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Differentiating Gene Expression profiles in Staphylococcal Enterotoxin B and Lipopolysaccharide induced human PBMCs

Megan Linebach[1], Meghan Murphy[1], Mark Hiner[1], Rachel Staab[1], Nabarun Chakraborthy[2], Marti Jett[2] and Chanaka Mendis[1]

[1]*Department of Chemistry, University of Wisconsin-Platteville, Platteville, WI, USA,*
[2]*Division of Pathology, Walter Reed Army Institute of Research, Silver Spring, MD, USA*

Abstract

Staphylococcal enterotoxin B, an exotoxin produced by staphylococcal aureus, is commonly associated with food poisoning and capable of triggering flu-like symptoms resembling those of endotoxins such as lipopolysaccharide released from Gram negative bacterial cell walls. Even though both exhibit analogous symptoms, each has its unique gene expression profile. Here we have successfully distinguished two toxins that display similar symptoms in human peripheral blood mononuclear cells using multiple gene expression analysis. Human peripheral blood mononuclear cells are the most easily accessible bio fluid, and include informative transcripts as a first line of immune defense for many disease processes and toxin exposures. Thirty genes that provided a unique genetic signature to differentiate exposures to the two toxins were first identified through microarray analysis and then validated by using multiple gene and protein quantitation techniques such as reverse transcription polymerase chain reaction, real time polymerase chain reaction and Enzyme-linked immunosorbent assays. We believe that this study will provide valuable information to future investigations aimed at predicting, identifying and isolating various disease states and/or aberrant exposures in a short time period.

Keywords: Staphylococcal enterotoxin B; Lipopolysaccharide; Human-PBMCs; Gene expression patterns

Introduction

Initially identified in 1884 as a cause of boils impetigo and other minor skin disorders, staphylococcal enterotoxins (SEs) constitute a family of proteins that bind to T-cell receptors and major histocompatability antigen complexes. In addition, SE's induce massive activation of T cells in the host, food poisoning, autoimmune disorders, and toxic shock [1,2]. Staphylococcal Enterotoxin B (SEB), which is known to impinge on cellular pathways controlling apoptosis [3] induce symptoms such as vomiting, diarrhea, vertigo and muscle weakness. Following 24 hours of exposure, these symptoms may further develop to induce hypotension and vasodilation of blood vessels in kidneys and other organs [4].

Lipopolysaccharide (LPS), an endotoxin frequently seen in the cell wall of Gram-negative organisms, stimulates various immune responses including secretion of cytokines and activation of macrophages and lymphocytes [4]. PBMCs provide the first line of defense for many processes induced by diseases or toxin exposures and reflect each of these induced processes through characteristic molecular signatures that differentiate and predict the type/strength of induction in a rapid manner [5]. To study the molecular expression patterns induced by both SEB and LPS, we further investigated the gene expression patterns induced by both toxins in a time dependent manner. Since many pathogenic agents ultimately lead to lethal shock, the study presented here was designed to compare and contrast gene expression patterns induced in the host via systematic gene expression analysis techniques such as microarrays, reverse transcription polymerase chain reactions (RT-PCR) and real time PCR. The study identified a set of genes that showed a differentiated expression when exposed to the two toxins. These results are some-what useful in explaining the reason for two toxins (SEB and LPS) that incur similar symptoms, especially related to vascular collapse, proceed with vastly different initiation steps as well as mechanisms/pathways that in turn induce unique effects during the course of illness [4]. Ability to differentiate exposure to SEB and LPS that induce a set of initial symptoms through gene expression is more significant as conventional analysis compromising fluctuations in the body, symptoms, histopathological examination, and blood biochemical analysis have so far been unsuccessful in differentiating similar exposures. However, in the current study we were able to show an unique gene expression pattern for each toxin after validating though multiple gene analysis techniques. In addition, we were able to show a consistent expression both at gene and protein level for some of the genes. We believe that gene and protein expression patterns are powerful tools in differentiating toxins or diseases that show similar initial symptoms.

Materials and Methods

Cells and cell cultures

Exposure of human PBMCs to SEB in vitro: Human PBMCs were collected from a leukopack from a single since donar as described in Jett et al., [6]. Human PBMCs, with or without SEB or LPS, were used at a final density of 2.5×10^6 cells/ml in RPMI 1640 medium supplemented with 10% human AB serum for 2, 6 and 24 hours under 5% CO_2 at 37°C.

Extraction of RNA and protein: Total RNA were isolated using Trizol reagent (Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. Proteins were extracted after a brief sonication using a lysis buffer that contained 20 mM HEPES, 10 mM

***Corresponding author:** Chanaka Mendis, Department of Chemistry, College of EMS, UW-Platteville, 1 University Plaza, Platteville, WI 53818 USA, Tel: 608-342-1692; Fax: 608-342-1559; E-mail: mendisc@uwplatt.edu

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EGTA, 40 mM β-glycerophosphate, 2.5 mM $MgCl_2$, 1 mM DTT, 150 mM NaCl, 2 mM Sodium Orthovanadate, 1 mM PMSF, 10 µg/ml Leupeptin, 10 µg/ml Aprotinin, 1% NP40 and 0.5% Deoxycholate.

Quantification and analysis of RNA and protein: RNA preparations were routinely examined by 1% agarose gel electrophoresis to verify the integrity of the samples (twice the intensity of the 28S upper ribosomal band than lower band (18S). In addition, nano-drop (NanoDrop Technologies; Wilmington, DE) was used to measure the 260/280 ratios to further confirm the above results. All samples were aliqouted and were stored in 10 µL aliquots at -80°C. RNA and protein samples were quantified using a NanoDrop and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.; Santa Clara, CA).

Toxins: SEB from S. aureus strain 10-275 was purified by the method of Schantz and co-workers (Schantz et al., 1965) and provided in the lyophilized form by the U. S. Army Research Institute of Infectious Diseases (Ft. Detrick, Frederick, MD, USA). The stock solution was prepared in sterile, pyrogen-free deionized water at a concentration of 5 mg/ml and stored at -80°C. All dilutions were done using cell culture medium to obtain the desired concentration. Lipopolysaccharide (LPS); LPS (catalog # L2360-10 mg) was purchased from Sigma as a lyophilized powder that was purified by phenol extraction (Sigma, St Louis MS).

ELISA: Equal quantities of proteins obtained from human PBMCs, with or without treatment of 100 ng/ml SEB or LPS, for a time period ranging from 2-24 hours were subjected to ELISA analysis to quantitate TNF-α protein according to manufacturer's instructions (Bethyl Laboratories, Montgomery, TX). Proteins were quantified using a Ceres UV 900-Hdi plate reader (Bio-Tek Instruments Inc., Winooski, VT) and expressed as mean values together with standard deviations. Mouse Anti-Human CD39 Monoclonal Antibody (CAB-637MHCD Biosciences, Inc, NY, USA) was used at 0.5 - 1 µg/mL with the appropriate secondary reagents to detect human CD69 using the Bethyl ELISA detection kits as described in detail under TNF- α ELISA analysis.

Microarray analysis: Microarray experiments were performed to differentiate the gene expression patterns of the two toxins (SEB and LPS). 2000 well characterized cDNA from a custom cDNA library (WRAIR, Silver Spring, MD) were re suspended in replicates of two in 3X SSC at a concentration of 100-150 ng/µl and deposited at 200 µM center-to-center spacing at 60% humidity on optically flat 25×76 mm glass slides. These glass slides were coated with covalently attached linear primary amines (TeleChem International Inc., Sunnyvale, CA) using a SDDC-2 microarrayer (Engineering Sciences, Inc, Toronto, Canada) equipped with a surface contact print head SPH 48 (TeleChem Sunnyvale, CA) and 90-100 µM diameter quill SMP3 Stealth Micro Spotting Pins from TeleChem International Inc.

Total RNA from Control and SEB or LPS treated human PBMCs were reverse transcribed and the cDNAs were labeled with Cy3 (Control) or Cy5 (Treated) using the NEN Micromax TSA labeling and detection kit (Perkin Elmer, Boston, MA). The resulting cDNAs were hybridized at 65°C to microarrays, scanned using Gene Pix 4000B Microarray Scanner (Axon Instruments Inc., Foster City, CA) and analyzed using Gene Pix 3.0 software package.

Clustering analysis: Average linkage hierarchical clustering of an uncentered Pearson correlation similarity matrix was carried out using the program Cluster, and the results were visualized with the program TreeView. The data was analyzed using GeneSpring TM version 4.1 (Agilent, Inc., San Carlos, CA) to identify patterns of gene regulation in PBMCs exposed to SEB or LPS. To normalize for staining intensity variations among arrays, the average difference values for all genes on a given array were divided by the sum of all measurements on that array. In addition, the average difference value for each individual gene was then normalized to itself by dividing all measurements for that gene by the mean of the gene's expression values over all the samples. Normalized values below the background levels in both the control and treated were excluded. To identify genes that showed significant variations in expression between SEB compared to control cells and LPS compared to control cells, Student's t-test was performed by using $p<0.005$ as a threshold

Real time PCR: Real time PCR was performed in triplicates using a uniprimer containing a 3' oligonucleotide tail (Z sequence) and a housekeeping gene (S15) in an I Cycler (BioRad, Hercules, CA). Total RNA extracted from SEB induced or control PBMCs were subjected to real time PCR using Amplifluor universal amplification kit (Intergen, Purchase, NY). Threshold cycle (cT) of each reaction was calculated, and normalized using the cT values of S15 (housekeeping gene).

RT-PCR: RT-PCR analysis was performed using an iScript TM cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA). Housekeeping gene primers (18S) were obtained from Clontech Corp. Palo Alto, CA, USA, and all other primers were designed using various primer-design software. Complimentary DNA (cDNA) were amplified using PCR master kits (Roche Diagnostics Indianapolis, IN). Amplified genes were then analyzed on 1% agarose gels, visualized through an in house imager and quantified using NIH Image J analysis software. All custom primers except 18S were purchased from Invitrogen Carlsbad, CA, USA, designed to have an annealing temperature of 60°C and were subjected to PCR for 35 cycles in a thermocycler (PerkinElmer, Waltham, MA, USA). All sequences are indicated in 5' to 3' direction and are abbreviated after the gene name as L or R for reverse or forward respectively. COPG2-L: CCA ATC ACA TCG TGT TCC AG, COPG2-R: CTG CAA CTG CTG TAG GGT CA, ST6GALNAC6-L: AGG AGG GTC AGA GGA GAA GC, ST6GALNAC6-R: CAG ACC CTG ACT GCA CAA GA, TRIAD3-L: CGA GTC ATA ACC AAG CAG CA, TRIAD3-R: CTT CGA TGG CCT GAT CAT CT, TRIM26-L: CAG AGT GGA GTC CAG GCT TC, TRIM26-R: TGG ATG TGC TGA TCA AGA GC, PHPRP3P-L: ACC TTG CGC TGA GTG AAT CT and HPRP3P-R: TCT CCC TTT GTC CCA GTG TC

Results and Discussion

Results

Genes expression changes identified by microarray analysis: The alterations in gene expression induced by SEB and LPS to human PBMCs at the outset were examined using microarray analysis. Even though most of the 2000 genes analyzed did not exhibit significant changes, 30 of those genes showed unique differences in expression (Figure 1a and 1b). Remarkably, all 30 of these genes showed opposite regulation patterns when exposed to each of the two toxins (genes were down regulated when exposed to SEB and upregulated when treated with LPS). All 30 genes are listed in Table 1 with their known functions. Out of these 30 genes, five genes [Homo sapiens ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminidealpha-2,6-sialyltransferase,6 ST6GALNAC6), TRIAD3 protein transcript variant 3, mRNA (TRIAD3), tripartite motif-containing 26 (TRIM26), coatomer protein complex, subunit gamma 2 (COPG2), and Homo sapiens PRP3 pre-mRNA processing factor 3 homolog (yeast) (HPRP3P)] that showed differential expression pattern though micro-

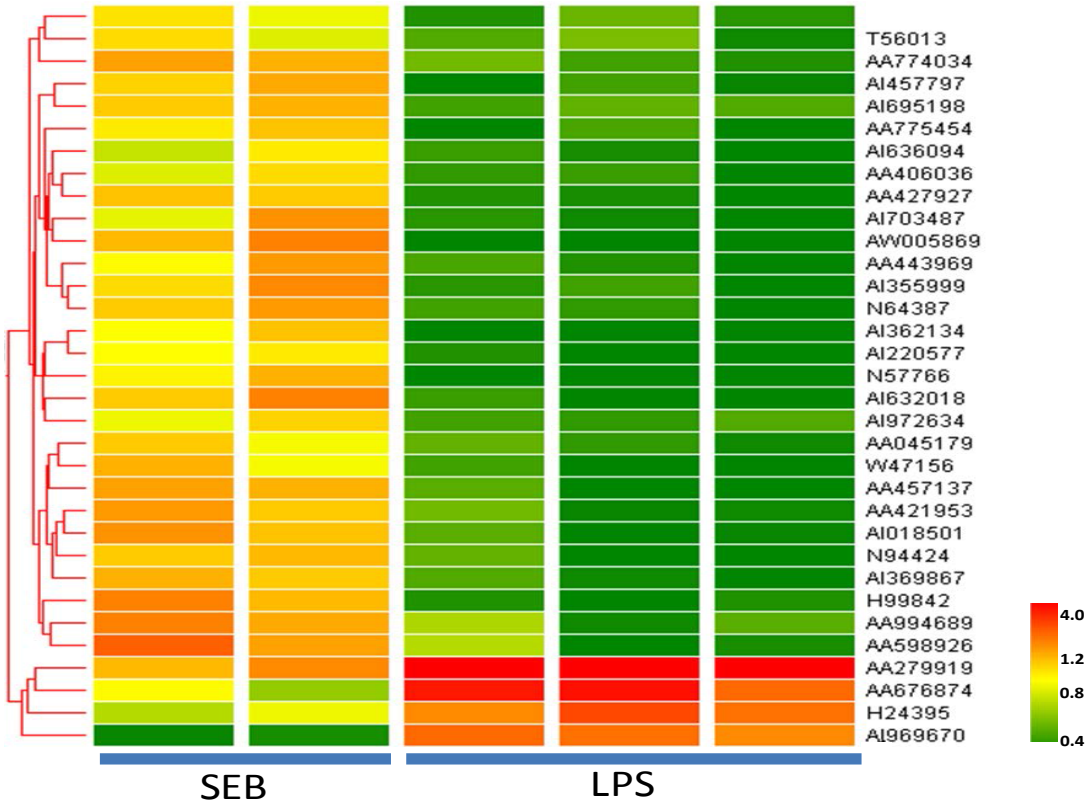

Figure 1A: Cluster analysis of SEB and LPS induced genes: Gene expression patterns of human PBMCs treated with SEB or LPS at 100 ng/ml for 24 hours were organized by unsupervised hierarchical clustering. After filtering, average linkage hierarchical clustering of an un-centered Pearson correlation similarity matrix was carried out using the program Cluster. The results were visualized with the program TreeView and the data analyzed using GeneSpring™ version 4.1 (Agilent, Inc.; San Carlos, CA) to identify patterns of gene regulation in PBMCs exposed to SEB and LPS analyzed in triplicates and duplicates respectively. Genes that showed significant variations in expression using Student's t-test with a $p < 0.005$ were selected from the total data set. Twenty three probes identified following the cutoff $p< 0.005$ is more than double of the amount, which could be mined by chance. The variations in gene expression are shown in matrix format. The scale extends from 0.4 to 4 fold changes compared to the control untreated samples.

array analysis were subjected to further analysis together with two other genes (CD-69 and TNF-α) that have previously been utilized as markers to distinguish exposure between the same two toxins. Quantitated expression of seven genes through microarray analysis at 24 hours are shown in Table 2. All seven genes exposed to LPS were up regulated except CD69 and TNF-α , which were expressed at control or just above control levels. When induced with SEB, all seven genes were down regulated except CD69 and TNF-α, which were up regulated 23.2 and 12.1 fold respectively.

Confirmation of microarray results by Real-Time PCR and RT-PCR analysis: To confirm the SEB and LPS induced gene expression patterns deduced from microarray analysis we carried-out a time dependent quantitative and semi-quantitative gene expression analysis for all seven genes as shown below.

Real-Time PCR Analysis; Real-time PCR analysis not only confirmed the previously observed expression patterns through microarray analysis, but also revealed a time dependent expression (Table 3, Figures 2a and 2b). The analysis showed a down regulation of all five genes beginning at 2 hours and continuing even after 24 hours of exposure to SEB except for HPRP3. HPRP3 regulation was still down regulated compared to control levels, but only very slightly. When exposed to LPS, all five genes showed a typical up regulation at both 2 hours and 24 hours, but the up regulation was much more evident at 24 hours compared to 2 hours of exposure. Out of the five genes, three (TRIAD3, HPRP-3 and ST6GALNAC6) showed only a modest up regulation (less than 2 fold) at 2 hours, while TRIAD3 expression was almost identical to control values (1.16 fold) at 2 hours.

RT-PCR analysis: RT-PCR was done as an additional confirmatory analysis. All analyzed genes except TNF-α and CD-69 showed a down regulated expression ranging from 4.35 to 1.56 fold when exposed to SEB. All genes except COPG-2 and ST6GALNAC6 showed a consistent down regulation at both at 6 hours and 24 hours. Both COPG-2 and ST6GALNAC6 were expressed close to control levels at 6 hours and 24 hours, respectively (Table 4). When exposed to LPS, all five genes except TNF-a and CD-69 showed an up regulation at all three time points even though the up regulation did not show a clear trend (increasing or decreasing) dependent on time. SEB induced CD69 expression pattern was consistently up regulated between 8-12 fold at all three time points while the expression when exposed to LPS was either similar to control levels (24 hours) or just above control levels for both 2 hours and 6 hours. Both SEB and LPS induced TNF-α expression was above control levels at all three time points (2, 6 and 24 hours) while the expression of SEB was at least 4-9 fold higher compared to LPS at all three of the above mentioned time points.

TNF-α and CD-69 protein expression analysis through ELISA: To correlate gene and protein expression pattern induced by the two toxins we further investigated CD69 and TNF-A using specific ELISA kits. Quantified protein levels of CD69 correlated with previously identified LPS and SEB induced gene expression patterns through

Gene Name	Abbreviation	Function
Mitogen-activated protein kinase kinase kinase kinase 3	MAP4K3	Activates JNK signaling pathway
Cofactor required for Sp1 transcriptional activation. subunit 6	CRSP6	Regulation of transcription
Potassium channel, subfamily K, member 4	KCNK4	Voltage insensitive outwardly rectifying potassium channel
tripartite motif-containing 26	TRIM26	DNA binding
PRP3 pre mRNA processing factor 3	HPRP3P	mRNA processing
Nup107-160 subcomplex subunit SEH1	SEC13L	protein transport
zinc finger protein 503	MGC2555	nucleic acid binding; zinc ion binding; metal ion binding
Ubiquitin conjugating enzyme 7 interacting protein 1	TRIAD3	Ligase activity
Small nuclear ribonucleoprotein D3 polypeptide	SNRPD3	mRNA processing
Coatomer protein complex, subunit gamma 2	COPG2	Transport protein from ER to Golgi network
ST6 -N-acetylgalactosaminide alpha-2,6-sialyltransferase 6	ST6GALNAC6	Alter cell-to-cell or cell-to-extracellular matrix interaction
Natriuretic peptide receptor B/guanylate cyclase B	NPR2	Integral membrane receptor
Crooked neck pre-mRNA splicing factor-like 1	CRNKL1	RNA processing
Transition protein 2	TNP2	DNA binding
Centaurin alpha 2	CENTA2	DNA binding
SUSHI repeat-containing protein	SRPUL	Involved in cellular migration
Dopamine Receptor D2	DRD2	G-protein coupled receptor activity
Chemokine (C-C motif) ligand 22	CCL22	Activated T-lymphocyte physiology
Tubulointerstitial nephritis antigen	TINAG	Adhesion of epithelial cells via integrins
Potassium inwardly-rectifying channel, subfamily J, member 1	KCNJ1	Produce potassium channels
Serologically defined colon cancer antigen 1	SDCCAG1	Colon cancer antigen
Periplakin	PPL	Interaction with plasma membrane
Polymerase 3	P3	bile acid:sodium symporter activity
Family with sequence similarity 189, member A2	X123	X
Bruton agammaglobulinemia tyrosine kinase	BTK	Development and maturation of B cells
La ribonucleoprotein domain family, member 7	HDCMA18P	Prevents RNA polymerase phosphorylation
Retinoic acid receptor responder (tazarotene induced) 1	RARRES1	Tumor-suppressor activity
3-hydroxy-3-methylglutaryl-CoA synthase 1	HMGCS1	acetyl-CoA metabolism
SEC22 vesicle trafficking protein homolog A	SEC22L2	ER to Golgi transport
Mitogen-activated protein kinase kinase kinase kinase 3	MAP4K3	Activates JNK signaling pathway
Cofactor required for Sp1 transcriptional activation. subunit 6	CRSP6	Regulation of transcription
Potassium channel, subfamily K, member 4	KCNK4	Voltage insensitive outwardly rectifying potassium channel
tripartite motif-containing 26	TRIM26	DNA binding
PRP3 pre mRNA processing factor 3	HPRP3P	mRNA processing
Nup107-160 subcomplex subunit SEH1	SEC13L	protein transport
zinc finger protein 503	MGC2555	nucleic acid binding; zinc ion binding; metal ion binding
Ubiquitin conjugating enzyme 7 interacting protein 1	TRIAD3	Ligase activity
Small nuclear ribonucleoprotein D3 polypeptide	SNRPD3	mRNA processing
Coatomer protein complex, subunit gamma 2	COPG2	Transport protein from ER to Golgi network
ST6 -N-acetylgalactosaminide alpha-2,6-sialyltransferase 6	ST6GALNAC6	Alter cell-to-cell or cell-to-extracellular matrix interaction
Natriuretic peptide receptor B/guanylate cyclase B	NPR2	Integral membrane receptor
Crooked neck pre-mRNA splicing factor-like 1	CRNKL1	RNA processing
Transition protein 2	TNP2	DNA binding
Centaurin alpha 2	CENTA2	DNA binding
SUSHI repeat-containing protein	SRPUL	Involved in cellular migration
Dopamine Receptor D2	DRD2	G-protein coupled receptor activity
Chemokine (C-C motif) ligand 22	CCL22	Activated T-lymphocyte physiology
Tubulointerstitial nephritis antigen	TINAG	Adhesion of epithelial cells via integrins
Potassium inwardly-rectifying channel, subfamily J, member 1	KCNJ1	Produce potassium channels
Serologically defined colon cancer antigen 1	SDCCAG1	Colon cancer antigen
Periplakin	PPL	Interaction with plasma membrane
Polymerase 3	P3	bile acid:sodium symporter activity
Family with sequence similarity 189, member A2	X123	X
Bruton agammaglobulinemia tyrosine kinase	BTK	Development and maturation of B cells
La ribonucleoprotein domain family, member 7	HDCMA18P	Prevents RNA polymerase phosphorylation
Retinoic acid receptor responder (tazarotene induced) 1	RARRES1	Tumor-suppressor activity
3-hydroxy-3-methylglutaryl-CoA synthase 1	HMGCS1	acetyl-CoA metabolism
SEC22 vesicle trafficking protein homolog A	SEC22L2	ER to Golgi transport

Table 1: Functional description of 30 genes identified through microarray analysis. Each of the 30 genes was identified according to microarray procedure as described in detail in materials and methods. All 30 genes were part of a profile that differentiated the SEB and LPS exposures. The genes are listed together with their known functions. Unknown functions are shown as "X".

Gene Names	SEB-24	LPS-24
TRIAD3	0.32 ± 0.01	4.5 ± 0.18
TRIM-26	0.65 ± 0.02	6.8 ± 0.2
HPRP-3	0.28 ± 0.03	7.8 ± 0.35
COPG-2	0.16 ± 0.02	6.3 ± 0.20
ST6GALNAC6	0.30 ± 0.02	3.8 ± 0.19
CD69	23.2 ± 2.3	1.12 ± 0.01
TNF-α	12.1 ± 0.5	2.1 ± 0.45

Table 2: Expression of seven genes identified through microarray analysis

Total RNA was extracted from SEB or LPS (100 ng/ml each) induced human PBMCs (2.5×10^6/ml) for twenty four hours and identical quantities of RNA samples were subjected to microarray analysis as described in detail in materials and methods. All reactions were repeated twice except for LPS (replicates of three), and the results are reported as mean values relative to the control together with the standard deviation values. TRIAD3 protein transcript variant 3(TRIAD3), tripartite motif-containing 26 (TRIM26), Homo sapiens PRP3 pre-mRNA processing factor 3 homolog (yeast) (HPRP3P), coatomer protein complex, subunit gamma 2 (COPG2), Homo sapiens ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1, 3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 6 (ST6GALNAC6), Cluster of Differentiation 69 (CD69) and Tumor necrosis factor alpha (TNF-α).

Gene Names	SEB 2 hrs	SEB 24 hrs	LPS 2hrs	LPS 24hrs
TRIAD3	0.19 ± 0.01	0.63 ± 0.01	1.16 ± 0.01	3.35 ± 0.18
TRIM-26	0.40 ± 0.01	0.22 ± 0.04	2.16 ± 0.08	4.48 ± 0.23
HPRP-3	0.57 ± 0.01	0.86 ± 0.14	1.53 ± 0.09	2.71 ± 0.20
COPG-2	0.02 ± 0.01	0.19 ± 0.06	2.11 ± 0.28	6.04 ± 0.40
ST6GALNAC6	0.70 ± 0.04	0.29 ± 0.02	1.41 ± 0.13	2.77 ± 0.25

Table 3: Validation of differential gene expression identified through microarray analysis by Real-time PCR

Real time PCR was performed in triplicates using a uniprimer containing a 3' oligonucleotide tail and housekeeping gene in an I-Cycler (Bio-Rad, Hercules, CA) as described in detail in materials and methods. Total RNA extracted from SEB induced, LPS induced or control PBMCs were subjected to real time PCR using Amplifluor universal amplification kits (Intergen, Purchase, NY). Threshold cycle (cT) of each reaction was calculated, and normalized using the cT values of 18-S (housekeeping) gene. All reactions were repeated twice and the results are reported as mean values relative to the control together with the standard deviation values.

microarray and RT-PCR analysis (Figure 3a and 3b). When induced with SEB, CD-69 was expressed 8.57 &13.52 fold at both 2 and 24 hour time points, respectively, while at 6 hours no visible expression was seen over control levels. LPS had no visible change in expression at 2, 6 and 24hours over control levels. TNF-α protein expression when induced with SEB was 2.91 and 6.5 fold higher when compared to LPS induced expression at both 2 and 24 hours respectively. Both toxins did not show any expression over control levels at 6 hours.

Discussion

In an attempt to study the complex molecular events induced in the host during lethal shock, we performed a series of gene expression analysis to differentiate, identify and profile genes induced by SEB and LPS in human PBMCs. Such a study is also very attractive due to the unsuccessful treatment of lethal shock probably due to the early activation of numerous cascades of cellular mediators and the inability to successfully target cytokines and inflammatory mediators in clinical trials [6]. Further complicating the matter is the similar symptoms initiated by the two toxins at the onset of the exposure.

Systematic examination of gene expression profiles have shown to divulge qualitative and quantitative differences leading to a possible mechanism of action in human PBMC when induced by bacteria and bacterial products [7]. Global gene analyzing methods such as Differential Display (DD) has also been used in multiple studies to assess the gene expression pattern of vascular endothelial cells, which accompanies the inflammatory reaction of atherosclerotic lesions [8] as well as microarray analysis that has differentiated a set of toxins from each other [9]. SEB and LPS are two toxins that show a comparable progression of symptoms that may ultimately lead to shock but has shown a distinct gene expression profile from each other. Here we have utilized multiple gene expression analysis techniques, each contributing at different strengths to clearly differentiate the exposure to two toxins that induce similar initial symptoms.

Initially, microarray analysis was employed to deduce a profile specific to each toxin by looking at 2000 characterized cDNA. This type of analysis is high throughput yet sensitive enough to identify expression patterns unique to each of the two toxins (Figure 1a). Cluster analysis performed on genes altered by SEB and LPS indicated similar (up, down or at control levels) as well as distinct expression patterns (Figure 1a). This observation makes sense as both toxins initially show similar symptoms and yet the eventual outcome is distinct from each other. It is then prudent to take a closer look at the genes that are part of a set of genes that would differentiate the two toxins (Table 1). If the above is true then any one of the 30 genes that is part of a unique gene profile for each toxin could potentially provide valuable information to distinguish the two toxin exposures. Even though microarray analysis is an excellent technique for simultaneously measuring genetic responses in a large number of genes, the precision of the data requires additional methods of confirmation. In order to further analyze a set of genes that showed differential expression through microarray analysis we utilized RT-PCR. RT-PCR is a fast semi quantitative endpoint gene expression analysis technique that allowed us to evaluate the contributory strength and extent of five differentially expressed genes identified by microarray analysis. We also included two more well characterized gene markers to reconfirm our analysis. We were confident that including a confirmatory analysis will not only provide the extent of each gene's contribution to the unique profile but also provide a better understanding of the time dependent expression pattern of each gene. Together, Real-Time PCR and RT-PCR not only allowed us to validate the regulation pattern identified though micro-array analysis but also provided us valuable information about the time dependent expression of each gene. The expression patterns obtained for all 5 genes through both techniques were similar to each other except for COPG-2 and ST6GALNAC6 expression analyzed through RTPCR. COPG2 showed an expression slightly above control levels (1.40 fold) when treated with SEB for 6 hours and ST6GALNAC6 showed an expression of over control levels (1.44 fold) at 24 hours (Table 4).

In order to further complement the analysis of the five genes we also employed real-time PCR. Real-time PCR analysis provided us another quantitative analysis technique to confirm the expression of these genes at both time points (2 and 24 hours). Down regulated gene expression levels of all 5 genes seen at 2 hours continued even after 24 hours of exposure to SEB except for HPRP3 (slightly up regulated over control levels) (Table 3). When exposed to LPS all 5 genes showed a typical upregulation at both 2hours and 24hours, but the upregulation was much more evident at 24 hours compared to 2 hours of exposure except TRIAD3 which was almost identical to control values (1.16 fold). The data clearly show a correlation of the differential expression profile observed through microarray analysis and Real-Time PCR. Additionally, the data is also reflective of the potential these gene analysis techniques may possess in identifying changes, diseases, exposures in similar studies. The genes utilized in this study contain functions ranging from inducing T-cell expression to cell death and some of the functions are shown below in detail.

Gene Names	SEB-2hrs	SEB-6hrs	SEB-24hrs	LPS-2hrs	LPS-6hrs	LPS-24hrs
TRIAD3	0.41 ± 0.06	3.90 ± 0.54	0.70 ± 0.04	4.75 ± 0.64	1.28 ± 0.90	0.86 ± 0.30
TRIM-26	0.30 ± 0.08	0.85 ± 0.22	0.40 ± 0.03	6.95 ± 0.35	1.46 ± 0.18	0.79 ± 0.63
HPRP-3	0.25 ± 0.02	0.93 ± 0.16	1.07 ± 0.33	1.33 ± 0.11	4.47 ± 0.22	6.82 ± 0.57
COPG-2	0.41 ± 0.02	1.40 ± 0.25	0.37 ± 0.18	0.80 ± 0.04	2.03 ± 0.26	2.43 ± 0.28
ST6GALNAC6	0.50 ± 0.20	0.68 ± 0.04	1.44 ± 0.40	2.98 ± 0.12	1.66 ± 0.10	2.59 ± 0.33
CD69	8.15 ± 0.52	11.5 ± 1.4	9.43 ± 0.71	1.88 ± 0.08	1.87 ± 0.49	0.89 ± 0.13
TNF-α	5.92 ± 0.42	9.10 ± 0.16	13.8 ± 0.57	1.83 ± 0.22	2.36 ± 0.08	1.26 ± 0.08

Table 4: Comparison of gene responses in SEB and LPS using RT-PCR

Total RNA was extracted from SEB or LPS (100 ng/ml each) induced human PBMCs (2.5 x 10^6/ml) exposed for 2, 6 and 24 hours and identical quantities of RNA samples were subjected to reverse transcription polymerase chain reactions (RT-PCR) analysis as described in detail in materials and methods. All reactions were repeated twice after normalizing with a housekeeping gene (18S), and the results are reported as mean values relative to the control together with the standard deviation values.

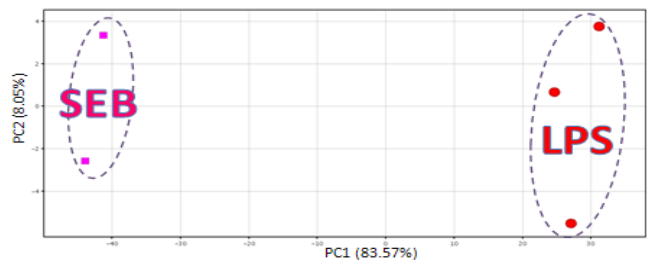

Figure 1B: Principal component (PCA) analysis; PCA was carried out using Partek Pro (Partek, Inc, MI). Results indicate that the first axis explains around 84% of cumulative variance. The pattern shows that this significant variation is attributed to the unique gene expression profiles of LPS- and SEB-treated family. The pattern further indicates that the internal deviation among the individual family of treating agents is referred to the residual variation explained by the second and third principal components, which are statistically insignificant. Overall, the majority of the variation among the data cloud is realized from the differed genomic response towards the two toxins (SEB and LPS); experimental variation among individual treatment group is insignificant.

COPG2 is a required complex for Golgi membranes and is essential to the transport of proteins from the Golgi to ER [10]. ST6GALNAC6 is responsible for the biosynthesis of DSGG (disialylgalactosylgloboside) from MSGG (monosialylgalactosylgloboside) in normal and malignant kidney [11]. HPRP3P is known to code for proteins which associate with U4 and U6 spliceosomes. Spliceosomes are the small nuclear riponucleoproteins (smRNPs) which remove non-gene intron sequences from DNA. This process is called intron removal or pre-mRNA splicing [12]. It is conceivable that malfunction of spliceosomes can lead to buildup of these useless fragments of DNA which can trigger cell death. TRIM26 is a member of the tripartite motif (TRIM) family and is strongly expressed within the thymus and peripheral CD 4+ T-cells and dispensable for T-cell development as well as peripheral immune functions [1]. Previous studies suggested that TRIM is an integral component of the T-cell receptor (TCR)/CD3 complex and might be involved in regulating TCR cycling [13]. TRIAD3 has been directly linked to apoptosis, a naturally occurring mechanism where a cell undergoes programmed death in response to external or internal signals [14]. SEB improperly activates the pathway controlling this process in a variety of cell types and the resulting large-scale cell death leads to irrevocable shock for the infected individual [14].

TNF-α and CD69 were also included in the analysis as both genes are known to be differentially expressed by the two toxins. Both genes clearly showed a differential expression when exposed to each of the toxins through both microarray and RT-PCR analysis. To confirm that the expression values observed at the genetic level did not result from obscure expression we also investigated the protein expression pattern of these two genes. Figure 3 confirms a similar expression pattern observed at both protein and gene level for TNF-α and CD69. Previous work done using human PBMCs in our laboratory has clearly indicated higher than normal T-cell activation followed by high expression levels of number of cytokines (including TNF-α) when exposed to SEB [4]. We have also seen a similar increase in CD 69 expression when exposed to SEB (unpublished data), However, when exposed to LPS both of these markers were not significantly induced over control levels. Such

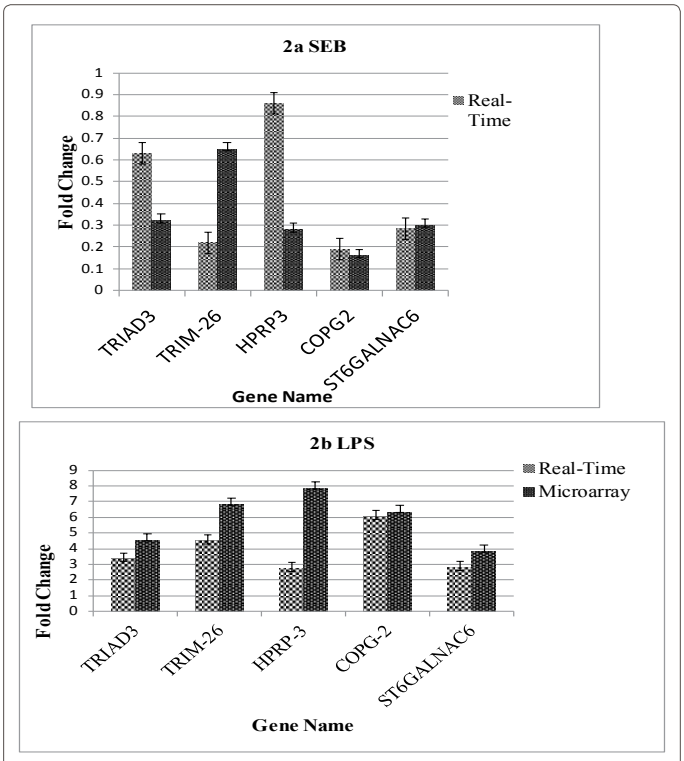

Figure 2: Comparison of genes induced by SEB and LPS in human PBMCs using microarrays and real-time PCR analysis. Expression of five genes that showed differential expression though micro-array analysis in human PBMCs treated with 100ng/ml SEB and LPS for 24 hours were determined as described in materials and methods. The microarray data was the average of three separate experiments (solid bars) while the real-time PCR results were from two experiments (empty bars) analyzed in duplicates as described in materials and methods. TRIAD3 protein transcript variant 3(TRIAD3), tripartite motif-containing 26 (TRIM26), Homo sapiens PRP3 pre-mRNA processing factor 3 homolog (yeast) (HPRP3P), coatomer protein complex, subunit gamma 2 (COPG2), and (Homo sapiens ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1, 3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 6 (ST6GALNAC6).

2a. Expression analysis of five genes that were differentially expressed though micro-array analysis and Real-time PCR in SEB induced human PBMCs. **2b.** Expression analysis of five genes that showed differential expression though micro-array analysis and Real-time PCR in LPS induced human PBMCs.

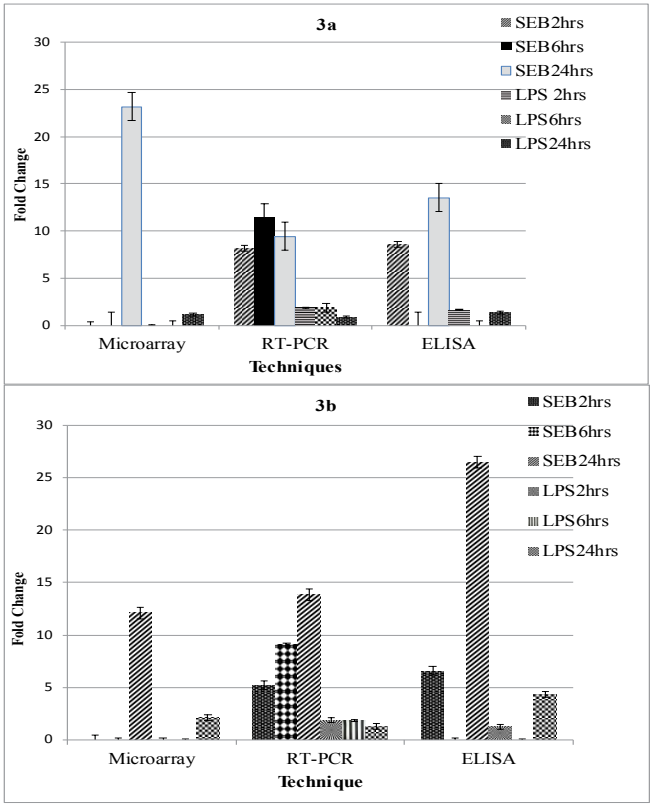

Figure 3: Comparison of gene and protein expression analysis of TNF-a and CD-69 induced by SEB and LPS; Equal amounts of RNA from SEB or LPS treated human PBMCs together with control RNA were reverse transcribed, analyzed by microarray analysis and RT-PCR, and quantified using NIH image software. All results are shown as mean values of three different experiments together with the standard deviations. Equal amounts of proteins were also extracted from the same samples as listed above by ELISA. Protein expression was determined from human PBMCs treated with SEB and LPS for 2 hours and 24 hours by ELISA and compared to the gene expression as depicted in detail in experimental procedures. The protein data was the average of three separate experiments (unfilled bars) while the gene expression results were from two experiments analyzed in duplicates on high resolution gels (dark, filled bars) Standard errors for all samples were calculated and presented together with fold changes. 3a. Quantification of CD69 using microarray, RT-PCR and ELISA analysis; 3b. Quantification of TNF-α using microarray, RT-PCR and ELISA analysis.

an analysis is significant as it confirms the expression pattern at both protein and gene level while eliminating any doubts about the quality of the observed values of gene expression.

Genetic expression analysis and profiling is a multi-million dollar industry that is highly utilized in multiple resources including pharmaceuticals to maximize therapeutic efficacy, improve prediction of disease stage, help prevent clinical onset of toxicity, identify quantitative biomarkers for animal and early human trials and assist in drug positioning due to its fundamental advantages in accuracy and predictability [15]. Here we have utilized a combination of gene analysis techniques to distinguish two toxins that often display similar initial symptoms and cause problems in the initial identification process. In addition, we have further verified gene expression patterns of two genes utilizing protein expression.

The work presented here may be useful to a number of studies, pursuing a rapid diagnostic approach to detect differences, diseases and exposures. Our approach heavily relies on reproducing expression pattern of a given gene profile through multiple analysis techniques. Furthermore, each gene should be able to be reproducible and utilized as a marker in identifying and treating aberrant fluctuations that may ultimately be detrimental to the cell. Even though we believe that a gene expression pattern can accurately predict an exposure or a disease state, there could be several other possible genomic signatures that might be used including epigenetic patterns, microRNA profiles, disease associations, protein expression and metabolite profiles to provide a stronger explanation [16]. We believe the study reinforces the strength of profiling genes and illustrates the importance of correlating time with strength of each gene expression pattern.

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"Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army."

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