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GeneChip Analyses of Global Transcriptional Responses of Murine Macrophages to the Lethal Toxin of *Bacillus anthracis*

Jason E. Comer,[1] Cristi L. Galindo,[2] Ashok K. Chopra,[2]*† and Johnny W. Peterson[2]*†

Departments of Experimental Pathology[1] and Microbiology and Immunology,[2] The University of Texas Medical Branch, Galveston, Texas

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We performed GeneChip analyses on RNA from *Bacillus anthracis* lethal toxin (LeTx)-treated RAW 264.7 murine macrophages to investigate global effects of anthrax toxin on host cell gene expression. Stringent analysis of data revealed that the expression of several mitogen-activated protein kinase kinase-regulatory genes was affected within 1.5 h post-exposure to LeTx. By 3.0 h, the expression of 103 genes was altered, including those involved in intracellular signaling, energy production, and protein metabolism.

Bacillus anthracis, the etiological agent of anthrax, causes fatal infections in humans and has gained notoriety as a bioterrorism weapon (10). *B. anthracis* produces two bipartite proteinaceous toxins, which are associated with virulence of the bacterium (6, 7, 41). Lethal toxin (LeTx) is comprised of protective antigen (PA), which is responsible for binding and entry into host cells (6), and lethal factor (LF), a zinc protease that cleaves mitogen-activated protein kinase kinases (MAPKKs) (11, 48, 63). LeTx is specifically cytotoxic for macrophages from certain inbred mouse strains (15, 55), which is likely physiologically relevant to human infection, since necropsy of anthrax inhalation victims revealed extensive macrophage apoptosis (22).

Resistance of some murine macrophages to LeTx-induced cytotoxicity was shown to be associated with alterations in the kinesin motor protein Kif1C (64), which was up-regulated in LeTx-sensitive macrophages and down-regulated in resistant cells (60). Further, it was shown by using DNA membrane arrays that genes under the regulation of glycogen synthase kinase 3β (GSK-3β) were down-regulated after LeTx treatment in macrophages (60). GSK-3β is implicated in cell fate determination and differentiation and is also involved in energy metabolism. GSK-3β is inhibited via phosphorylation by protein kinase A (PKA), PKB (also called Akt), PKC, and 90-kDa ribosomal subunit S6 kinase (p90RSK) (13, 31). LeTx-induced inhibition of MAPKKs blocked induction of certain NF-κB target genes, such as p38, allowing apoptosis of activated macrophages (13, 31).

Although an earlier study suggested that LeTx-mediated lethality of the host involved hyperproduction of cytokines by macrophages (24), more recently it was demonstrated that LeTx impaired host immune responses (12). Additionally, LeTx was reported to cause cell lysis via an increase in permeability to ions and rapid depletion of ATP (25), which might involve reactive oxygen species intermediates (26). Protein synthesis and proteasome activity are also required for LeTx cytotoxicity for macrophages, which suggests that degradation of survival factors might also be a potential mechanism of toxin-induced macrophage killing (4, 59).

To determine global host cell responses to intoxication, we employed Affymetrix Mouse Genome 430A 2.0 GeneChips (Santa Clara, Calif.) to investigate the expression profile of LeTx-treated macrophages and to identify potentially important genes involved in the mechanism of action of LeTx. GeneChips contained ~22,600 probe sets representing transcripts and variants from over 14,000 well-characterized mouse genes.

Expression profiles of LeTx-treated RAW 264.7 murine macrophages. RAW 264.7 cells were grown to 60% confluence (19) and treated with 1.0 μg of PA/ml and 0.2 μg of LF/ml (List Biological Laboratories, Campbell, Calif.) for 0, 1.5, or 3.0 h in three independent experiments. This 5:1 ratio of PA to LF provided optimal cytotoxic responses in macrophages in a 4-h period (unpublished data). The toxin components were analyzed for lipopolysaccharide (LPS) contamination by using Pyrogent Plus (BioWhittaker, Walkersville, Md.); no LPS was detected in PA, and LPS contamination (1 ng/μg) of LF was noted. To compensate for the potential effect of LPS, a control experiment was performed to examine alterations in cellular transcription with 0.2 ng of LPS/ml, which corresponded to the concentration present in 0.2 μg of LF/ml. We also performed an experiment using 1 μg of PA/ml alone in order to determine the contribution, if any, of the toxin-binding moiety to macrophage gene expression. LF alone is nontoxic to host cells, as it requires PA to bind to eukaryotic cells (14).

There was no detectable cell death at 1.5 h in LeTx-treated macrophages; however, by 2 h we could detect cell lysis (20 to 30%), which increased to 80% by 4 h, as determined by the release of lactate dehydrogenase enzyme from host cells by using the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega Corporation, Madison, Wis.) (data not shown). Therefore, these two time points (1.5 and 3.0 h) provided us with early and late samples, respectively, during the intoxication process. By 4 h, it was not possible to obtain enough RNA from macrophages to perform GeneChip analysis. After toxin challenge, RNA was isolated and applied to GeneChips and the data were analyzed using four separate techniques: GeneChip Operating Software (GCOS; Affymetrix), Significance of Analysis of Microarrays software (SAM; Stanford University,

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Medical Research Building, 301 University Blvd., Galveston, TX 77555-1070. For Ashok K. Chopra: Phone: (409) 747-0578. Fax: (409) 747-6869. E-mail: achopra@utmb.edu. For Johnny W. Peterson: Phone: (409) 772-4910. Fax: (409) 747-6869. E-mail: jpeterso@utmb.edu.

† A.K.C. and J.W.P. contributed equally to the manuscript.

TABLE 1. Significant genes up- or down-regulated by LeTx in RAW 264.7 murine macrophages from 0 to 1.5 h as determined by four separate analysis methods (grouped by function)[a]

Function and GenBank identification	Gene or gene product name	Result by method: GCOS (fold change)	Spotfire (fold change)	SAM (fold change)	ANOVA (*P* value)	LPS (GCOS; fold change)	PA (GCOS; fold change)
Inflammation							
L38281.1	Immune-responsive gene 1 (Irg1)	3.9	8.8	2.4	0.0251154		
NM_030612.1	Molecule possessing axkyrin repeats induced by LPS (mail; pending)	4	3.2	3.4	0.0305235	2.5	
NM_009140.1	Mip-2	6	6	3.4	0.037161	4.3	
Signaling							
NM_026268.1	Dual-specificity phosphatase 6 (Dusp6)	−3.5	−3.4	−3.2	9.10E−06		
BC011193.1	Prostaglandin E_2 receptor; EP4 subtype	2.3	2.3	2.3	0.0003018		
U72881.1	Regulator of G-protein signaling 16 (Rgs16)	3	3.1	1.9	0.003012		
AF282255.1	Calcipressin 1	3.3	4.6	3.4	0.0003941		
AF260717.1	Calcipressin 1	4.3	3.3	3.7	6.06E−07		
U94828.1	Regulator of G-protein signaling 16 (Rgs16)	11.2	20.3	9.4	1.48E−06		
Transcription factor							
AV026617	c-Fos	−3.4	−3.8	−3.5	0.0035431		
BC002081.1	c-Jun	−2.2	−2.7	−2.6	0.023096		
NM_007913.1	Early growth response 1 (Egr1)	−2.1	−2.8	−4.1	9.00E−05	1.9	
NM_008452.1	Kruppel-like factor 2	2.2	2.5	2.6	0.0003795		
AY061760.1	Nuclear factor, interleukin 3, regulated, (NFil3)	2.5	2.2	2.2	0.0004621		
X06746.1	Early growth response 2 (Egr2)	3.8	3	3	6.98E-07		
X06746.1	Early growth response 2 (Egr2)	4.1	3.5	3.4	0.0059769		
Other							
BB533903	Histone H1.2	2.1	2.1	2.1	0.011142		
NM_015786.1	Histone H1.2	2.4	2.5	2.3	0.0005401		
Unknown							
BB183628	cDNA	2	2	1.7	0.0023644		
BC025169.1	cDNA	2.7	3	3	0.0139313		
BG066184	cDNA	3.4	4.2	3.7	0.0020878		−3.2

[a] Genes listed twice were represented on the GeneChips by more than one probe set, and each was determined separately to be significantly up- or down-regulated by LeTx. A minus sign before the value indicates down-regulation of the gene.

Palo Alto, Calif.), Spotfire DecisionSite 7.3 software (Spotfire Inc., Somerville, Mass.), and analysis of variance (ANOVA).

Principal component analysis with Spotfire DecisionSite 7.3 software was also employed to describe general trends in gene expression induced by LeTx in macrophages (19). Expression of 98.7% of the genes was not altered by LeTx treatment. A total of 1.2% of the genes (270 probe sets) showed altered expression between 1.5 and 3.0 h during the intoxication process, while the expression of only 0.1% of the genes (22 probe sets) was changed from 0 to 1.5 h (data not shown). These data indicated that the major effects of LeTx on macrophage gene transcription occurred just prior to cellular death.

We compared the 0-h time points to 1.5 and 3.0 h, and a change in gene expression was considered significant if it met the following criteria: at least twofold alteration compared to control samples, a *P* value of 0.05 or less, and the occurrence of the change for at least two out of the three independent experiments. We further expected that each significantly altered gene would be identified by all of the analysis techniques that were used. Based on these stringent criteria, 17 genes were up- or down-regulated at 1.5 h and included those involved in inflammation, intracellular signaling, and regulation of transcription. Inflammation-related genes were also up-regulated in LPS (0.2 ng/ml)-treated macrophages, suggesting that residual LPS contamination of LF might have been responsible for the induction of these genes. Therefore, we very cautiously interpreted our data and focused on genes that were not induced by LPS.

Two probe sets representing the gene coding for regulator of G-protein signaling 16 (Rgs16) were up-regulated by 1.5 h in LeTx-treated macrophages (Table 1). Rgs proteins inhibit signal transduction by increasing the GTPase activity of G protein α subunits, thereby driving them into their inactive GDP-bound form. Rgs16 specifically inhibits $G\alpha_{i3}$ and subsequently antagonizes RhoA signaling (30), which perturbs several intracellular signaling processes, including regulation of the cytoskeleton (47, 52), phospholipid metabolism (2, 51, 65), cell migration (18), and gene expression through c-*fos* serum response element sites (28). Rgs16 also preferentially attenuates the activation of p38 (66), which, in addition to LeTx-mediated cleavage of MKK3 and MKK6 (62), might contribute to negative regulation of MAPKK signaling.

LeTx up-regulated prostaglandin E_2 receptor, subtype 4 (EP_4), by 1.5 h (Table 1). EP_4 stimulates cyclic AMP (cAMP) formation and can activate extracellular signal-regulated kinases 1 and 2 (ERK1/2) by way of phosphatidylinositol 3-kinase (16). EP_4 may also activate cAMP-independent signaling pathways, such as phosphatidylinositol 3-kinase-dependent stimulation of T-cell factor/lymphoid enhancer factor (Tcf/Lef) reporter activity (17), which is associated with agonist-dependent phosphorylation and inactivation of GSK-3 (8, 13, 16, 31, 57). LeTx-induced up-regulation of EP_4 correlates with pre-

vious observations (i.e., LeTx-induced down-regulation of GSK-3β target genes) (60).

Early growth response factor 2 (Egr2) was significantly up-regulated by 1.5 h in response to LeTx treatment of macrophages (Table 1). Egr2 is involved in the regulation of proliferation and differentiation of host cells (32). Egr2 plays a key role in the PTEN (phosphatase and tensin homolog on chromosome 10)-induced apoptotic pathway by altering the permeability of the mitochondrial membrane, thereby releasing cytochrome *c* and activating caspases 3, 8, and 9. Erg2 could also directly induce the expression of the proapoptotic proteins Bcl-2/E1B 19-kDa interacting protein-3-like (BNIP3L) and Bcl-2 homologous antagonist/killer (BAK) protein (61).

Genes that were down-regulated at 1.5 h were the gene that coded for dual-specificity phosphatase 6 (Dusp6), the FBJ osteosarcoma oncogene (c-Fos), and the Jun oncogene (c-Jun), with c-Fos having the greatest reduction in expression (Table 1). Dusp6 is a phosphatase that preferentially inactivates ERKs (43). The AP-1 transcription factor subunits c-Fos and c-Jun lie downstream of MAPK signaling, and since LeTx cleaves activators of MAPKs, their down-regulation was not surprising. Additionally, GSK-3β activates transcription of c-Jun (33), so down-regulation of c-Jun, as determined by our GeneChip analyses, correlates with previous studies that demonstrated LeTx-induced down-regulation of GSK-3β target genes (60).

By 3 h, expression of 103 genes was altered by at least twofold compared to the 0-h control as determined by the various statistical methods used (Table 2). Most of the up-regulated genes encoded inflammatory and signal transduction proteins, as well as several transcription factors. Of the 62 down-regulated genes, 35% were involved in protein metabolism, including genes that coded for 15 ribosomal subunits (represented by 19 probe sets). Several genes involved in the electron transport chain were also down-regulated, including ATP synthase epsilon chain, cytochrome *c* oxidase subunit VIIa polypeptide 2-like, NADH dehydrogenase (ubiquinone) 1 alpha subcomplexes 2 and 7, ubiquinol-cytochrome *c* reductase subunit, and ubiquinol-cytochrome *c* reductase complex 11-kDa protein, which indicated a decrease in energy production in intoxicated cells.

There were several cytoskeletal genes that were down-regulated by toxin treatment at 3 h (Table 2): thymosin beta 10 (Tmsb10), thymosin beta 4 (Tmsb4x), troponin I skeletal fast 2 (Tnni2), and cytoplasmic dynein light chain. Tmsb10 and Tmsb4x are actin-binding proteins involved in cytoskeleton organization and biogenesis (45). Tnni2, another actin-binding protein, is a structural constituent of the cytoskeleton (44), and cytoplasmic dynein light chain is a molecular motor involved in intracellular transport along microtubules (21).

There were three immune response-associated genes that were specifically up- or down-regulated by LeTx and not by LPS or PA: CD137 ligand, Toll-like receptor 2 (TLR2), and plasminogen activator inhibitor type I (PAI-1) (Table 2). CD137 ligand (also called 4-IBB ligand) was down-regulated by the toxin. This protein is a tumor necrosis factor (TNF) receptor (ligand) superfamily member that stimulates both primary and secondary responses of $CD4^+$ and $CD8^+$ T cells (9). Additionally, CD137 is constitutively expressed on primary monocytes and, upon cross-linking with immobilized CD137 ligand, mediates adherence, activation, proliferation, survival, expression of proinflammatory cytokines, and down-regulation of the anti-inflammatory cytokine interleukin-10 (35–37, 40, 53, 56). Furthermore, it has also been demonstrated that CD137 ligand is required for initiation of proliferation of monocytes in response to LPS (34).

TLR2 was up-regulated by LeTx at 3 h (Table 2). TLR2 is a receptor that binds components of gram-positive bacteria and is essential for the recognition of peptidoglycan (58). Engagement of TLR2 has also been shown to result in activation of NF-κB, cytokine production, and apoptosis (1). Alternatively, recent data have also suggested that several pathogens might utilize TLR2 as an immune escape mechanism via the production of anti-inflammatory cytokines (3, 20, 46, 50, 54).

PAI-1, which was significantly up-regulated by LeTx (3.8- to 9.6-fold by 3 h; Table 2) is the main physiological inhibitor of tissue-type and urokinase-type plasminogen activators. Up-regulation of PAI-1 results in local tissue fibrin deposition during severe inflammation (29, 38) and massive imbalance of the coagulation and fibrinolytic systems during bacterial infection-induced sepsis, which ultimately leads to multiorgan failure (5, 49). In fact, the actual level of PAI-1 increase correlates with the severity of disseminated intravascular coagulation and sepsis, which is a 100% predictor of lethality (5, 42). Induction of PAI-1 and subsequent fibrinolysis could also interfere with migration of immune cells. LeTx-induced down-regulation of CD137 and up-regulation of TLR2 and PAI-1 could have profound consequences on host immune responses during *B. anthracis* infection and will be pursued in future.

In addition to the previously mentioned Dusp6, which dephosphorylates ERKs, there were three signaling molecules that were preferentially down-regulated by LeTx at 3 h but were not altered by LPS or PA treatment alone. These included G protein, gamma 2 subunit (Gng2); cAMP-dependent protein kinase inhibitor gamma (PKIγ); and protein tyrosine phosphatase 4a2. On the other hand, protein tyrosine phosphatase non-receptor type 8 was up-regulated by LeTx (Table 2). The consequences of LeTx-mediated alteration in the expression of these genes are unclear but might be important for LeTx-mediated host responses to infection and will be further investigated.

There were several transcription factors that were up-regulated by LeTx by 3 h (Table 2), most notably activating transcription factor 3 (ATF3). ATF3 is a stress-inducible member of the ATF/CREB family of transcription factors (23), and induction of ATF3 is strongly associated with apoptosis (27). LeTx also induced alteration in the expression levels of zinc finger proteins, Kruppel-like factor 2, interleukin-3-regulated nuclear factor, orphan nuclear receptors, and TSC22-related transcription factor, which could contribute to LeTx-mediated host cell responses.

Confirmation of GeneChip data by real-time RT-PCR of selected genes. In order to confirm the GeneChip data, we performed real-time reverse transcription-PCR (RT-PCR) (39) on selected genes using the ABI Prism 7000 sequence detection system. TaqMan reverse transcriptase (Applied Biosystems, Foster City, Calif.) was used to synthesize cDNA from total RNA isolated from LeTx-treated cells. Experiments were performed in parallel, and fold values were determined after normalization of each gene to glyceraldehyde-3-phosphate dehydrogenase by the comparative threshold method.

TABLE 2. Significant genes up- or down-regulated by LeTx in RAW 264.7 murine macrophages from 0 to 3 h as determined by four separate analysis methods (grouped by function)[a]

Function and GenBank identification	Gene or gene product name	Result by method:					
		GCOS (fold change)	Spotfire (fold change)	SAM (fold change)	ANOVA (*P* value)	LPS (GCOS; fold change)	PA (GCOS; fold change)
Cell adhesion							
NM_020008.1	C-type (calcium-dependent, carbohydrate recognition domain) lactin, superfamily member 12	2.1	2	2	1.68E−05		
Cell cycle							
BC003290.1	Cyclin 1	−2	−2	−1.6	0.000866		
Cytoskeletal regulation							
AV148480	Thymosin, beta 10	−2.1	−2.2	−5	5.58E−05		
BB096368	Thymosin, beta 10	−2.4	−2.3	−3.5	0.000787		
NM_025284.1	Thymosin, beta 10	−2.4	−2.5	−2.5	0.001843		
NM_021278.1	Thymosin, beta 4	−2	−2	−4.7	0.000101		
NM_009405.1	Troponin I, skeletal, fast 2	−3.1	−2.3	−1.8	0.000574		
BG094946	Cytoplasmic dynein light chain	−2	−2.1	−6.3	4.98E−06		
Energy production							
NM_025983.1	ATP synthase epsilon chain, mitochondrial	−2.5	−2.7	−3.5	0.000783		
NM_025650.1	Ubiquinol-cytochrome *c* reductase (6.4-kDa) subunit	−2.5	−2.8	−2.3	0.022367		
AW106975	Ubiquinol-cytochrome *c* reductase complex 11-kDA protein (cDNA)	−2.3	−2.5	−2	0.00247		
C88880	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 7	−2.2	−2	−3.6	0.000554		
AK009614.1	Cytochrome *c* oxidase subunit VIIa polypeptide 2-like	−2.1	−2	−6.4	3.75E−06		
NM_010885.1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 2	−2	−2	−1.8	0.042225		
Immune response-inflammation							
NM_009404.1	CD137 ligand	−2.6	−2.3	−1.8	0.006252		
AF128218.1	Mip-1 beta	2.2	2.2	1.7	0.030223	3	
NM_011905.1	Toll-like receptor 2	2.3	2.1	2.1	0.001		
NM_009856.1	CD83 antigen	3.5	4.1	4.5	0.028976	2.5	1.6
NM_030612.1	Molecule possessing ankyrin repeats induced by LPS (mail; pending)	4.3	3.7	4	0.030523	9.8	
L38281.1	Immune-responsive gene 1 (Irg1)	6.5	7.3	3.5	0.025115	18.4	
NM_009140.1	Mip-2	9.4	8.9	8.4	0.037161	21.1	2
NM_008871.1	Plasminogen activator inhibitor, type I	9.6	6.1	3.8	0.00995		
Protein metabolism							
AV216370	Ribosomal protein L36	−2.4	−2.2	−1.9	0.004075		
NM_009084.1	Ribosomal protein L37a	−2.4	−2.4	−1.9	0.012705		
NM_018730.1	Ribosomal protein L36	−2.3	−2.3	−2	0.002914		
NM_009079.1	Ribosomal protein L22	−2.1	−2.1	−1.8	0.017672		
NM_025592.1	Ribosomal protein L35	−2.1	−2.2	−2.2	0.002808		
NM_018860.1	Ribosomal protein L41	−2.1	−2	−2	0.003252		
NM_023133.1	Ribosomal protein S19	−2.1	−2.2	−1.9	0.026968		
BF237074	Ubiquitin A-52 residue ribosomal protein fusion product 1 (Uba52)	−2	−2.4	−2.2	0.002938		
BC004625.1	Ubiquitin-like protein Nedd8	−2	−2.2	−3.9	0.000394		
NM_016738.1	Ribosomal protein L13	−2	−2.2	−1.8	0.003931		
AV124660	Ribosomal protein L26	−2	−2	−1.7	0.021368		
NM_009082.1	Ribosomal protein L29	−2	−2	−1.8	0.02696		
AV124739	Ribosomal protein L35	−2	−2	−1.9	0.010129		
AV124680	Ribosomal protein L35	−2	−2	−1.7	0.027161		
NM_018730.1	Ribosomal protein L36	−2	−2.1	−2.1	0.002701		
NM_019865.1	Ribosomal protein L44	−2	−2.3	−2.2	0.006207		
NM_009091.1	Ribosomal protein S15	−2	−2.1	−1.8	0.038687		
AV151252	Ribosomal protein S21	−2	−2.7	−1.9	0.018089		
AV123577	Ribosomal protein S21	−2	−2.6	−2	0.054336		
AA208652	Ribosomal protein S27	−2	−2	−1.7	0.015819		
AA030209	Ribosomal protein S17	2	2	1.9	7.50E-06		
Protein traffic king							
NM_024171.1	Sec61 beta subunit	−2.1	−2	−2	0.017967		
BO010487.1	ADP ribosylation factor	2.1	2.1	2.1	0.000341	−4.3	
Signaling							
AF260717.1	Calcipressin 1	6.1	6.6	5.8	6.06E−07		
BC021599.1	Guanine nucleotide binding protein (G protein), gamma 2 subunit	−5.4	−5.9	−3.8	0.017082		

Continued on following page

TABLE 2—*Continued*

Function and GenBank identification	Gene or gene product name	Result by method: GCOS (fold change)	Spotfire (fold change)	SAM (fold change)	ANOVA (*P* value)	LPS (GCOS; fold change)	PA (GCOS; fold change)
NM_026268.1	Dual-specificity phosphatase 6 (Dusp6)	−3.3	−3.5	−5.2	9.10E−06		
BC026550.1	Protein kinase inhibitor gamma (PKI gamma)	−2.6	−2.3	−2.1	0.021481		
BB780848	Pleckstrin homology domain-containing, family A (phosphoinositide-binding-specific) member 3 (Fapp1)	−2.2	−2.4	−1.8	0.001432	−1.6	
AW495875	Protein tyrosine phosphatase 4a2	−2	−2.1	−1.9	0.04473		
BC011193.1	Prostaglandin E_2 receptor, EP4 subtype	2.1	2	2	0.000302		
NM_008979.1	Protein tyrosine phosphatase, non-receptor type 8	2.1	2.3	2.3	0.000359		
NM_010907.1	NF-κB inhibitor alpha	2.2	2	1.9	8.61E-06	2.3	
AF326555.1	Phosphodiesterase 4B, cAMP specific	2.2	2.6	2.4	1.23E-06	4.3	
BB100249	Regulator of G-protein signaling 16 (Rgs16)	3.3	3.6	3.1	9.93E-07	1.5	
U94828.1	Regulator of G-protein, signaling 16 (Rgs16)	15.4	18	14.9	1.48E−06	3.5	
Stress-apoptosis							
AF274027.1	Glutathione peroxidase 4	−2.4	−2.1	−7.2	1.50E-06		
BF658806	Ribosome-associated membrane protein 4 (Ramp4)	−2.4	−2.7	−2.1	0.024068		
AK010420.1	Growth arrest and DNA damage-inducible 45 beta (GADD45 beta)	2.8	4.3	3.5	2.48E-06	3.7	
AI323528	Growth arrest and DNA, damage-inducible 45 beta (GADD45 beta) (cDNA)	3	2.9	2.8	6.43E−05	3.2	
Transcription factor							
NM_011980.1	Zinc finger protein 146 (Zfp146)	−10.4	−8.4	−4.1	0.00246		
BC002081.1	c-Jun	−2.8	−2.6	−2.6	0.023096	1.9	
BB031791	Zinc finger protein 36, C3H type-like 2	−2.4	−2.2	−2.2	0.004184		
NM_007913.1	Early growth response 1 (Egr1)	−2.2	−2.7	−4.5	9.00E−05	2.8	
BC019946.1	Activating transcription factor 3 (ATF-3)	2.1	2.7	1.7	0.030924		
AF155372.1	Nuclear factor κB subunit p100 (Nfkb2)	2.1	2.1	1.8	0.000173	3	
NM_011498.1	Basic helix-loop-helix domain, containing, class B2	2.3	2	1.7	0.019764		
NM_009046.1	Oncogene-related B (Rel-B)	2.5	2.6	2.3	2.01E−05	2.5	
NM_008452.1	Kruppel-like factor 2	2.6	3	3	0.00038		
AY061760.1	Nuclear factor, interleukin-3, regulated (Nfil3)	3.7	3.9	3.3	0.000462		
X06746.1	Early growth response 2 (Egr2)	3.8	7.8	3.9	0.005977	2	
BE824605	Orphan nuclear receptor Nr4a2	4.5	3.9	3.5	0.000169		
AF201289.1	TSC22-related inducible leucine zipper 3c (TSC-22R)	4.5	4.6	2.9	0.001233		
X06746.1	Early growth response 2 (Egr2)	5	5.4	4	6.98E−07	2.5	
NM_010444.1	Orphan nuclear receptor Nr4a1	9.3	6.9	6.8	0.0016022		
NM_013613.1	Orphan nuclear receptor Nr4a2	11.3	10	3	0.001257		
Transporter							
BB277461	Neutral amino acid transporter A	2.4	3.1	1.8	0.006518		
Other							
NM_007763.1	Cysteine-rich intestinal protein (Crip)	−2.4	−2.4	−3.5	0.000937		
NM_010240.1	Ferritin light chain 1	−2.1	−2.1	−5.5	2.45E-05		
NM_015786.1	Histone H1.2	2.2	2.2	2.2	0.00054		
BB533903	Histone H1.2	2.2	2.3	2.3	0.011142	−1.5	
W91024	Histone H2A	−2.1	−2.1	−4.8	7.56E−05		
U29539.1	Lysosome-associated protein transmembrane 5	2	2	2	4.63E-07		
AK004120.1	Nucleolar protein family A, member 3 (Nola3)	−2.1	−2.3	−2.1	0.019914		
BM210485	Plectin 1	2.1	2	1.6	0.009902	1.5	
U37500.1	RNA polymerase II largest, subunit (RPB1)	2	2.6	1.6	0.009562		
AA709993	Small EDRK-rich factor 1 (Serf1)	−2.2	−2.4	−1.8	0.011101		
NM_009121.1	Spermidine supermine N_1-acetyltransferase	−2.1	−2.1	−1.7	0.002182		1.5
NM_009169.1	Split hand foot deleted gene 1 (Shfdg1)	−2.1	−2	−1.9	0.016858		1.5
Unknown							
BE945188	cDNA	−2.2	−2.4	−2	0.010897		
AF353245.1	D12 protein	−2.1	−2.2	−2.2	0.001657		
AF353245.1	D12 protein	−2.1	−2.6	−2.1	0.001966		
AV012102	cDNA	−2.1	−2.2	−1.9	0.021583		
BC006603.1	cDNA	−2	−2.1	−1.8	0.004157		
W42220	cDNA	−2	−2	−1.9	0.008602		
BQ044335	cDNA	−2	−2.5	−1.8	0.009801		
AV170241	cDNA	−2	−2.1	−1.9	0.041788		
NM_138743.1	cDNA	−2	−2.2	−1.9	0.048444		
NM_022332.1	Suppression of tumorigenicity	2	2.5	2	0.000302		
AA049040	cDNA	2.1	2	1.9	0.009861	1.6	
BB408147	cDNA	2.4	2.1	2	5.01E−07		
BB183628	cDNA	2.4	2.1	2.1	0.002364		
BC025169.1	cDNA	2.8	3.2	2.5	0.013931		
BG066184	cDNA	9.2	9.4	4.2	0.002088		
NM_008383.1	Centrosomal protein 2	10.7	14.4	3.5	0.000368		

[a] Genes listed twice were represented on the GeneChips by more than one probe set, and each was determined separately to be significantly up- or down-regulated by LeTx. A minus sign before the value indicates down-regulation of the gene.

TABLE 3. Real-time RT-PCR confirmation of altered expression levels of selected genes at 3.0 h postchallenge[a]

GenBank identification	Gene or gene product name	GeneChip analysis				Real-time RT-PCR (fold change)
		Fold change			*P*	
		GCOS	Spotfire	SAM		
NM016738	Ribosomal subunit L13	−2	−2.2	−1.8	0.003931	−1.6
NM009079	Ribosomal subunit L22	−2.1	−2.1	−1.8	0.017672	−1.7
NM010885	NADH dehydrogenase (abiquinone) 1 alpha subcomplex 2	−2	−2	−1.8	0.042225	−1.5
BC002081	c-Jun	−2.8	−2.6	−2.6	0.023096	−2.4
NM026268	Dual-specificity phosphatase 6	−3.3	−3.5	−5.2	9.10E−06	−3.6
BC021599	Guanine nucleotide binding protein-(G protein), gamma 2 subunit	−5.4	−5.9	−3.8	0.017082	−1.6
NM008979	Protein tyrosine phosphatase, non-receptor type 8	2.1	2.3	2.3	0.000359	2.2
NM010907	NF-κB inhibitor alpha	2.2	2	1.9	8.61E−06	1.6
U94828	Regulator of G-protein signaling 16 (Rgs16)	15.4	18	14.9	1.48E−06	2.1

[a] A minus sign before the value indicates down-regulation of the gene.

We chose nine representative genes from the functional groups affected by LeTx for confirmation (Table 3). The ribosomal subunit genes L13 and L22, as well as NADH dehydrogenase 1 alpha subcomplex 2, were confirmed to be down-regulated at 3 h (Table 3), reinforcing the GeneChip data showing that LeTx inhibited protein synthesis and energy production in intoxicated cells. Alterations in the transcription of genes involved in MAPK and other signaling pathways (G; protein gamma 2 subunit and protein tyrosine phosphatase non-receptor type 8) were also confirmed by this technique.

According to GeneChip analysis and real-time RT-PCR confirmation, LeTx altered expression of genes involved in MAPK signaling cascades, including genes up- and downstream of the toxin's cleavage targets. LeTx also down-regulated electron transport chain genes and genes involved in protein synthesis. In our model, we were not able to confirm the observations of Tucker et al. (60) that expression of Kif1C was up-regulated in a LeTx-sensitive macrophage cell line. We did, however, observe decreases in c-Jun and c-Fos expression, confirming the suppression of GSK-3β-regulated genes. Although the exact mechanism of LeTx-mediated macrophage cytolysis remains a mystery, these data provided for the first time a comprehensive expression profile of intoxicated cells. More studies are required to determine how the altered gene expression induced by LeTx causes macrophage-specific cytolysis.

Considered together, the data provided potential intracellular targets of LeTx-mediated macrophage signaling. Additionally, the results suggested avenues through which LeTx might induce macrophage cytotoxicity, resulting in apoptosis or necrosis, which, based on our analyses, appears to depend upon the energy status of host cells. There were several genes that were up- or down-regulated by LeTx that were not previously known to play a role in LeTx-mediated host cell responses. These newly discovered genes are prospects for future investigations of the mode of action of LeTx, which, given the lethality of anthrax and its potential as a biological weapon, are important for developing better diagnostic and treatment strategies.

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