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Evaluating Gene Expression and Signaling Activities in Lipopolysaccharide Induced Human Peripheral Blood Mononuclear Cells

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Abstract: Lipopolysaccharide (LPS) is an endotoxin that induces the secretion of pro-inflammatory cytokines in multiple cell types that ultimately could lead to septic shock in humans. This study is aimed at identifying a set of functionally relevant genes induced by LPS and evaluating the effect of signal transduction pathway inter-connector, cJun N-terminal Kinase (JNK), on LPS induced gene and protein expression in human Peripheral Blood Mononuclear Cells (hPBMCs). A specific gene expression pattern induced by LPS was identified through microarray analysis and was confirmed in a time dependent manner by Reverse Transcription Polymerase Chain reaction (RT-PCR). In the presence of JNK specific inhibitor also known as anthrapyrazolone inhibitor and/or SP600125 both analyzed gene and protein expression that somewhat explained LPS induced cellular activities were altered. We believe the study will allow us to not only identify a set of functionally relevant genes induced by LPS but also better understand the role played by the complex interactions of multiple signal transduction pathways induced by LPS.

Keywords: Lipopolysaccharide, Signaling Activities, cJun N-terminal Kinase, Gene Expression

1. Introduction

Lipopolysaccharides (LPS) are cellular structures located in the outer membrane of a gram-negative bacterial cell wall. The structure is comprised of three parts: a hydrophobic domain (lipid A), a core polysaccharide, and an O-antigen side chain. The lipid A portion of LPS is embedded in the outer membrane and is responsible for the endotoxicity of the molecule. The remainder of the molecule extends beyond the cell surface. The bacteria's survival strategy is to protect itself from the host's defenses by changing the length and composition of the O-antigen. The LPS molecule, by consequence of containing lipid A, becomes most toxic when it is released due to damage to the cell membrane, bacterial lysing, or multiplication [1, 2].

LPS is frequently present in bacteria responsible for food borne illnesses. *Escherichia coli* and *Salmonella enteritidis* are two of the most common and well known of these bacteria. According to the Centers for Disease Control and Prevention, approximately 48 million people fall ill due to food borne illnesses each year in the United States.

The symptoms caused by LPS may include mild symptoms like fever, diarrhea, and vomiting, or more severe symptoms including septic shock, and even death. LPS induce septic shock by binding to toll-like receptors, CD14 and stimulating multiple innate immune responses including secretion of cytokines, activation of macrophages, and lymphocytes. Symptoms of bacterial infections (such as LPS) are similar to

those of the stomach flu, often causing an incorrect or delayed diagnosis. The likelihood of delays in diagnosis and treatment, and the lethality of the disease, makes LPS a potential threat as a biotoxin. When LPS is ingested, an individual has the possibility for treatment, but if used as an aerosol, the toxin can be almost immediately lethal to a large number of people.

Human PBMCs provide the first line of defense for many processes induced by diseases or toxin exposures by posing characteristic molecular signatures for each induced process and allowing to discriminate and predict the type/strength of induction in a rapid manner [3]. The above-mentioned factors made us further investigate the gene expression patterns induced by LPS at multiple exposure times in hPBMCs. Since many pathogenic agents ultimately lead to lethal shock, the study presented here was designed to investigate gene expression patterns induced in the host and compare these patterns through systematic gene expression analysis techniques such as microarrays and RT-PCR. By comparing host cell responses to these two toxins at a genetic level, a set of genes was identified to facilitate a clear differentiation in exposure to the two toxins [4]. Although SEB and LPS toxins incur similar outcomes, especially related to vascular collapse, they proceed with individual initiation steps as well as mechanisms/pathways that in turn induce somewhat different effects during the course of illness [4]. However, gene expression analysis of blood cells carried out by microarrays resulted in a characteristic molecular signature for each of the toxins as shown in Figure 1 [4].

As some of the signaling events seen in LPS induced cells was similar to signaling events observed for staphylococcal enterotoxin B (SEB) induced cells, we wanted to take a closer look at JNK, which played a crucial role in transmitting the SEB induced signal [3-5]. JNK pathway, when induced by LPS, has shown to have similar symptoms and patho-mechanisms that lead to stimulation of various immune responses including secretion of cytokines and activation of macrophages and lymphocytes [6]. Our approach included a dual confirmatory approach at a gene and protein level of a functionally relevant set of genes induced by LPS, which were identified by microarray analysis carried out in out laboratory [4]. Finally, JNK specific inhibitor (SP600125), an inhibitor of a previously identified signaling inter-connector (JNK) was used to evaluate the effect on signaling activity [4]. This Conducted inhibitor study revealed the crucial role played by JNK in LPS induced signaling and gene expression. We hope the work done in this study will help better understand the complex signaling and gene expression activities induced by LPS.

2. Materials and Methods

2.1. Micro-Array

Human PBMC's from normal human donors were treated with LPS (100 ng/ml) for 1 to 24 hours at 37°C. Total RNA from Control and LPS treated hPBMCs 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 custom microarrays or AtlasTM glass microarrays, scanned using Gene Pix 4000B Microarray Scanner (Axon Instruments Inc., Foster City, CA) and the data analyzed using Gene Pix 3.0 software package (Figure 1). 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 (Figure 1). The data was analyzed using GeneSpring TM version 4.1 (Silicon Genetics, San Carlos, CA).

2.2. Microarray Analysis

Microarray experiments were performed to differentiate the gene expression patterns of the two toxins, SEB and LPS. PCR products were obtained from a PCR library (WRAIR, Silver Spring, MD), resuspended 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 x 76 mm glass slides 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 hPBMCs 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 the data analyzed using Gene Pix 3.0 software package.

2.3. 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 (Silicon Genetics, San Carlos, CA) to identify patterns of gene regulation in hPBMCs 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, an

ANOVA test was performed by using $P < 0.005$ as a threshold.

2.4. Cells and Cell Cultures

Exposure of hPBMCs to SEB in vitro; Human PBMCs were collected from leukopacks from normal donors as previously described [16]. Human PBMCs, with or without LPS and SEB were used at a final density of 2.5x 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. Prior to the treatment with 100 ng/ml of LPS for different time periods, all cell mixes were incubated with 10 μM JNK specific inhibitor (SP600125), at room temperature for 30 minutes under 5% CO_2 at 37°C.

2.5. Extraction of RNA and Proteins

Cells were harvested and total RNA isolated using TRIzol reagent (Life Technologies, Grand Island, NY) while proteins were isolated with Lysis buffer (Cell Signaling Technology, Danvers, MA).

2.6. Quantification of RNA and Protein Samples

Extracted RNA and protein samples were quantified using a NanoDrop (NanoDrop Technologies; Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.; Santa Clara, CA). Agilent Technologies 2100 Expert software was used to quantify the RNA and proteins while NanoDrop, ND-1000 Spectrophotometer (NanoDrop Technologies; Wilmington, DE), was used to verify the Agilent quantification data.

2.7. Visualization & Quantification

Visualization of gels was done using an in-house set-up. Analyzed genes of interest were quantified by using imageJ program. All results were normalized and are expressed as fold change values.

2.8. Toxin

SEB from S. aureus strain 10-275, purified and was 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 the concentration of 5 mg/ml and stored at −80°C [4]. When used, the stock solution was diluted with cell culture medium to the desired concentration. Lipopolysaccharide (LPS); LPS (catalog # L2360-10mg) was purchased from Sigma as a lyophilized powder purified by phenol extraction (Sigma, St Louis MS).

2.9. ELISA

Equal quantities of proteins obtained from hPBMCs, with or without treatment of 100 ng/ml LPS, for a time 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.

2.10. RT-PCR

RT-PCR analysis were performed using an iScriptTM cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA). Housekeeping gene primers (18S) were obtained from Clontech Corp, Palo Alto, CA, and all other primers were designed using various primer-design software. Complimentary DNA (cDNA) was amplified using PCR master kits (Roche Diagnostics Indianapolis, IN). Amplified genes were then analyzed on 1% agarose gels, visualized through an imager and quantified using NIH ImageJ analysis software. All custom primers except 18S were purchased from Invitrogen Carlsbad, CA, USA. All primers were 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.

MAD2L2-L: CAC TGT TGG ATG GTC ACA GG, MAD2L2-R: CAG TGT CAG CTG ATG GGA GA, HSPTP-L: GCA GGA GCT CAG CAT AGA CC, HSPTP-R: GAA CTA GCC TGA GCC AGG TG, HSPH1-L: TCC GGA AAG ATG AAC AGG TC, HSPH1-R: TGC ACA TCC TCT GGC TAC TG, RPSK-L: GCT TTT CAA GCG AAA TCC TG, RPSK-R: ACT AGG TGG AAT GCC AGG TG, DUSP1-L: CTG CCT TGA TCA ACG TCT CA, DUSP1-R: ACC CTT CCT CCA GCA TTC TT, SH2D2A-L: CAA AGA AGC CTG GAG GTC AG, SH2D2A-R: CCC AGA CTC AAA AGG CTC AG, CIRBP-L: CTT TTT GTT GGA GGG CTG AG, CIRBP-R: CTT GCC TGC CTG GTC TAC TC, APOBEC-L: ACC ATT TGG AGC TGG ACA AC, APOBEC-R: CCT TGG GTG AGC AAT TAG GA.

Table 1. *Abbreviations and the names of genes that have been investigated in this paper through multiple gene expression analysis techniques.*

Symbol	Gene Name	Function
MAD2L2	Mitotic arrest deficient-like 2	Cell Proliferation
HSPTP	Heat Shock Protein family H, member 1	Cell Stress/Shock
HSPH1	Heat shock 105kDa/110kDa protein 1	Effects Cell stress
RPS6K	Ribosomal protein S6 kinase, 70kDa, polypeptide 2	Protein Synthesis during Cell proliferation
DUSP1	Dual specificity phosphatase 1	Oxidative cell shock
SH2D2A	SH2 domain protein 2A	Protein interactions crucial to cell function
CIRBP	Cold inducible RNA binding protein	Cell Proliferation

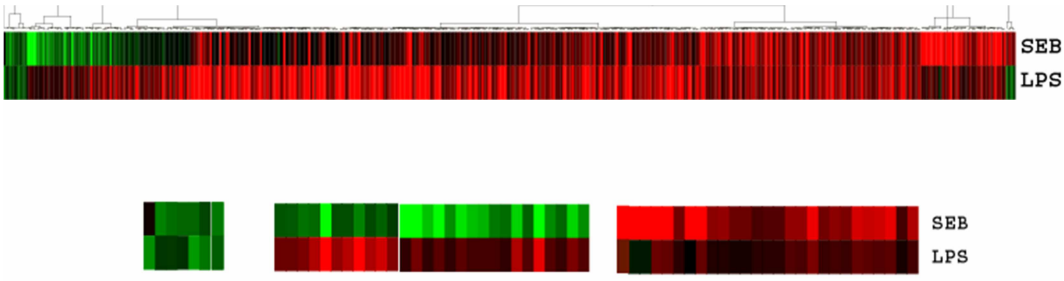

Figure 1. *Clustering Analysis of LPS induced genes using Micro-array. Gene expression patterns of hPBMCs treated with SEB or LPS at 100 ng/ml for 24 hours were organized by unsupervised hierarchical clustering. After filtering, average linkage hierarchical clustering of an un-centered Pearson correlation 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 (Silicon Genetics; San Carlos, CA) to identify patterns of gene regulation in hPBMCs exposed to SEB and LPS analyzed in triplicates and duplicates respectively. Genes that showed significant variations in expression using ANOVA with a $P < 0.005$ were selected from the total data set. The variations in gene expression are shown in matrix format. The scale extends from 0.125- to 8-fold compared to the control untreated cells [4].*

Table 2. *Comparison of the expression of 8 genes from genetic signature in LPS induced hPBMCs using microarrays and RT-PCR analysis. Total RNA was extracted from LPS (100 ng/ml) induced hPBMCs (2.5 x 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 and the results are reported as mean values relative to the control (fold change) together with the standard deviation values. RT-PCR reactions were performed on samples treated with LPS (100 ng/ml) for 24 hours as described in Materials and Methods. Identical total RNA samples were used for all analysis, and the bands of PCR products were digitized after normalizing with a house keeping gene (18S rRNA) and quantitated using NIH image software as described in materials and methods. All RT-PCR analysis of seven genes that have been implicated in LPS related activities such as cell proliferation and cellular stress were done in triplicates and their expression reported as mean values (fold change) together with standard deviations. Down-regulation of gene expression is represented by values less than 1.0; up-regulation is represented by values greater than 1.0; and a value equal to 1.0 is indicative of no change in gene expression.*

Gene	RT-PCR	Microarray
MAD2L2	0.14 +/- 0.06	0.5
HSPTP	4.82 +/- 0.10	2.6
HSPH1	0.63 +/- 0.01	0.4
RPS6K	0.75 +/- 0.06	0.4
DUSP1	11.10 +/- 0.24	6.0
SH2D2A	0.42 +/- 0.02	0.7
CIRBP	0.50 +/ -0.01	0.4

Table 3. *Effect of JNK specific inhibitor (SP600125) on a set of known gene expression pattern of LPS induced hPBMCs. A set of genes specific for LPS induction previously identified by Microarrays and RT PCR were further examined with or without the JNK specific inhibitor (SP600125) through RT-PCR. After designing specific primers for each gene that was analyzed, RT-PCR were performed on samples treated with LPS (100 ng/ml) with or without the JNK specific inhibitor (SP600125) for 2, 6 and 24 hours as described in Materials and Methods. Identical total RNA samples were used for all analysis, and the bands of PCR products were digitized after normalizing with a house keeping gene (18S rRNA) and quantitated using NIH image software as described in materials and methods. All reactions with LPS with or without the inhibitors were done in triplicates and the results are reported as mean values (fold change) together with the standard deviations.*

Gene Name	With LPS	With LPS and JNK Inhibitor (2 hours)	With LPS and JNK Inhibitor (24 hours)
MAD2L2	0.14 +/- 0.06	3.01 +/- 0.02	1.4 +/- 0.06
HSPTP	4.82 +/- 0.10	0.95 +/- 0.05	0.80 +/- 0.10
RPS6K	0.75 +/- 0.06	2.00 +/- 0.04	4.46 +/- 0.06
CIRBP	0.50 +/ -0.01	4.00 +/- 0.01	3.26 +/- 0.02

Table 4. *Comparison of protein expression of TNF-a marker induced by LPS with and without the JNK specific inhibitor (SP600125); Equal amounts of proteins were extracted from human PBMCs that were induced by LPS with or without JNK specific inhibitor (SP600125) and subjected to ELISA as described in detail under materials and methods. TNF-a protein expression is the average of three separate experiments (fold change) together with the standard deviations.*

Time	LPS	LPS + Inhibitor
2 Hours	5.4 +/- 0.08	1.85 +/- 0.04
6 Hours	11.37 +/- 0.14	1.71 +/- 0.01
24 Hours	12.14 +/- 0.24	2.53 +/- 0.05

3. Results and Discussion

3.1. Results

3.1.1. Genes Expression Changes Identified by Microarray Analysis

The alterations in gene expression induced by LPS in hPBMCs 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 had unique differences in expression as anticipated (Figure 1). Out of these genes seven genes that showed unique expression pattern to LPS and directly or indirectly effected cell proliferation and cellular stress, were further evaluated using both gene and protein analysis. Functions of each of the seven genes are listed on Table 1.

3.1.2. Confirmation of Microarray Results by RT-PCR Analysis

To confirm the LPS induced gene expression patterns deduced from microarray analysis we carried-out a time dependent gene expression analysis of seven function specific genes (as described elsewhere) using RT-PCR. A comparison of the values obtained by both techniques are summarized in Table 2. Similar expression patterns were observed for all 7 genes by both RT-PCR and microarray analysis. While MAD2L2, HSPH1, RPS6K, SH2D2A and CIRBP genes showed downregulation HSPTP and DUSP1 showed upregulation.

3.1.3. Effect of JNK Specific Inhibitor (SP600125) on a Set of LPS Specific Genes

We used JNK specific inhibitor (SP600125) to better understand the signaling activities induced by LPS. For this

evaluation we used 4 genes that were known to explain some of the known functions of LPS. The expression pattern of these 4 genes were analyzed by RT-PCR after inducing hPBMCs with LPS for 2 and 24 hours (Table 3).

All four genes (MAD2L2, HSPH1, RPS6K, and CIRBP) showed opposite expression when exposed to the JNK specific inhibitor (SP600125) at both evaluated time points. While MAD2L2 showed an upregulation with JNK specific inhibitor (SP600125) at 24 hours, its upregulation was close to control levels (1.4-fold) at 24 hours.

3.1.4. Effect of JNK Specific Inhibitor (SP600125) on TNF-Alpha Expression

To evaluate the effect of JNK specific inhibitor (SP600125) on TNF-alpha protein expression we carried out ELISA analysis. Quantified protein levels of LPS induced TNF-α was similar to previously observed protein expression levels in our laboratory [4] at all three evaluated times (2, 6, 24 hours) as shown on Table 4. When exposed to JNK specific inhibitor (SP600125) the expression levels decreased at least over 3-fold for all three time points.

3.2. Discussion

It is widely known that systematic examination of gene expression profiles in hPBMC induced by bacteria and bacterial products divulge qualitative and quantitative differences leading to a possible mechanism of action [7]. Global gene analyzing methods such as Differential Display (DD) [8] and microarray analysis are used in multiple studies to assess gene expression pattern in many organisms including vascular endothelial cells, which accompanies the inflammatory reaction of atherosclerotic lesions and differentiate a set of toxins from each other [5, 9-14].

Previously, we have used microarray analysis to acquire a distinctive gene expression profile for two toxins (SEB and LPS) that show a comparable progression of symptoms, yet each has an eventual outcome that is vastly distinct from each other [4]. Previous studies done in our laboratory has also shown that LPS induces cell shock as well reduction of cell proliferation [6]. To analyze part of the gene profile that allowed us to distinguish the two toxins and explain some of LPS induced activities, we utilized RT-PCR to further investigate the functionally relevant LPS specific set of genes.

The gene expression results obtained through microarray and RTPCR for seven genes that show LPS specific function are shown in Table 2. All seven genes are either involved in cell proliferation or directly affected by cellular stress. The expression patterns (up or down regulation) obtained for all 7 genes through both techniques were somewhat similar. The genes that were directly involved in impeding cell proliferation or has a function that effects cell proliferation, were all down regulated (MAD2L2, HSPH1, RPS6K, SH2D2A and CIRBP) while the genes that induce cellular stress that could ultimately lead to cell shock were upregulated (HSPTP and DUSP1) [15-19]. These results are very encouraging because impeded cell proliferation and cell shock are key events that has known to be implicated with LPS induction.

As previous work done in our laboratory suggested key signaling pathways induced by SEB [4, 5] were connected through multiple interconnectors, we hypothesized that LPS could also induce similar signaling activities in human PBMCs. As JNK pathway is known to control responses of multiple types of shock [20] we used JNK specific inhibitor (SP600125) to further validate the involvement of JNK pathway in LPS induction. The study was done to evaluate the effect on the expression pattern of functionally relevant genes at both two hours and 24 hours. As shown in Table 3, JNK specific inhibitor (SP600125) altered LPS specific gene expression pattern at both time points. These results suggest the ability of JNK specific inhibitor (SP600125) to reverse cell proliferation and cell shock, two key cellular events that intimately associated with identifying LPS induction and lead to LPS induced symptoms. Even though a more comprehensive functional analysis is needed the results observed in this study shows the reversal of early cellular events like cell shock and cell proliferation, that has shown to lead ultimately to multiple LPS induced symptoms including cell shock.

Additionally, we have also seen that LPS induced TNF-α, gene and protein expression levels were above control levels irrelevant of the time cells are exposed to the toxin [5]. So we extended the study by further evaluating the effectiveness of JNK specific inhibitor (SP600125) on TNF-a protein expression. Our results indicated that JNK specific inhibitor (SP600125) suppressed TNF-a expression when compared to control levels (Table 4). These results confirm the crucial role played by pathway interconnector JNK and the effect it has on cellular stress and cell proliferation, two key cellular responses induced by LPS that can ultimately lead to progression of symptoms including septic shock.

4. Conclusions

Knowledge of alterations to early molecular events observed by blocking a key pathway inter-connector, JNK, investigated in this study, will have a tremendous impact on understanding the signaling pathways induced by LPS in hPBMCs. Early identification of such key elements will help in determining exposure to deadly toxins such as LPS before significant cellular damage occurs and designing strategies to block such deadly cellular events. The study collectively aims to elucidate the complexity of signal transduction pathways induced by LPS, cross-talk of pathways, and inhibitory effect of JNK specific inhibitor (SP600125) on known cellular and genetic expression associated with LPS as illustrated in Table 3. Further work will be necessary at both in vivo and in vitro level to pinpoint the efficiency and the efficacy of this crucial pathway inter-connector to better understand the complex cellular activities and pathways induced by LPS.

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Conflicts of Interest

The authors declare no conflicts of interest.

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