA for KIAA1479 protein, partial cds	1.85	3.40	1.84
AK026720	cDNA: FLJ23067 fis, clone LNG04993	2.57	4.71	1.83
AK025971	cDNA: FLJ22318 fis, clone HRC05303	2.75	5.01	1.82
NM_004688	N-myc (and STAT) interactor (NMI)	2.84	5.12	1.80

NOTE. A regulation of at least 1.8-fold was used as the criterion to consider a gene to be differentially regulated.

[a] Average of the ratio of expression in cells infected with H37Rv vs. noninfected cells (significance analysis of microarrays [SAM]).

[b] Average of the ratio of expression in cells infected with the *sigE* mutant vs. noninfected cells (SAM).

[c] Ratio of gene expression in macrophages infected with the *sigE* mutant vs. H37Rv.

[d] $P < .05$ (*t* test).

PCRs for *M. tuberculosis* RNA samples were performed as described elsewhere [8]. For macrophage RNA, reactions were done using the 1-step QuantiTect SYBR Green Kit (Qiagen) and the Prism 7900H sequence detection system (Applied Biosystems) with SDS software (version 2.1). Cycles consisted of an initial incubation at 95°C (15 min), followed by 35 cycles at 94°C (15 s), 58°C (30 s), and 75.5°C (30 s). All determinations were performed in duplicate or triplicate. Nontemplate controls run with every assay consistently had no cycle-threshold values before 35 cycles of PCR. The abundance of each mRNA was normalized to β-actin expression and was compared with that in untreated cells to calculate the relative induction. Sequences of primers are available on request.

ELISA. Supernatants from macrophages at 24 h after infection were harvested for determination of cytokine protein levels by means of commercial kits, in accordance with the manufacturer's instructions (R&D Systems).

RESULTS AND DISCUSSION

Genes dependent on SigE for induction during macrophage infection. We compared the transcriptional profile of the intracellular strain H37Rv and the *sigE* mutant after macrophage infection at a time (24 h) when the growth of the mutant is not differentially affected compared with the wild-type strain. Our analysis indicated 16 *M. tuberculosis* genes that are under SigE regulation during macrophage infection (table 1). Among these genes, we identified *fadD23* and *fadE23* [12]. The gene *rmlB2* codes a putative galactose epimerase that is possibly involved in the synthesis of galactofuran (essential to the linking of peptidoglycan and mycolic acid) [13]; *Rv2276 (cyp121)* codes for a cytochrome P450 that may be also related to detoxification of fatty acids [14]. *Rv2094c (tatA)* codes for one of the components of the twin arginine translocation (TAT) system. This system is involved in translocation of folded proteins [15]. We have ob-

Table 3. Genes differentially regulated after infection of resting bone marrow macrophages with H37Rv or the *sigE* mutant.

Gene ID	Description	H37Rv[a]	*sigE* mutant[b]	*sigE* mutant/ H37Rv[c,d]
Genes up-regulated after infection with the *sigE* mutant				
NM_008176	GRO1 oncogene (Gro1)	1.71	5.32	3.11
M83218	Intracellular calcium-binding protein (MRP8)	1.43	4.23	2.96
NM_010766	Macrophage receptor with collagenous structure (MARCO)	1.68	3.73	2.22
NM_021293	CD33 antigen (Cd33)	1.15	2.39	2.07
NM_010119	EH-domain containing 1 (Ehd1)	1.14	2.10	1.83
Genes up-regulated after infection with H37Rv or the sigE mutant				
M17790	SAA4 gene encoding serum amyloid A	3.64	11.75	3.23
X89690	mRNA for TPCR52 protein	2.71	7.94	2.93
NM_011414	Secretory leukocyte protease inhibitor (Slpi)	3.26	8.25	2.53
NM_030720	G protein–coupled receptor 84 (Gpr84)	3.60	8.04	2.23
NM_011905	Toll-like receptor 2 (TLR2)	2.60	4.88	1.88
NM_011171	Protein C receptor, endothelial (Procr)	2.36	4.41	1.87

NOTE. A cutoff of >1.8-fold or <0.56-fold was used.

[a] Average of the ratio of expression in cells infected with H37Rv vs. noninfected cells (significance analysis of microarrays [SAM]).
[b] Average of the ratio of expression in cells infected with the *sigE* mutant vs. noninfected cells (SAM).
[c] Ratio of gene expression in macrophages infected with the *sigE* mutant vs. H37Rv.
[d] $P < .05$ (*t* test).

served that *tatA* is up-regulated in macrophages and is under the control of SigE. The other components of the TAT system in *M. tuberculosis* are encoded by *tatC,* which is downstream of *tatA,* and *tatB,* in an operon downstream of *sigE.* This latter operon includes *Rv1222,* which codes for a putative anti–sigma factor that may control SigE and a gene that encodes the serine protease HtrA. The observation that the TAT system of secretion is under SigE regulation may also indicate that a cell envelope defect is associated with the attenuation of the *sigE* mutant. In *Mycobacterium smegmatis,* the TAT system is involved in the secretion of β-lactamases [16, 17]. The differential expression of some of the *M. tuberculosis* genes determined in the DNA array experiments was validated by RT-PCR (figure 1).

We have also observed that the expression of *Rv2745c, Rv2744c, Rv2743c, sigB, htpX, acr2, Rv2053c,* and *Rv2052c* genes are constitutively under SigE regulation. It has been described previously that these genes are under SigE control when *M. tuberculosis* is exposed to SDS stress [6].

Effect of the SigE regulon on the macrophage's transcriptome. To study the impact of the SigE regulon on the response of macrophages to *M. tuberculosis* infection, we compared the transcriptional profile of host cells infected with H37Rv or the *sigE* mutant. For these experiments, we used human THP-1 cells and resting and IFN-γ-activated mouse BMMs.

Infection of macrophages with the wild-type strain H37Rv or the *sigE* mutant resulted in differential expression of numerous genes, compared with that in uninfected cells. Although many genes were similarly regulated by either *M. tuberculosis* strain, THP-1 cells infected with the *sigE* mutant up-regulated 12 genes that were not significantly up-regulated during infection with H37Rv (table 2). Among these genes, we observed up-regulation of T-bet (T-box 21) gene expression. T-bet is a transcriptional regulator associated with the induction of IFN-γ production and the inhibition of interleukin (IL)–10 production that leads to a long-lasting Th1 response [18]. Interestingly, T-bet knockout mice are more sensitive than wild-type mice in an aerosol model of *M. tuberculosis* infection [19]. Eleven genes were up-regulated in THP-1 cells after infection with either H37Rv or the mutant strain, but the expression of these genes was significantly higher after infection with the *sigE* mutant than with H37Rv. Many of theses genes have been shown to be relevant for the host response to other pathogens. The expression of the ubiquitin-specific protease 18 (USP18/Ubp43) is induced by type 1 IFN, suggesting a relevant role for this molecule in the innate immune response [20]. We have observed the up-regulated expression of defensins after infection with the *sigE* mutant. Interestingly, an antimycobacterial effect has been demonstrated previously for defensin β2 [21]. Production of defensins has been proposed as an alternative host defense mechanism in human macrophages to the nitric oxide mechanism in murine macrophages [22]. Monocyte chemotactic protein (MCP)–3 (CCL7) is a chemokine that has been shown to be induced by mycobacterial lipoarabinomannan [23]. MCP-3 has a broad spectrum of action, and its ability to attract dendritic cells can be of special importance for the control

Table 4. Genes differentially regulated after bacterial infection of interferon-γ–treated mouse bone marrow macrophages.

Gene ID	Description	H37Rv[a]	*sigE* mutant[b]	*sigE* mutant/ H37Rv[c,d]
Genes up-regulated after infection with the *sigE* mutant				
NM_020562	Putative TNF-resistance–related protein	1.24	4.34	3.49
X14607	SV-40 induced 24p3	1.56	4.18	2.67
NM_013652	Small inducible cytokine A4 (CCL4)	1.48	3.83	2.59
AK020041	13 days embryo forelimb	1.30	2.78	2.14
AB041997	PGES mRNA for prostaglandin E synthase	1.29	2.60	2.01
AB020974	mRNA for MAIL	1.30	2.50	1.93
Genes up-regulated after infection with H37Rv or the *sigE* mutant				
NM_008361	Interleukin 1 beta (Il1b)	5.61	22.34	3.98
NM_009117	Serum amyloid A 1 (Saa1)	7.50	24.28	3.24
NM_009252	Serine protease inhibitor 2-2 (Spi2-2)	8.69	27.98	3.22
NM_011315	Serum amyloid A 3 (Saa3)	4.52	14.53	3.22
NM_011337	Small inducible cytokine A3 (CCL3)	2.46	7.54	3.06
NM_010554	Interleukin 1 alpha (Il1a)	2.40	7.06	2.94
NM_007534	Bcl2a1b	3.52	10.10	2.87
NM_013693	Tumor necrosis factor (Tnf)	3.15	8.24	2.62
NM_011198	Prostaglandin-endoperoxide synthase 2 (Ptgs2)	1.97	4.91	2.50
NM_007752	Ceruloplasmin (Cp)	2.27	5.66	2.49
NM_007535	Bcl2a1c	4.72	11.38	2.41
NM_007376	Alpha-2-macroglobulin (A2m)	2.12	4.70	2.22
NM_025834	RIKEN cDNA 1300015B06 gene	2.14	4.74	2.22
NM_016921	ATPase, H+ transporting, lysosomal I (Atp6i)	2.67	5.83	2.18

NOTE. A cutoff of >1.8-fold or <0.56-fold was used.

[a] Average of the ratio of gene expression in cells infected with H37Rv vs. noninfected cells (significance analysis of microarrays [SAM]).
[b] Average of the ratio of gene expression in cells infected with the *sigE* mutant vs. noninfected cells (SAM).
[c] Ratio of gene expression in macrophages infected with the *sigE* mutant vs. H37Rv.
[d] $P < .05$ (*t* test).

of *M. tuberculosis* infection. N-myc Stat 1 interactor, or NMI, is a molecule involved in STAT-dependant transcription, particularly in response to IFN-γ [24]. These results from THP-1 cells suggest that SigE function in the wild-type strain confers an ability to avoid the induction of host defenses.

We have also compared the effect of infection with *M. tuberculosis* and the *sigE* mutant in resting BMMs or BMMs activated with IFN-γ. Genes in resting BMMs differentially regulated after infection with the wild-type strain H37Rv or the *sigE* mutant are described in table 3. Five genes were up-regulated after infection with the *sigE* mutant but not after infection with the H37Rv strain in resting cells. Among these were genes coding for intracellular calcium protein (MRP8), the MARCO receptor, and the CXCL1 chemokine (GRO1). In IFN-γ–treated cells, these genes were also up-regulated after infection with the wild-type strain, although expression in the macrophages infected with the mutant strain was still significantly higher than in cells infected with the wild-type strain. Another group of 6 genes were up-regulated in resting cells infected with either the wild-type or the mutant strain. However, increased expression was observed in macrophages infected with the mutant strain, compared with the expression observed in cells infected with H37Rv. The expression of the genes coding for protein C receptor, Toll-like receptor 2 (TLR2), and the secretory leukocyte protease inhibitor (SLPI) was up-regulated equally for both strains in IFN-γ–activated cells. It is worth noting that the antimycobactericidal activity of SLPI produced by BMMs was recently reported [25]. The magnitude of the response of resting macrophages to infection with the *sigE* mutant is comparable only to the response of these cells to H37Rv after activation with IFN-γ. Serum amyloid A and G protein–coupled receptor 84 (Grp84) were up-regulated for both strains in IFN-γ–activated macrophages, but the effect caused by the *sigE* mutant was significantly higher in resting as well as in IFN-γ–activated macrophages. Serum amyloid A induces the synthesis of proteoglycans by macrophages, which might antagonize the effect of transforming growth factor β, avoiding the negative effect that this molecule can have during *M. tuberculosis* infection in mice [26]; it has been also observed

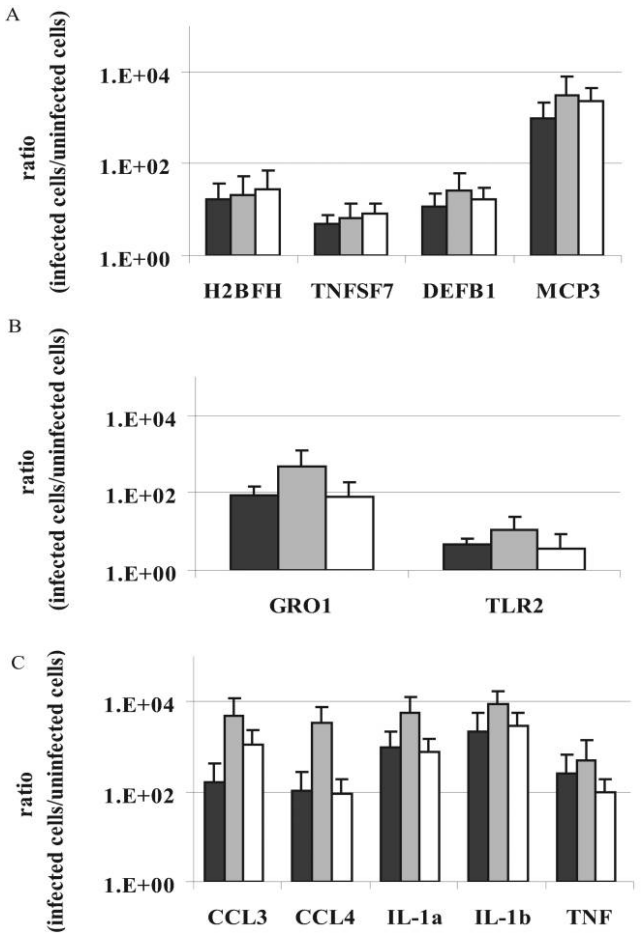

Figure 2. Expression of macrophage genes induced after infection with the *Mycobacterium tuberculosis* strains. The expression of genes found to be differentially regulated by infection of macrophages with the H37Rv strain *(dark gray columns),* the *sigE* mutant *(light gray columns),* or the *sigE* complemented strain *(white columns)* by DNA microarrays analysis was confirmed by quantitative reverse-transcription polymerase chain reaction with SYBR green. The expression of each gene was normalized to the housekeeping gene β-actin and compared with that in noninfected cells. The figure shows the arithmetic mean $\pm$ SD for 3 biological replicates. Results are expressed in logarithmic scale. *A,* THP-1 cells. *B,* Resting mouse bone marrow macrophages. *C,* Interferon-γ–treated mouse bone marrow macrophages.

that T cells in mice deficient in Grp84 produce more Th2 cytokines [27]. Genes particularly up-regulated after IFN-γ treatment are shown in table 4. Six genes, including those encoding the CCL4 chemokine and prostaglandin E synthase, were up-regulated by infection with the *sigE* mutant in IFN-γ–treated cells. The CCL4 chemokine could be involved in the recruitment of regulatory T cells that can modulate the magnitude of the host response to the pathogen [28]. It has been reported that prostaglandins play a dual role during *M. tuberculosis* infection. The production of this inflammatory mediator can be important for inducible nitric oxide synthase activation at an early phase of infection, although a high concentration of prostaglandins at chronic stages of the infection can be detrimental to the host [29]. It has also been determined that lipid bodies that characterize the foamy macrophages observed in the lungs of mice infected with bacillus Calmette-Guérin are particularly rich in prostaglandins [30]. Interestingly, histopathological analysis of lungs infected with the *sigE* mutant indicated an increased number of foamy macrophages [31]. The presence of foamy macrophages during *M. tuberculosis* infection has been associated with the mycolic acid components of the *M. tuberculosis* cell wall [32]. Twenty-three other genes were up-regulated by infection with one or the other strain, compared with gene expression in noninfected cells; however, significantly higher up-regulation was observed in IFN-γ–activated cells infected with the mutant strain. These genes included those encoding IL-1β, IL-1α, tumor necrosis factor (TNF), and CCL3 and genes related to the process of apoptosis. The up-regulation of these molecules induced by the *sigE* mutant may have important consequences in the modulation of the immune response. Thus, as for THP-1 cells, the *sigE* mutant causes a far more robust immune defense response than does the wild-type strain in murine macrophages—that is, it fails to limit host defense. The differential activation of macrophages induced by H37Rv or the *sigE* mutant

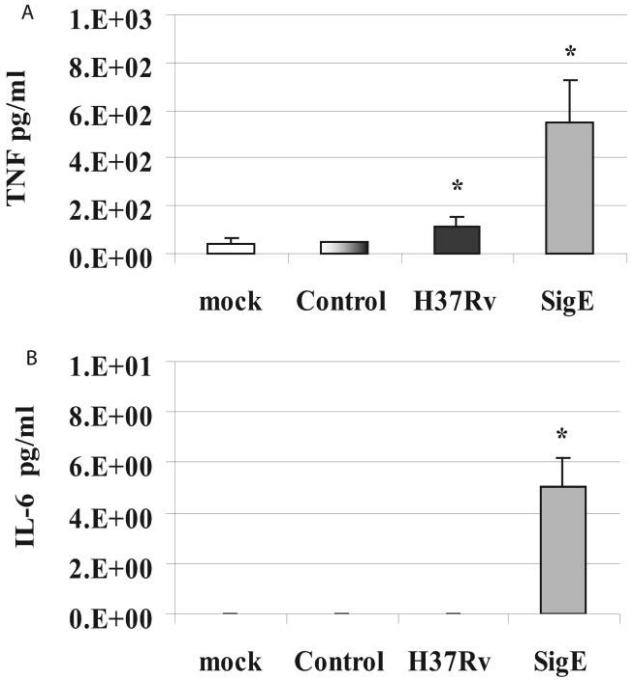

Figure 3. Determination of cytokine production. Levels of tumor necrosis factor (TNF) *(A)* and interleukin (IL)–6 *(B)* were determined in the supernatants of interferon-γ–activated bone marrow macrophages. The figure shows the levels of the indicated cytokine (arithmetic mean $\pm$ SD for 3 biological replicates) produced by noninfected macrophages (control) and by macrophages infected with H37Rv or the *sigE* mutant. $^*P < .01$ (for TNF, H37Rv or *sigE* mutant vs. control; for IL-6, *sigE* mutant vs. control and H37Rv vs. *sigE* mutant) or $^*P < .05$ (for TNF, H37Rv vs. *sigE* mutant) (*t* test for all comparisons).

was verified by quantitative RT-PCR (figure 2). In a random selection of genes, we confirmed a higher gene expression induced by the *sigE* mutant in comparison with the wild-type strain. The effect of the *sigE* mutant strain was restored in most cases to levels similar to the those of the wild-type strain when the macrophages were infected with a complemented *sigE* mutant. Sensitivity differences between both methodologies used to determine gene expression account for the disparity in fold inductions or detection above baseline.

Gene expression analysis in BMMs indicated that cells infected with the *sigE* mutant had more transcripts for TNF and IL-1β than did cells infected with the wild-type strain. To determine whether the cytokine mRNA detected in these experiments correlated with the level of these proteins, the production of cytokines by BMMs infected with the *sigE* mutant or the wild-type strain was measured. We evaluated levels of TNF, IL-1β, IL-6, IL-10, IL-12, RANTES, and IP-10. We found that the *sigE* mutant induced statistically significantly higher levels of TNF and IL-6 than did the wild-type strain in cells pretreated with IFN-γ after 24 h of infection (figure 3). These results at the protein level are in agreement with our DNA microarray results, indicating a central role for TNF in the BMM response to infection with the *sigE* mutant. We were not able to detect changes in mRNA levels of cytokines in THP-1 cells, although we observed the induction of other components of the innate immune response of the host by the *sigE* mutant in these macrophages. These observations can be associated with particular characteristics of each type of cell. It has been previously demonstrated that infection of dendritic cells with the *sigE* mutant induced higher production of IL-10 and reduced production of IP-10, compared with that in cells infected with the wild-type strain [33]. Our evaluations of these molecules in macrophages did not show differences between the cells infected with the wild-type and those infected with the mutant strain. These results are not surprising, given that it has been observed that dendritic cells and macrophages infected with *M. tuberculosis* have a different response at the level of cytokine production [34].

Analysis of our results indicated that the *M. tuberculosis sigE* mutant induced the up-regulation of macrophage gene expression, which correlated with an increased innate immune response. Many of these components have been described previously as part of the common defense program that the host elicits after infection with many pathogens [35]. Although *M. tuberculosis* is able to induce a strong innate immune response by the host, which is fundamental for the formation of the granuloma [36], recent experimental results have demonstrated that *M. tuberculosis,* in order to survive in the host, down-regulates the host immune response [37]. Several studies have indicated that *M. tuberculosis* strains with different degrees of virulence elicit inflammatory responses that are quantitatively and qualitatively different [38–40].

In conclusion, our results demonstrate that *M. tuberculosis* SigE regulates the expression of bacterial components important to the maintenance of the cell envelope, which helps the microorganism cope with environmental stress and suppress the host immune system and antibacterial response. Future experiments are planned to evaluate the ability of the *sigE* mutant to stimulate the host's immune system to generate a protective response against a subsequent *M. tuberculosis* infection.

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