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Global Transcriptional Response of Macrophage-Like THP-1 Cells to Shiga Toxin Type 1▽†

Dinorah Leyva-Illades,[1] Rama P. Cherla,[1] Cristi L. Galindo,[2] Ashok K. Chopra,[3] and Vernon L. Tesh[1]*

Department of Microbial and Molecular Pathogenesis, Texas A&M Health Science Center, College Station, Texas 77843[1]; Bioinformatics Institute, Virginia Polytechnic and State University, Blacksburg, Virginia 24061[2]; and Department of Microbiology and Immunology, The University of Texas Medical Branch, Galveston, Texas 77555[3]

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Shiga toxins (Stxs) are bacterial cytotoxins produced by the enteric pathogens *Shigella dysenteriae* serotype 1 and some serotypes of *Escherichia coli* that cause bacillary dysentery and hemorrhagic colitis, respectively. To date, approaches to studying the capacity of Stxs to alter gene expression in intoxicated cells have been limited to individual genes. However, it is known that many of the signaling pathways activated by Stxs regulate the expression of multiple genes in mammalian cells. To expand the scope of analysis of gene expression and to better understand the underlying mechanisms for the various effects of Stxs on host cell functions, we carried out comparative microarray analyses to characterize the global transcriptional response of human macrophage-like THP-1 cells to Shiga toxin type 1 (Stx1) and lipopolysaccharides. The data were analyzed by using a rigorous combinatorial approach with three separate statistical algorithms. A total of 36 genes met the criteria of upregulated expression in response to Stx1 treatment, with 14 genes uniquely upregulated by Stx1. Microarray data were validated by real-time reverse transcriptase PCR for genes encoding early growth response 1 (Egr-1) (transcriptional regulator), cyclooxygenase 2 (COX-2; inflammation), and dual specificity phosphatase 1 (DUSP1), DUSP5, and DUSP10 (regulation of mitogen-activated protein kinase signaling). Stx1-mediated signaling through extracellular signal-regulated kinase 1/2 and Egr-1 appears to be involved in the increased expression and production of the proinflammatory mediator tumor necrosis factor alpha. Activation of COX-2 is associated with the increased production of proinflammatory and vasoactive eicosanoids. However, the capacity of Stx1 to increase the expression of genes encoding phosphatases suggests that mechanisms to dampen the macrophage proinflammatory response may be built into host response to the toxins.

Shiga toxins (Stxs), also known as verotoxins, are genetically and functionally related bacterial cytotoxins primarily produced by enteric pathogens. *Shigella dysenteriae* serotype 1 expresses Shiga toxin, while Stx-producing *Escherichia coli* (STEC) may produce one or more antigenically related toxins categorized as Shiga toxin type 1 (Stx1) or Stx2 (30, 44). Stx-producing bacteria continue to constitute a global health problem. When contaminated food or water is ingested, it can result in bloody diarrhea and potentially lead to life-threatening complications such as hemolytic-uremic syndrome (HUS) and central nervous system complications (36, 46). Stxs have an AB_5 structure, consisting of a single enzymatic A-subunit in noncovalent association with a pentameric ring of B subunits (9–10). The toxins bind the glycolipid receptor globotriaosyl ceramide (Gb_3) on the cell surface by interaction with the toxin B subunits. They are then internalized to early endosomes and transported through the *trans*-Golgi network and Golgi apparatus to reach the endoplasmic reticulum (ER) (34). In the ER, the toxin A subunit is proteolytically processed and the A_1 fragment retrotranslocates into the cytoplasm (45). Once in the cytoplasm, the A_1 fragment catalytically cleaves a single adenine residue in the 28S rRNA component of 60S eukaryotic ribosomal subunits, resulting in protein synthesis inhibition (40).

Despite possessing the capacity to inactivate ribosomes, Stxs have also been shown to have different biological effects on different cell types. Stxs can activate host cell signaling pathways that result in cytokine and chemokine expression (5, 8, 13–14, 38). It has been shown that interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) sensitize endothelial cells to the action of Stx *in vitro* by increasing Gb_3 expression (43, 54). Therefore, the innate immune response elicited by Stxs may contribute to the development of vascular lesions by sensitizing endothelial cells to the action of toxins (37). We have found that when macrophage-like THP-1 cells are stimulated with Stx1, they secrete TNF-α, IL-1β, IL-6, and a number of CC and CXC chemokines. Alterations in expression of cytokines and chemokines by Stxs are regulated through transcriptional and translational mechanisms. Transcriptional regulation involves prolonged activation of stress-associated protein kinases JNK (c-Jun N-terminal kinases) and p38, transient activation of mitogen-activated protein kinases (MAPKs) extracellular signal-activated kinase 1/2 (ERK1/2), and activation of transcription factors NF-κB (nuclear factor-κB) and activation protein 1 (AP-1). Translational control involves, in part, stabilization of mRNA transcripts and activation of eukaryotic translation initiation factors (5, 13–14, 38–39, 49).

* Corresponding author. Mailing address: Department of Microbial and Molecular Pathogenesis, 407 Reynolds Medical Building, Texas A&M Health Science Center, College Station, TX 77843-1114. Phone: (979) 845-1313. Fax: (979) 845-3479. E-mail: tesh@medicine.tamhsc.edu.

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Our approach to studying the capacity of Stxs to activate cytokine and chemokine gene expression to date has been limited to these individual genes. However, the myriad signaling pathways activated by Stxs may potentially regulate the expression of large numbers of genes (6). Therefore, we carried out comparative microarray analyses to help explore global transcriptional responses of macrophage-like THP-1 cells treated with Stx1 or the well-characterized bacterial outer membrane constituent lipopolysaccharides (LPS). Previous studies have explored global gene expression induced by Stx2 in whole murine kidneys (20) and by Stx1 and Stx2 in human vascular endothelial cells (27). Our findings support these earlier studies in that Stxs elicited limited, but reproducible, changes in gene expression in macrophage-like THP-1 cells. The major category of genes that we found upregulated by Stx1 treatment of macrophage-like cells encode transcription regulatory factors, although genes involved in signaling, apoptosis, and inflammation were also activated.

MATERIALS AND METHODS

Reagents. Antibodies directed against human early growth factor 1 (Egr-1), cyclooxygenase 2 (COX-2), and actin were obtained from Cell Signaling Technology, Beverly, MA. ERK1/2 inhibitor PD 98059 was obtained from Calbiochem (La Jolla, CA). Except where noted, all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Toxins. Stx1 used in the present study was prepared as previously described (47). Briefly, Stx1 was purified from cell lysates prepared from *E. coli* DH5α(pCKS112) by sequential ion exchange and chromatofocusing chromatography. Purity of toxins preparations was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining and Western blot analysis with anti-Stx1 antibodies. Toxin preparations contained <0.1 ng of endotoxin per ml as determined by the *Limulus* amoebocyte lysate assay (Associates of Cape Cod, Falmouth, ME). Purified LPS derived from the enterohemorrhagic *E. coli* serotype O111 was purchased from Sigma Chemical Co (St. Louis, MO).

Cell lines. The human myelogenous leukemia cell line THP-1 (53) was obtained from the American Type Culture Collection, Manassas, VA. The cells were maintained in RPMI 1640 (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO_2 in a humidified incubator.

Macrophage differentiation and stimulation. The mature macrophage-like state was induced by treating THP-1 cells (10^6 cells/ml) for 48 h with phorbol 12-myristate 13 acetate (PMA) at 50 ng/ml. Plastic-adherent cells were washed twice with cold, sterile Dulbecco phosphate-buffered saline (PBS), and incubated with fresh RPMI 1640 lacking PMA but containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). The medium was then changed every 24 h for 3 additional days. Experiments were performed on the fourth day after removal of PMA. To isolate total RNA, differentiated THP-1 cells (2.5×10^6 cells/ml) were washed once with cold PBS and fresh RPMI 1640 medium containing 10% FBS, with no antibiotics added prior to stimulation with Stx1 (400 ng/ml) or LPS (200 ng/ml) for various times. We have previously demonstrated that these stimulant doses produced maximal cytokine protein secretion in differentiated THP-1 cells *in vitro* (38). To prepare cellular lysates, THP-1 cells (2.5×10^6 cells/ml) were serum starved by growth in RPMI 1640 medium containing 0.5% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) for 18 h to reduce background kinase signaling. Cells were washed as described above and stimulated with Stx1 (400 ng/ml) or LPS (200 ng/ml) in RPMI 1640 plus 0.5% FBS for various times. For MAPK inhibitor studies, cells were treated with the ERK1/2 inhibitor (PD98059; 50 μM) for 1 h prior to challenge with Stx1 (400 ng/ml) in RPMI 1640–10% FBS for various times.

Microarray analysis. Total RNA from macrophage-like THP-1 cells was isolated by using the RNAqueous kit (Ambion, Austin, TX), and 20 μg of total RNA was processed for microarray analysis. Briefly, cDNA synthesis, *in vitro* transcription, labeling and fragmentation to produce the oligonucleotide probes were performed according to the GeneChip manufacturer's instructions (Affymetrix, Santa Clara, CA). The probes were first hybridized to a test array (Affymetrix) and then to the GeneChip human genome HG-U133 Array (containing 54,675 probe sets representing more than 39,000 transcripts derived from ~33,000 well-substantiated human genes) using the GeneChip Hybridization Oven 640. The chips were washed in a GeneChip Fluidics Station 400 (Affymetrix), and the were results visualized with a Gene Array scanner using Affymetrix software. The data were analyzed by using the following software: GeneSifter (VizX Labs, Seattle, WA), Significance Analysis of Microarrays (SAM; Stanford University, Stanford, CA), and Spotfire DecisionSite 9.0 (Spotfire, Inc., Somerville, MA). Genes that were considered as significantly differentially expressed between untreated and Stx1-treated cells were identified separately using each of the three analysis methods. Those deemed as uniquely altered by Stx1 were not differentially expressed between untreated and LPS-treated cells using any of the three different analysis techniques. Raw and processed data (a total of nine arrays) were deposited in the Gene Expression Omnibus (GEO) online (www.ncbi.nlm.nih.gov/geo) database (accession no. GSE19315).

TABLE 1. Real-time PCR primers[a]

Target	Reference[b]	Orientation[c]	Primer sequence (5′–3′)
Egr1	16	F	GCCTGCGACATCTGTGGAA
		R	GCCGCAAGTGGATCTTGGTA
COX2[d]		F	GAATCATTCACCAGGCAAATTG
		R	TCTGTACTGCGGGTGGAACA
DUSP1	26	F	GGCCCCGAGAACAGACAAA
		R	GTGCCCACTTCCATGACCAT
DUSP5	15	F	CATCAGCCAGTGTGGAAAACC
		R	GGCCACCCTGGTCATAAGC
DUSP10	11	F	AAGAGGCTTTTGAGTTCAT TGAG
		R	CAAGTAAGCGATGACGATGG
GAPDH	13	F	CAACGGATTTGGTCGTATTGG
		R	GGCAACAATATCCACTTTACCA GAGT
TNF-α	57	F	CCAGGCAGTCAGATCATCTTCTC
		R	AGCTGGTTATCTCTCAGCTCCAC

[a] Primers were synthesized by Integral DNA Technologies (Coralville, IA).
[b] Published reference for each primer set.
[c] F, forward; R, reverse.
[d] Designed using IDT's Primer Quest.

Real-time reverse transcription-PCR (RT-PCR). Total RNA was isolated by using a TRIzol Plus kit (Invitrogen, Carlsbad, CA) with an RNase-free DNase (Invitrogen) treatment according to the manufacturer's instructions. RNA was reverse transcribed to cDNA by using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA), and real-time PCR was performed on the resulting cDNAs using SYBR green I double-stranded DNA binding dye (Applied Biosystems). Real-time specific primer sequences for Egr-1, COX-2, dual-specificity phosphatase 1 (DUSP1), DUSP5, and DUSP10, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in Table 1 (13, 15–16, 22, 26, 57). The real-time PCRs were carried out with 100 nM concentrations (each) of forward and reverse primers in a final volume of 25 μl. To control for the presence of contaminating DNA in the real-time PCRs, reverse transcriptase-negative reactions were included. Nontemplate controls were run to test for DNA-contaminated primers. Real-time reactions were run and analyzed by using an ABI Prism 7500 sequence detection system (Applied Biosystems). Dissociation curves for PCR samples were made to guarantee amplification of the correct genes. The amount of mRNA fold induction was determined from the change in threshold cycle (ΔC_T) values normalized for GAPDH expression and then normalized to the value derived from cells at time zero prior to medium change or treatment. Statistical analyses of real-time PCR data were performed using ΔC_T values.

Preparation of cellular lysates. Cells were harvested and lysed at 4°C in modified radioimmunoprecipitation assay buffer (1.0% Nonidet P-40, 1.0% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 0.25 mM sodium pyrophosphate, sodium vanadate and sodium fluoride [2 mM each], 10 μg of aprotinin/ml, 1.0 μg of leupeptin and pepstatin/ml, and 200 mM phenylmethylsulfonyl fluoride). Extracts were collected and cleared by centrifugation at

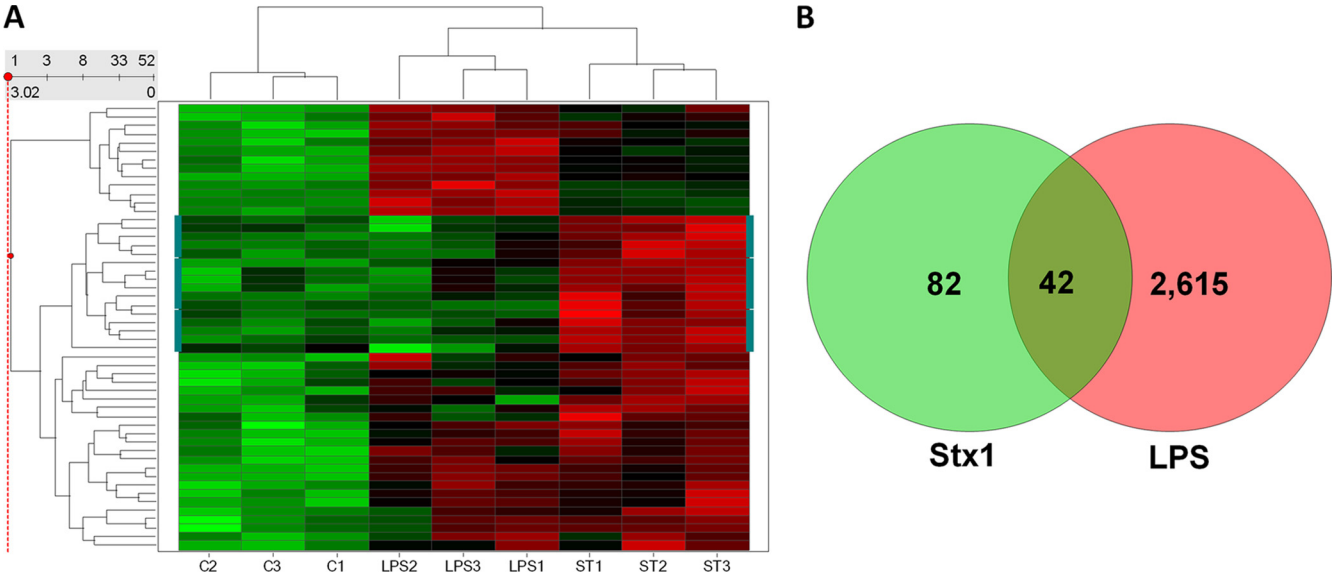

FIG. 1. (A) Hierarchical clustering (performed using Spotfire DecisionSite) shows subsets of genes that are specifically upregulated in response to LPS or Stx1 (marked on the sides by a blue-green line) or are coregulated by LPS and Stx1. Bright green indicates very low signal values, bright red represents very high signal values, and black represents median signal values. C, control (untreated); LPS, LPS-treated; ST, Stx1-treated cells. Each replicate is indicated by the numbers 1 to 3. Each of the sample types (C1 to -3, LPS1 to -3, and ST1 to -3) clustered together and apart from the other two sample types, and LPS- and Stx1-treated macrophages were more similar to each other than to untreated control cells. One subset of genes (indicated by the blue-green line on either side) was specifically upregulated in response to Stx1 treatment but not LPS treatment. (B) Venn diagram showing upregulated gene probes after Stx or LPS treatment. GeneSifter software was used to perform RMA normalization, followed by pairwise comparisons and Student *t* test with a Benjamini and Hochberg correction; the cutoffs used were a fold change of at least 1.5 and an adjusted *P* value of <0.05.

15,000 × *g* for 10 min. Cleared extracts were stored at −80°C until further use for Western blot analysis.

Western blot analysis. Cell extracts prepared from stimulated THP-1 cells were used for determination of protein concentration using the Micro BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (100 μg per gel lane) were separated by SDS-PAGE using 8% acrylamide gels and transferred to nitrocellulose membranes, which were blocked with 5% nonfat milk prepared in Tris-buffered saline (TBS)/Tween 20 (200 mM Tris [pH 7.6], 1.38 M NaCl, 0.1% Tween 20) and incubated overnight at 4°C with various primary antibodies specific for Egr-1, COX-2, and actin in 5% bovine serum albumin made with TBS–0.1% Tween 20. Membranes were then incubated with secondary antibody (rabbit immunoglobulin G coupled to horseradish peroxidase) for 1 h at room temperature. Bands were visualized using the Western Lightning chemiluminescence system (NEN-Perkin Elmer, Boston, MA). The intensities of protein bands captured on autoradiography film were quantitated using ImageJ software (Bio-Rad, Hercules, CA). The fold induction was calculated as stimulated protein band intensity values divided by unstimulated control protein band intensity values after normalizing for loading controls. The data shown are from at least three independent experiments.

Measurement of eicosanoids. Differentiated THP-1 cells (2.5×10^6 cells/ml) were treated with Stx1 (400 ng/ml) for various time points. Supernatants were then collected to measure soluble eicosanoid production. Prostaglandin E_2 (PGE_2) concentrations were determined by using a PGE_2 EIA kit (Assay Designs, Ann Arbor, MI), and thromboxane B_2 (TXB_2) was measured by using a TXB_2 enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's instructions. PGE_2 and TXB_2 standards were provided with each kit and used in each assay. Absorbance was measured at 450 nm (PGE_2) or 420 nm (TXB_2) by using an enzyme-linked immunosorbent assay (ELISA) reader (Dynatech MR5000, Chantilly, VA). The results were expressed as pg of PGE_2 or TXB_2/ml in supernatants. The sensitivities of these assays were 8 pg/ml (PGE_2) and 11 pg/ml (TXB_2).

Statistical analysis. Quantitative RT-PCR, ELISA, and Western blot data were analyzed by one-way analysis of variance (ANOVA) with Dunnett's post test, and quantitative RT-PCR data of ERK1/2 inhibitor-treated cells were analyzed by two-way ANOVA with the Bonferroni post test using GraphPad Prism version 5.00 for Windows (San Diego, CA).

RESULTS

Stx induces upregulation of gene transcription in macrophage-like THP-1 cells. In three separate experiments, THP-1 cells were untreated or treated with Stx1 (400 ng/ml) or LPS (200 ng/ml) for 6 h. RNA was then isolated and applied to HG-U133 GeneChips. The data were analyzed by using a combinatorial approach utilizing different software programs, statistical algorithms, and biological relevance measured in order to obtain the most robust list of genes representing highly consistent and reproducible results. Signal values were averaged, and the fold change determined using GeneSifter. Genes were deemed as significantly differentially expressed if the observed average fold change was at least 1.5 and the Benjamini and Hochberg-corrected *P* value was <0.05. For Stx1-treated cells, SAM analysis (Stanford University) was also performed, using the two-class unpaired option with the additional criteria of at least 1.5-fold differential expression. Individual pairwise comparisons were also carried out by using Spotfire DecisionSite 9.0 software; a fold change of at least 1.5 was expected. For LPS-treated samples, only the average fold change and Student *t* test (obtained using GeneSifter) were used to determine significance. Hierarchical clustering of the normalized signal values corroborated the statistical analyses (Fig. 1A). Each of the sample types (untreated cells [control], LPS-treated cells, and Stx1 treated cells) clustered together and apart from the other two sample types. LPS- and Stx1-treated cells were more similar to each other than to untreated control cells. Significantly, although we identified genes regulated by both Stx1 or LPS (depicted by the color red in lanes labeled LPS1 to LPS3

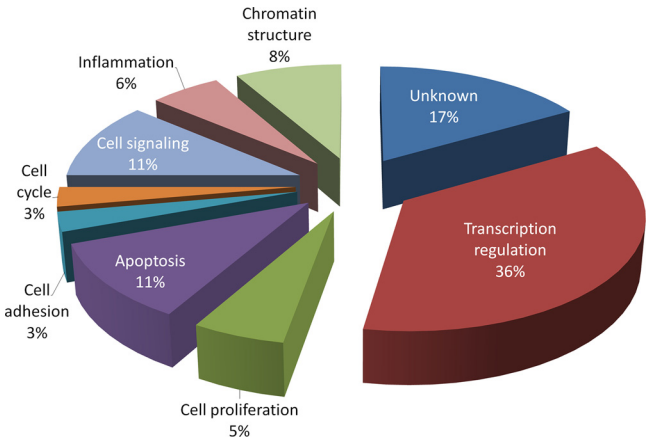

FIG. 2. Genes upregulated by Stx1. The 36 genes uniquely upregulated by Stx1 or coregulated by LPS treatment were categorized by function.

and ST1 to ST3 in Fig. 1A), we also identified genes uniquely regulated by Stx1 (depicted by the color red only in lanes labeled ST1 to ST3) or LPS (red only in lanes labeled LPS1 to LPS3). Using only GeneSifter, 2,615 probes were found to be upregulated by LPS treatment, 42 by both LPS and Stx1 and 82 by Stx1 alone as shown in Fig. 1B. Further statistical analysis of Stx1 treated samples showed that 52 probe sets (representing 36 genes) were significantly differentially expressed between untreated and Stx1 treated macrophage-like THP-1 cells. Of these 52 probe sets, 31 were also altered by LPS treatment; the remaining 21 probe sets represented 14 different genes that were specifically altered by Stx1 treatment (i.e., were not altered by LPS treatment). Interestingly, there were no genes downregulated by Stx1 treatment across all three replicate samples. The 36 genes significantly upregulated by Stx1 treatment compared to untreated controls (the complete list is shown in Table S1 in the supplemental material) were then categorized by function. As shown in Fig. 2, the majority of the genes either uniquely upregulated by Stx1 or coregulated by LPS encoded proteins involved in transcriptional regulation, followed by genes encoding products with unknown functions, apoptosis, cell signaling, chromatin structure, inflammation, cell proliferation, cell cycle, and finally cell adhesion. The listing of 149 gene probe sets that were upregulated by Stx1 but failed one or more of the statistical tests is shown in Table S2 in the supplemental material.

Validation of Stx1 induced upregulation of genes in macrophage-like THP-1 cells by real-time RT-PCR. Based on earlier studies suggesting that Shiga toxins induce the expression of cytokines and chemokines (13) and trigger signaling cascades (5), we elected to further analyze the expression of five select genes involved in transcriptional regulation, inflammation and cell signaling (Table 2). In order to confirm the microarray data, real-time RT-PCR was performed. Fold change values were determined by first normalizing each gene to GAPDH and then to the untreated control by using the comparative threshold method (25). The microarray analyses revealed that Egr-1, COX-2, DUSP1, DUSP5, and DUSP10 were upregulated 10.7-, 3.2-, 6.9-, 3.6-, and 1.7-fold, respectively. We confirmed upregulation of these genes by real-time RT-PCR, with fold-increases of 51 for Egr-1, 3.6 for COX-2, 15.9 for DUSP1, 2.0 for DUSP5, and 2.4 for DUSP10. Differences in fold increases between microarray analysis and real-time RT-PCR may be due to the different nature of the two techniques; for example, the two methods require and use different normalization procedures (28). However, the trend of upregulation is confirmed by both assays.

Egr-1 is upregulated by Stx1 and LPS treatments. Egr-1 is a zinc finger transcription factor that belongs to a group of early response genes. Many environmental stimuli including growth factors, hormones, and neurotransmitters dramatically and rapidly induce Egr-1 expression. It has been proposed that Egr-1 couples extracellular signals to long-term responses by altering expression of genes that are Egr-1 sensitive (48). Egr-1 is known to be a pleiotropic inflammatory transactivator whose transcriptional activity has been shown to play a major role in more than 300 target genes, including TNF-α, intercellular adhesion molecule 1 (ICAM-1), macrophage chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 (MIP-1), IL-1β, IL-6, transforming growth factor β (TGF-β), CD44, plasminogen activator inhibitor 1, and tissue factor (21, 35). Interestingly, many of these Egr-1-regulated target genes have been shown to be induced by Stxs (13–14). To better understand the kinetics of Egr-1 expression induced by treatment with Stx1 and LPS, macrophage-like THP-1 cells were stimulated for various times, and Egr-1 mRNA and protein levels were measured by real-time RT-PCR and Western blot analysis, respectively. Egr-1 mRNA was detected as early as 30 min (1.7-fold) after stimulation with Stx (400 ng/ml), reaching maximum expression by 4 h (52.3-fold). The levels then decreased but did not return to basal levels, as at 24 h, there was still a 20-fold increase in Egr-1 mRNA expression (Fig. 3A). Upregulated expression of Egr-1 mRNA by LPS (200 ng/ml) was not detected at the 6-h time point, based on microarray analysis. However, a more complete analysis of the kinetics of *egr-1* induction using real-time RT-PCR suggested that LPS treatment of macrophage-like THP-1 cells induced Egr-1 mRNA with very different expression kinetics compared to Stx1. Egr-1 mRNA was detected 15 min after stimulation with LPS (17-fold), reaching maximal expression by 1 h (280-fold) and then steadily decreasing. There was, however, elevated transcript expression (13.6-fold) detected after 6 h, the last time point

TABLE 2. Validation by real-time RT-PCR of selected genes determined to be upregulated by treatment of THP-1 macrophage-like cells with Stx1 (400 ng/ml) or LPS (200 ng/ml) for 6 h

GenBank accession no.[a]	Gene	Fold change[b] as determined by:			
		Microarray		qRT-PCR	
		Stx1	LPS	Stx1	LPS
NM_001964*	Egr1	10.7	<1.5	51	13.6
NM_000964*	COX2	3.2	44.5	3.6	82.19
NM_004417	DUSP1	6.9	6.1	15.9	62.7
U16996	DUSP5	3.6	21.2	2	1117
N36770	DUSP10	1.7	<1.5	2.4	3

[a] Genes denoted by an asterisk (*) were represented on the microarray by more than one probe set, and each was determined separately to be significantly upregulated by Stx1.
[b] The values shown are means of three or more experiments.

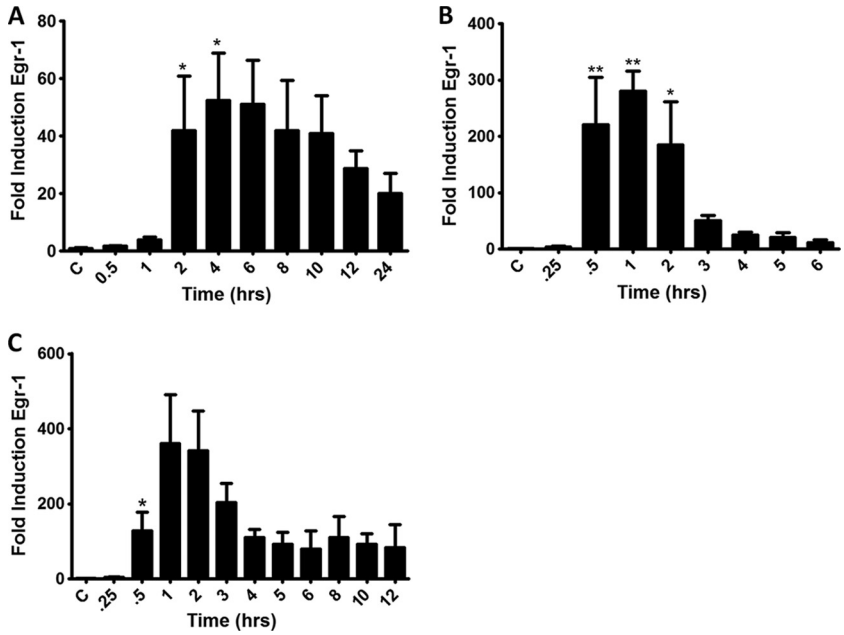

FIG. 3. Egr-1 mRNA expression by macrophage-like THP-1 cells. Cells were stimulated with Stx1 (400 ng/ml) (A), LPS (200 ng/ml) (B), or Stx1+LPS (400 and 200 ng/ml, respectively) (C) for different times. Total RNA was isolated, DNase treated, and cDNA synthesized. Quantitative real-time PCR was performed with primers specific for Egr-1 and GAPDH. Relative expression was calculated by using the ΔC_T method. The data shown are the mean fold induction ± the standard error of the mean (SEM) derived from at least three independent experiments. Statistical significance was calculated by using one-way ANOVA (*P* values: *, <0.05; **, <0.01).

collected in these stimulation experiments (Fig. 3B). Thus, LPS elicited a rapid and extensive increase of Egr-1 mRNA that decayed very quickly (within 2 h), whereas Stx1 caused a sustained, lower level of Egr-1 mRNA expression over 24 h. When cells were treated with both Stx1 and LPS, a combination of the individual treatment kinetics was observed (Fig. 3C). Egr-1 transcript was detected as early as 15 min (5.2-fold), increased rapidly peaking at 1 h (202-fold increase) and gradually decreased to a 23-fold increase at 12 h after stimulation. This was the last time point measured in these experiments because, as previously shown (12), macrophage-like THP-1 cells treated with both Stx1 and LPS undergo rapid apoptosis.

At the protein level, elevated Egr-1 production was observed by Western blot analysis at 2 h (2.0-fold) after Stx treatment, reaching maximum levels at 8 h (3.2-fold) and slowly decreasing to near basal levels by 24 h (Fig. 4A and C). When cells were stimulated with LPS, elevated Egr-1 protein level was detected as early as 1 h and peaked at 2 h (27.2-fold), gradually decreasing to undetectable levels by 6 h (Fig. 4B and D). Thus, the kinetics of Egr-1 protein production induced by Stx1 or LPS was well correlated with mRNA induction kinetics, with the latter preceding protein production.

ERK1/2 signaling is involved in Stx1 induced Egr-1 and TNF-α mRNA expression. It has been shown that activation of the MEK-ERK1/2 pathway by LPS can induce *egr-1* expression (11). Furthermore, we have shown that Stx1 transiently activates ERK1/2 signaling in THP-1 cells (5). Therefore, we investigated the role of ERK1/2 in Stx1 induced Egr-1 mRNA expression. Cells were treated with the ERK1/2 inhibitor PD98059 for 1 h before Stx1 stimulation for various times. We found that treatment with Stx1 plus ERK1/2 inhibitor almost completely abolished Egr-1 transcript, significantly so at the 2- and 6-h time points (Fig. 5A). Numerous studies suggest that nuclear recruitment of NF-κB, Egr-1, and c-Jun are required for full activation of TNF-α in macrophages and macrophage-like cell lines (51, 56). Therefore, the capacity of Stx1 to increase TNF-α mRNA production through the ERK1/2-Egr-1 pathway was explored. When THP-1 cells were treated with Stx1 plus ERK1/2 inhibitor, the TNF-α transcript levels dropped dramatically compared to Stx1-treated cells, a change in expression that was statistically significant at 6 h after intoxication (Fig. 5B).

COX-2 is upregulated by Stx1 and LPS treatments. COX-2 catalyzes the rate-limiting step in the formation of eicosanoids. COX-2 expression is inducible, and yet the enzyme remains undetectable in most mammalian tissues under basal conditions. Many stimuli can induce COX-2 expression and production such as LPS, cytokines, nitric oxide, growth factors, and UVB irradiation, among others (52). COX-2 upregulation was detected in the microarray study in cells treated with Stx1 (400 ng/ml) or LPS (200 ng/ml) for 24 or 6 h, respectively. Using real-time RT-PCR, we noted a slow and constant increase in COX-2 transcript expression during the 24-h time course after Stx1 treatment. Significant increases in COX-2 transcript were detected after 1 h (1.5-fold) and slowly increased, reaching a maximum after 24 h (5.3-fold), as shown in Fig. 6A. LPS is known to be a potent inducer of *cox-2* gene transcription (17). When macrophage-like THP-1 cells were stimulated with LPS, we observed an increase in COX-2 transcript, but with very different induction kinetics and quantities compared to Stx1 (Fig. 6B). Increased transcript levels were detected as early as 15 min after LPS stimulation (11-fold) and reached maximum levels at the 3-h time point (4,407-fold). COX-2 transcript levels then decreased to an 82-fold induction after 6 h of

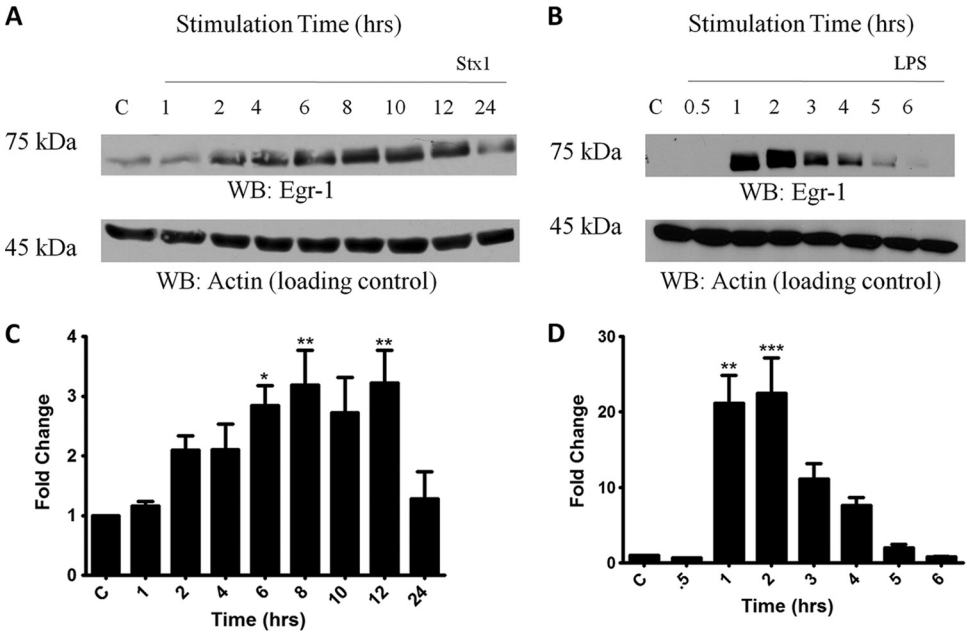

FIG. 4. Egr-1 protein levels in macrophage-like THP-1 cells. Cells were stimulated with Stx1 (400 ng/ml; panels A and C) or LPS (200 ng/ml; panels B and D) for various times. Whole-cell lysates (100 μg) were subjected to SDS–4 to 20% PAGE and probed with a polyclonal antibody against Egr-1. An antibody against actin was used to normalize protein loading. The blots shown in panels A and B are representative of at least three independent experiments. Panels C and D show the mean fold change ± the SEM calculated from densitometric scanning of at least three individual experiments. Statistical significance was calculated by using one-way ANOVA (*P* value: *, <0.05; **, <0.01; ***, <0.001).

treatment. Therefore, we show that while Stx1 caused a slow, constant increase of low levels of COX-2 expression, LPS triggered a rapid, transient increase of a much greater magnitude. When cells were treated with both stimulants, we expected to see a pattern similar to what was observed for the Egr-1 transcript, that is, the rapid induction of high levels of mRNA expression. However, when THP-1 cells were treated with both Stx1 and LPS, COX-2 mRNA was detected as early as 15 min (2.6-fold) but peaked at 2 h (721.5-fold) with much less magnitude compared to that seen with the LPS treatment (Fig. 6C), a phenomenon not observed in the Egr-1 transcript. At longer time points, the fold induction of COX-2 expression was increased in Stx1+LPS-treated cells. For example, at 6 h after stimulation, COX-2 expression was increased 3.5-fold by Stx1, 86-fold by LPS, and 156-fold by Stx1+LPS. Thus, Stx1 appeared to dampen the effect of LPS on the induction of peak levels of COX-2 mRNA expression in macrophage-like THP-1 cells, but higher levels of COX-2 mRNA expression were maintained over time when the cells were simultaneously treated with both bacterial products.

When we studied protein levels of COX-2 in cells treated with Stx1 we found that COX-2 levels increased as early as 1 h (2.5-fold) and remained relatively constant, peaking at 6 h (3.8-fold) and later decreasing slightly (Fig. 7A and C). LPS is considered a potent activator of COX-2 expression (17). Surprisingly, when we measured protein levels of COX-2 in LPS

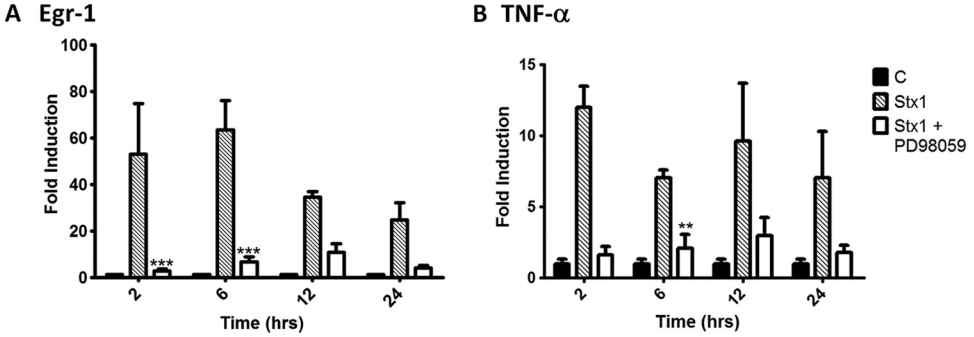

FIG. 5. Stx1-induced ERK1/2 signaling regulates Egr-1 and TNF-α mRNA expression in macrophage-like THP-1 cells. Cells were stimulated with Stx1 (400 ng/ml) in the presence or absence of the ERK1/2 inhibitor PD 98059 (50 μM) for different times. Total RNA was isolated, DNase treated, and cDNA synthesized. Quantitative real-time PCR was performed using primers specific for Egr-1 (A) and TNF-α (B) and normalized to GAPDH. Relative expression was calculated by using the ΔC_T method. The data shown are the fold induction values ± the SEM from at least three independent experiments. Statistical significance was calculated by using two-way ANOVA (*P* value: **, <0.01; ***, <0.001 [Stx1 versus Stx1+PD98059]).

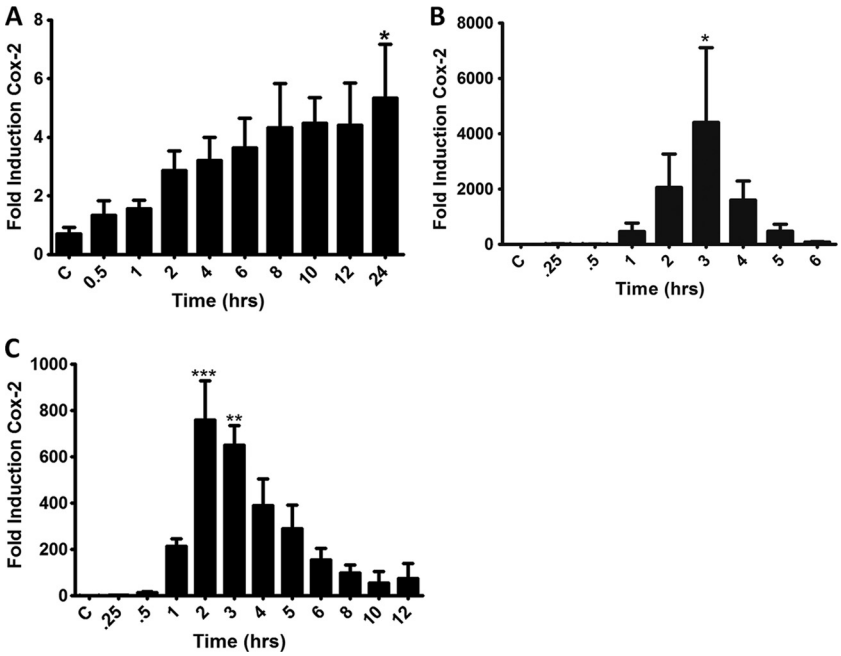

FIG. 6. COX-2 mRNA expression by macrophage-like THP-1 cells. Cells were stimulated with Stx1 (400 ng/ml) (A), LPS (200 ng/ml) (B), or Stx1+LPS (400 and 200 ng/ml, respectively) (C) for different times. Total RNA was isolated, DNase treated, and cDNA synthesized. Quantitative real-time PCR was performed with primers specific for COX-2 and GAPDH. The relative expression was calculated by using the ΔC_T method. The data shown are the mean fold induction ± the SEM from at least three independent experiments. Statistical significance was calculated by using one-way ANOVA (*P* value: *, <0.05; **, <0.01; ***, <0.001).

treated THP-1 cells, we found similar induction kinetics and quantities compared to those elicited by Stx1 (Fig. 7B and D). Thus, for reasons that remain to be explored, the high levels of COX-2 transcript induced by LPS did not correlate with equivalent increases in COX-2 protein levels.

Eicosanoid production in macrophage-like THP-1 cells treated with Stx1. COX-2 transforms arachidonic acid (AA) to eicosanoids. First AA is converted to prostaglandin G_2 and H_2, which are then transformed to prostaglandins, prostacyclins or thromboxanes. Induction of COX-2 leads to the production of eicosanoids, which may eventually cause an inflammatory reaction in the host (52). For this reason, we measured the levels of a key proinflammatory prostaglandin, PGE_2, and the vasoactive eicosanoid thromboxane, TXB_2, in culture supernatants collected from cells stimulated with Stx1 (400 ng/ml) or LPS (200 ng/ml) for various time points. We found a time-dependent increase of both PGE_2 and TXB_2 in cells treated with Stx1 (Fig. 8A and B). Levels above baseline were detected after 6 h (PGE_2) and 8 h (TXB_2) of treatment and continued to increase, reaching their maximum levels after 24 h. Thus, the synthesis of PGE_2 and TXB_2 correlates with COX-2 mRNA and protein expression/production patterns induced by Stx1 treatment. LPS treatment also resulted in a time-dependent increase of PGE_2 production with a greater magnitude than Stx1 treatment (Fig. 8C) that mirrored the COX-2 protein expression pattern seen in Fig. 7D, reaching maximal production at 8 h (678 pg/ml).

Dual-specificity phosphatase expression induced by Stx1 and LPS. We have previously shown that Stx1 triggers the activation of the MAPK cascades JNK, p38, and ERK (5). Also known as MAPK phosphatases (MKPs), DUSPs are enzymes which dephosphorylate and inactivate MAPK isoforms in mammalian cells. Significant upregulation of three DUSPs (DUSP1, -5, and -10) in cells treated with Stx1 was seen by the microarray analysis and validated by real-time RT-PCR. The observation that Stx1 induces DUSP transcripts suggests that signaling pathways to counterbalance MAPK activation by Stx1 may be operative in macrophage-like THP-1 cells. Therefore, we analyzed the mRNA kinetics of all three phosphatases. In cells stimulated with Stx1, DUSP1 mRNA levels increased as early as 30 min (2.6-fold) and continually increased, peaking at the 24-h time point (24.6-fold). When cells were stimulated with LPS, the kinetics of induction was different from Stx1 treatment; DUSP1 mRNA was detected at 15 min (5.4-fold), peaking at 3 h (154.6-fold), and decreasing slowly to 62.7-fold at 6 h. When cells were treated with both stimulants, a biphasic pattern was observed. DUSP1 mRNA was detected as early as 15 min (6.9-fold), peaking at 3 h (171.8-fold) decreasing to 57.7-fold at 4 h and peaking again at 12 h with more intensity than the first peak (501.5-fold) (Table 3).

DUSP5 mRNA expression in cells treated with Stx1, followed a similar pattern to DUSP1. Elevated DUSP5 mRNA was detected later, at 4 h (1.5-fold), steadily increasing until reaching a maximum of 7.6-fold after 24 h. LPS kinetics were faster and stronger but also sustained. Transcript was detected after 15 min of exposure to LPS (4.1-fold), increased in a time-dependent fashion and peaked after 6 h (1,117-fold), never decreasing. In cells treated with both Stx1 and LPS, DUSP5 transcript was detected after 15 min (1.8-fold) and increased steadily throughout the time course, peaking at 12 h (3,063-fold) (Table 3).

As for DUSP10 expression in cells treated with Stx1, tran-

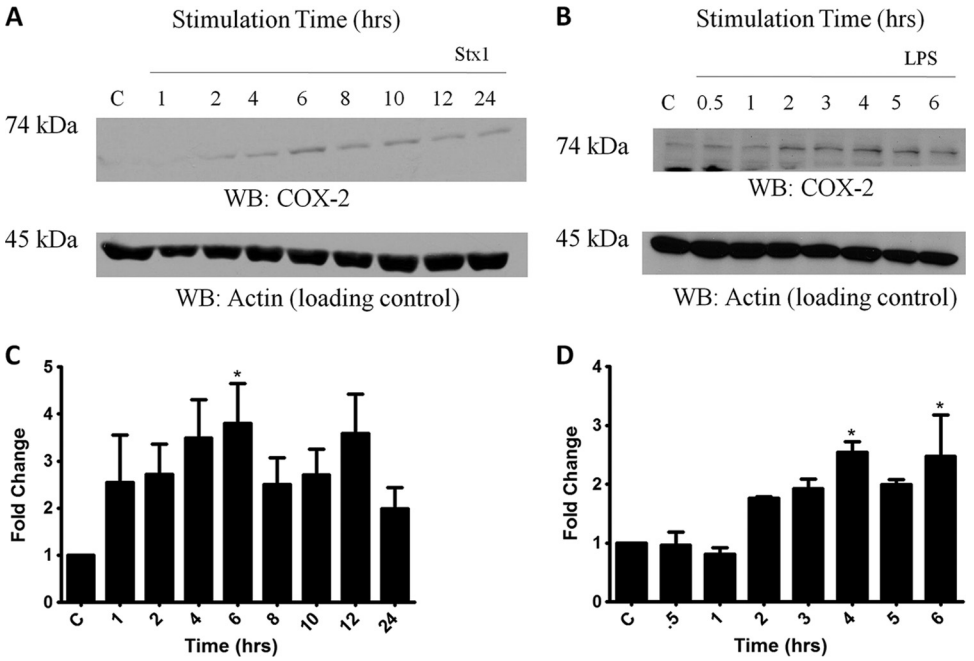

FIG. 7. COX-2 protein levels in macrophage-like cells. Cells were stimulated with Stx1 (400 ng/ml; panels A and C) or LPS (200 ng/ml; panels B and D) for various times. Whole-cell lysates (100 μg) were subjected to SDS–4 to 20% PAGE and probed with a polyclonal antibody against COX-2. An antibody against actin was used to normalize protein loading. The blots shown in panels A and B are representative of at least three independent experiments. Panels C and D show the mean fold change ± the SEM calculated by densitometric scanning of at least three individual experiments. Statistical significance was calculated by using one-way ANOVA (*P* value: ∗, <0.05).

script was detected after only 30 min (1.5-fold) and increased slowly, reaching its maximum after 24 h (10-fold). LPS was not a stronger inducer than Stx1 for DUSP10. Furthermore, the kinetics of DUSP10 induction by LPS was quite different from other genes analyzed. DUSP10 transcript was not detected until 1 h after treatment initiation (1.7-fold), slowly increased until peaking after 5 h (3.2-fold). When cells were treated with both Stx1 and LPS, we did not observe an additive effect on

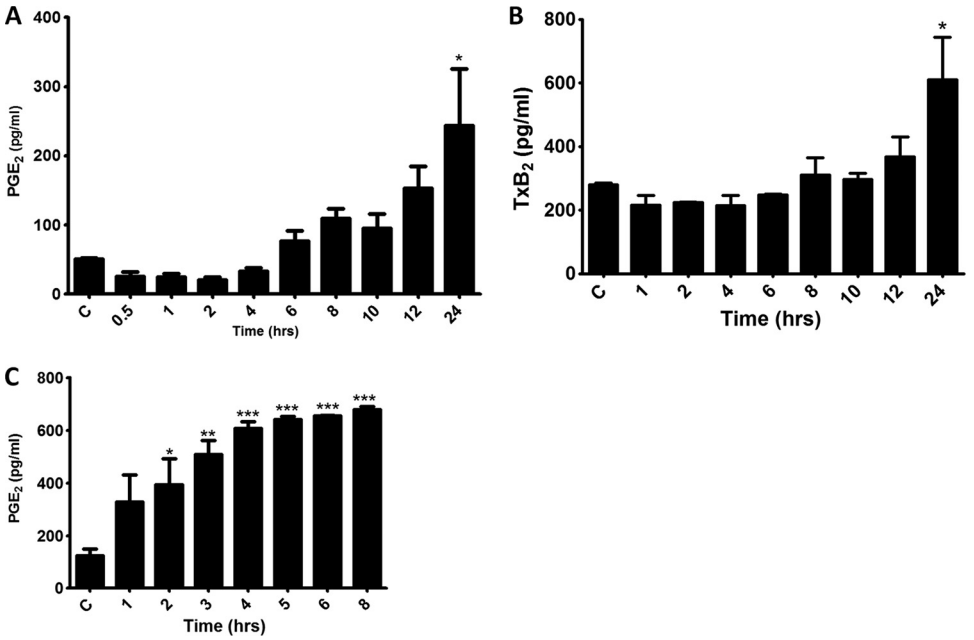

FIG. 8. PGE_2 and TxB_2 production in macrophage-like THP-1 cells stimulated with Stx1 (400 ng/ml; panels A and B) or LPS (200 ng/ml; panel C). PGE_2 and TxB_2 levels were measured in supernatants collected from cells stimulated with Stx1 or LPS for various times by using a sandwich ELISA. Eicosanoid standards were provided with the kits used. Values are given as pg/ml and are expressed as the mean ± the standard error of three independent experiments. Statistical significance was calculated by using one way ANOVA (*P* value: ∗, <0.05).

TABLE 3. Dual-specificity phosphatase mRNA expression in differentiated THP-1 cells treated with Stx1 (400 ng/ml), LPS (200 ng/ml), or Stx1+LPS (400 and 200 ng/ml, respectively) for various times

Time point (h)	Dual-specificity phosphatase mRNA expression (mean fold change ± SE)[a]								
	Stx			LPS			Stx+LPS		
	DUSP1	DUSP5	DUSP10	DUSP1	DUSP5	DUSP10	DUSP1	DUSP5	DUSP10
Control	0.7 ± 0.2	0.6 ± 0.3	0.8 ± 0.1	0.7 ± 0.4	1.4 ± 0.7	0.7 ± 0.4	0.7 ± 0.30	1.4 ± 0.7	0.8 ± 0.2
0.25	–	–	–	5.4 ± 3.7	4.1 ± 1.6	0.7 ± 0.1	6.9 ± 2.1	1.8 ± 0.9	1.2 ± 0.1
0.5	2.6 ± 0.8	0.6 ± 0.2	1.5 ± 0.3	50.5 ± 38.8	2.1 ± 1.9	1.0 ± 0.8	43.2 ± 11.0	2.0 ± 0.9	0.9 ± 0.3
1	2.9 ± 0.6	0.8 ± 0.2	1.8 ± 0.5	131.6 ± 56.5	12.8 ± 11.6	1.7 ± 1.4	141.3 ± 16.8	11.2 ± 6.6	1.8 ± 0.4
2	11.8 ± 4.0	1.0 ± 0.1	1.7 ± 0.8	141.2 ± 76.0	137.2 ± 108.4	1.2 ± 0.9	124.4 ± 47.5	234.7 ± 167.0	2.2 ± 0.6
3	–	–	–	154.6 ± 50.3	420.1 ± 248.5	2.0 ± 0.4	171.8 ± 119.2	839.8 ± 701.6	2.9 ± 1.8
4	18.5 ± 6.0	1.5 ± 0.2	1.9 ± 0.4	77.0 ± 6.5	416.6 ± 198.7	2.0 ± 0.5	57.67 ± 3.3	496.7 ± 323.6	1.8 ± 0.2
5	–	–	–	57.0 ± 18.4	705.7 ± 95	3.2 ± 0.1	142.3 ± 1.7	730.7 ± 314.8	2.8 ± 0.9
6	15.9 + 4.1	2.0 ± 0.3	2.4 ± 0.4	62.7 ± 45.3	1,117.0 ± 523.4*	3.0 ± 0.1	170.6 ± 49.9	979.0 ± 499.6	3.5 ± 1.8
8	14.6 ± 3.5	3.2 ± 0.6*	3.6 ± 1.2	–	–	–	292.7 ± 31.3**	1,214.0 ± 461.4	3.8 ± 0.5
10	19.8 ± 7.3*	3.8 ± 0.7*	3.1 ± 0.7	–	–	–	235.6 ± 24.0*	1,594.0 ± 489.3	6.9 ± 0.6
12	16.9 ± 5.4	3.3 ± 0.9*	4.2 ± 1.4	–	–	–	501.5 ± 8.7***	3,063.0 ± 1119.0*	15.3 ± 3.6*
24	24.6 ± 76.2**	7.6 ± 0.9*	10.0 ± 3.0***	–	–	–	–	–	–

[a] –, Data not available. *P* value: *, <0.05; **, <0.01; ***, <0.001 (compared to the control). Means and standard errors are shown based on at least three independent experiments.

DUSP10 transcription as seen in other genes. DUSP10 mRNA was detected after 15 min and increased slowly, peaking at 12 h (15.3-fold) (Table 3).

DISCUSSION

We have previously shown that human macrophage-like THP-1 cells are relatively resistant to the cytotoxic action of Stx1, with a 50% cytotoxic dose (CD_{50}) of >1.0 μg/ml (38). Furthermore, we used [^{3}H]leucine incorporation experiments to demonstrate that treatment of THP-1 cells with sublethal amounts of Stx1 (400 ng/ml) mediated a transient increase in protein synthesis (7). We showed that much of this increased synthetic capacity was directed to the production of cytokines (IL-1β, IL-6, and TNF-α) and chemokines (IL-8, MIP-1α, MIP-1β, MCP-1, and GRO-2) (13, 14). The purpose of the present study was to more fully evaluate the transcriptional response of macrophage-like cells in response to Stx1.

We found that 36 genes were upregulated by this treatment, with 14 genes being uniquely upregulated by Stx1. These genes fell into the following functional categories (listed in order of decreasing frequency): transcriptional regulation, unknown function, apoptosis, cell signaling, chromatin structure, inflammation, cell proliferation, cell cycle, and cell adhesion. A complete listing of these genes is found in Table S1 in the supplemental material. Interestingly, no genes were downregulated by Stx1 treatment of THP-1 cells. In comparison to LPS, Stx1 affected the transcription levels of a relatively limited number of genes. These findings appear to be consistent with earlier microarray analyses using Stxs. Keepers et al. (20) performed microarray analysis on unfractionated murine renal cells treated with Stx2, LPS, or Stx2+LPS and detected, respectively, 136, 737, and 722 significantly expressed genes. In murine cells, genes uniquely upregulated by Stx2 encoded proteins with functions related to transcriptional regulation, cell proliferation, and cell cycle regulation. Matussek et al. (27) treated human umbilical vein endothelial cells with sublethal amounts of Stxs for 24 h and showed that 20 genes were upregulated by Stx1 and 15 genes were upregulated by Stx2. Most of the genes altered by toxin exposure encoded cytokines and chemokines (IL-8, CSF2, GRO-1, GRO-2, and GRO-3).

A comparison of toxin-specific gene induction in endothelial cells revealed interesting differences. The genes encoding the cytokine IL-6 and the chemokine MCP-1 appeared to be uniquely activated by Stx2, whereas Stx1 selectively activated the expression of genes encoding the transcription factors Egr-1, Fos, and JunB and the phosphatase DUSP1. In accordance with these results, we have shown that both Egr-1 and DUSP1 are activated in Stx1-treated THP-1 cells. Epidemiological studies suggest that infection with STEC expressing Stx2 or Stx2 variant toxins constitutes a greater risk for progression to life-threatening extraintestinal complications (2, 46). It is intriguing to speculate that differences in the capacity of the toxin types to alter the expression of genes encoding inflammatory mediators or transcriptional regulators may be associated with differences in disease progression. However, more comparative analyses of gene expression using Stx1 and Stx2 with multiple cell types will be necessary to establish a correlation between toxin-mediated changes in gene expression and disease outcome.

It should be noted that the microarray data reported here were subjected to three independent, stringent statistical algorithms and the biological relevance was measured. Thus, there is a high level of confidence in the list of genes reported to be upregulated by Stx1. However, this approach is likely to underestimate the total numbers of genes whose expression may be altered by Stx1. In this regard, 149 probe sets were filtered out of the analysis based on failure to meet all of the criteria for statistical significance, including genes whose expression has previously been reported to be upregulated by Stxs: TNF-α, IL-8, MIP-1α (CCL3), MIP-1β (CCL4), and GRO-β (CXCL2) (see Table S2 in the supplemental material). Furthermore, the microarray analysis was limited to a single time point, 6 h of Stx1 (or LPS) stimulation. Our previous analyses of the kinetics of the THP-1 cell cytokine/chemokine response to Stx1 suggested that this time point should be near optimal for transcriptional activation (13–14); however, a more com-

plete time course of changes in gene expression triggered by Stx1 will be necessary to fully characterize the macrophage response to the toxins.

Since the majority of the upregulated genes fell into the transcriptional regulation category, and Egr-1 expression was shown to be upregulated by Stx2 in murine kidney cells (20) and by Stx1 in human endothelial cells (27), we decided to further explore the expression kinetics of Egr-1 in response to Stx1, LPS and Stx1+LPS treatment. Egr-1 is an immediate-early transcription factor that is known to play a major role in regulating the expression of TNF-α, ICAM-1, MCP-1, MIP-1, IL-1β, and IL-6, proteins that are known to be produced in response to Stxs (3, 13–14, 20, 27, 38–39). Thus, signaling through Egr-1 in macrophages may lead to the production of cytokines and chemokines, the promotion of tissue damage, and the induction of leukocyte recruitment and activation in the various target organs affected by Stxs. We detected upregulated expression of this gene with all treatments, albeit with different kinetics. Egr-1 mRNA and protein production were slowly induced by Stx1, compared to LPS, and the peak fold increase in Egr-1 protein level induced by Stx1 was only ~15% of that induced by LPS. LPS is a well-known stimulator of Egr-1 expression, and our results showing rapid mRNA and protein levels are in agreement with previous studies (11). Patients with bacillary dysentery may be endotoxemic (24) and those with hemorrhagic colitis may express elevated titers of antibodies directed against STEC O antigens (18), suggesting that LPS may access the circulation after intestinal epithelial barrier damage or perturbation. Therefore, we treated THP-1 cells with Stx1+LPS. Treatment of THP-1 cells with both stimulants resulted in a combination of the individual kinetic profiles; that is, Egr-1 transcripts were rapidly induced as seen after LPS treatment, and yet prolonged elevations in transcript levels were detected, as was the case after Stx1 treatment. This pattern of gene expression is a phenomenon that has been seen for other genes induced by Stx1+LPS treatment, including those that code for TNF-α and IL-1β (14).

Egr-1 is an 80-kDa phosphoprotein that contains multiple zinc-finger DNA-binding domains. In response to external stimuli such as LPS, cytokines, growth factors, hypoxia, or generation of reactive oxygen intermediates, Egr-1 is translocated to the nucleus and binds to *cis*-active CG-rich sequences located in many promoter elements (4). The promoter for the *egr-1* gene contains five sequential serum response elements (SREs) capable of binding serum response factor and ternary complex factors (TCFs) such as Elk-1, as well as binding sites for NF-κB, Sp1, and CRE (48). Stimuli that activate the JNK and p38 MAPK cascades phosphorylate TCFs, which subsequently facilitates TCF binding to SREs and *egr-1* gene activation (reviewed in reference 31). We have previously shown that Stx1 activates the NF-κB, JNK, p38, and ERK pathways in macrophage-like THP-1 cells (5, 39). To link Stx1-induced ERK activation with signaling through Egr-1 for increased TNF-α expression, we showed that the ERK inhibitor PD 98059 significantly reduced the rapid activation of *egr-1* expression and TNF-α production in response to the toxin.

We also explored the expression patterns of COX-2 in response to the different treatments. COX-2 catalyzes the rate-limiting step in the formation of eicosanoids from arachidonic acid that is released from the plasma membrane by phospholipase A_2. COX-2 is inducible and remains undetectable in most mammalian tissues under basal conditions (52). We found that Stx1 induced modest (~4-fold) increases in COX-2 mRNA and protein levels over a 24-h time course. In contrast, LPS dramatically increased COX-2 mRNA expression in a transient manner but elicited levels of COX-2 protein that were roughly comparable to that elicited by Stx1. The lack of correlation between LPS-induced COX-2 transcript expression and protein production in THP-1 cells is currently not understood. Treatment of THP-1 cells with both stimulants resulted in rapid COX-2 transcript induction similar to that seen with LPS treated cells; however, the magnitude of induction was only ~20% of that induced by LPS alone. This phenomenon of reduced COX-2 mRNA and protein production was also reported for human glomerular endothelial cells treated with Stx1+LPS compared to LPS alone (41). We also noted reduced levels of IL-8 after treatment of THP-1 cells with Stx1+LPS compared to LPS alone (13). These data suggest that Stxs may selectively dampen some components of the inflammatory response elicited by LPS when both bacterial products are present.

To explore the functional consequences of increased COX-2 expression, we measured PGE_2 and TxB_2 release by macrophage-like THP-1 cells treated with Stx1 or LPS. PGE_2 is synthesized by many cell types and is considered the principal prostaglandin in acute inflammation (33). Thromboxane (TxA_2) is a potent vasoconstrictor and platelet-aggregating eicosanoid that is produced by platelets and macrophages among other cell types and is measured indirectly by its stable metabolite TxB_2 (29). We found that Stx1 induces both PGE_2 and TxB_2 release at relatively late time points after intoxication, while LPS induces a rapid release of PGE_2 that increases in a time-dependent fashion and with higher magnitude. Adler et al. (1) and Schmid et al. (41) previously observed TxB_2 release in rat and human glomerular epithelial and endothelial cells treated with Stx1, and it is noteworthy that renal TxB_2 biosynthesis is increased in children suffering from HUS (50). Although we did not measure prostacyclin (PGI_2) production by THP-1 cells, Stx1 treatment of epithelial and endothelial cells has been reported to increase PGI_2 release (1, 41). The vasodilatory and platelet aggregation inhibitory activities of PGI_2 are thought to counterbalance the activities of TxA_2. Clinical studies have reported both increased and decreased serum and urinary PGI_2 levels in HUS patients; however, studies in the primate model of Stx1-induced acute renal failure did not show a clear correlation between increased renal PGI_2 production and the development of renal pathology (42).

Finally, we looked at dual specificity phosphatase expression in THP-1 cells treated with Stx1. These enzymes dephosphorylate and inactivate MAPK isoforms in mammalian cells by specifically dephosphorylating threonine and tyrosine residues (32). We have previously shown that in macrophage-like THP-1 cells treated with Stx1, multiple MAPK pathways are activated. Prolonged activation of JNK and p38 MAPKs was found when cells were treated with Stx1 alone, whereas ERK was only modestly activated for a short period of time (5, 8). We found that DUSP1 mRNA was rapidly induced by Stx1 treatment. DUSP1 mRNA expression in response to Stx1 treatment has also been shown in Caco-2 and human umbilical vein endothelial cells (23, 27). Similar to the other genes ana-

lyzed here, the magnitude of the DUSP1 response was much higher when cells were treated with LPS and even higher when both stimulants were used compared to DUSP1 expression induced by Stx1 alone. To our knowledge, ours is the first study to show that DUPS5 and DUSP10 transcript expression is increased in cells treated with Stx1. Like DUSP1, DUSP5 showed similar mRNA expression patterns with all of the treatments, but this was not the case for DUSP10, where Stx1 and LPS had very similar, rather modest effects on *DUSP10* transcription. Higher expression of these phosphatases at later time points after intoxication corresponds with the decline in MAPK activation we have previously reported (5, 8), suggesting that DUSPs may act as negative regulators of Stx1-mediated cytokine/chemokine expression. The precise balance of kinase and phosphatase expression and activation will be explored further in future studies.

In summary, our data suggest that a select subset of genes is activated in the macrophage after exposure to purified Stx1. Over one-third of these genes encode transcriptional regulators. Some of these genes are also activated by the bacterial outer membrane component LPS, while others appear to be uniquely activated by Stx1. The microarray data suggest that macrophages may play an important role in the pathogenesis of disease caused by Stxs. There is monocyte infiltration into the kidney during the course of HUS, and elevated levels of MCP-1 have been detected in the urine of HUS patients. These findings have been further confirmed using a murine HUS model (19, 55). The interaction of Stxs with macrophages (i) increases the expression of the immediate-early transcription factor Egr-1, which positively regulates cytokine and chemokine expression; (ii) increases the expression of COX-2, an enzyme involved in the generation of inflammatory and vasoactive eicosanoids; and (iii) increases the expression of DUSPs which may disrupt MAPK signaling and downregulate the inflammatory response.

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