Mol. Cells *29*, 585-594, June 30, 2010
DOI/10.1007/s10059-010-0073-4

Molecules and Cells

Protein Expression Changes in Human Monocytic THP-1 Cells Treated with Lipoteichoic Acid from *Lactobacillus plantarum* and *Staphylococcus aureus*

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Lipoteichoic acid (LTA) from *Staphylococcus aureus* (aLTA) and from *Lactobacillus plantarum* LTA (pLTA) are both recognized by Toll-like receptor 2 (TLR2), but cause different stimulatory effects on the innate immune and inflammatory responses, and their underlying cellular mechanisms are unknown. In this study, comparative proteome analysis was performed using two-dimensional gel electrophoresis and mass spectrometry on protein extracts from human monocyte THP-1 cells stimulated with either aLTA or pLTA. Differentially expressed proteins might be involved in innate immunity and inflammation. Cells treated with aLTA and with pLTA showed different protein expression profiles. Of 60 identified proteins, 10 were present only in treated cells (8 in aLTA-treated only, and 2 in pLTA-treated only), 1 protein (IMPDH2) was suppressed by pLTA, and 49 were up- or down-regulated more than three-fold by aLTA- or pLTA- stimulation. Several proteins involved in immunity or inflammation, antioxidation, or RNA processing were significantly changed in expression by aLTA- or pLTA-stimulation, including cyclophilin A, HLA-B27, D-dopachrome tautomerase, Mn-SOD, hnRNP-C, PSF and KSRP. These data demonstrated that aLTA and pLTA had different effects on the protein profile of THP-1 cells. Comparison of the proteome alterations will provide candidate biomarkers for further investigation of the immunomodulatory effects of aLTA and pLTA, and the involvement of aLTA in the pathogenesis of *Staphylococcus aureus* sepsis.

INTRODUCTION

Both *Staphylococcus aureus* and *Lactobacillus plantarum* are Gram-positive bacteria, but they exhibit very different physiological effects on the human immune response. *S. aureus* is recognized as a major pathogen, causing a variety of diseases ranging from superficial skin infections to severe life-threatening conditions such as bacteremia, endocarditis, pneumonia, abscesses, and post-operative wound infections (Diekema et al., 2001). In contrast, *L. plantarum* is non-pathogenic and is known as a health-promoting probiotic lactic acid bacterium that inhabits the normal human intestine. Increasing evidence suggests that lactic acid bacteria not only have anticarcinogenic and antioxidative activities (Grangette et al., 2005; Lin and Yen, 1999), but also play important roles in regulating the immune system, protecting against intestinal infection, and lowering total blood cholesterol levels (Bloksma et al., 1979; Ha et al., 2006; Lee and Lee, 2006; Mohama-dzadeh et al., 2005).

The major immune modulator of Gram-positive bacteria is lipoteichoic acid (LTA), an amphiphilic polymer that traverses the bacterial cell wall (Fischer, 1988; Seo et al., 2008), similar to the lipopolysaccharide (LPS) cell wall component of Gram-negative bacteria (Morrison and Ryan, 1987). Although LTA is expressed by both pathogenic and probiotic Gram-positive bacteria (Neuhaus and Baddiley, 2003), the immunomodulatory properties of LTA from the two types of bacteria are very different. LTAs from pathogenic Gram-positive bacteria, such as *S. aureus, S. pneumoniae* and *S. epidermidis*, efficiently activate monocytes and macrophages through the secretion of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-8 (Ellingsen et al., 2002; Mattsson et al., 1993; Standiford et al., 1994). In contrast, the LTA from the probiotic *L. plantarum* (pLTA) minimally induces TNF-α production compared to *S. aureus* LTA (aLTA), but effectively inhibits aLTA- or LPS-triggered TNF-α secretion, and suppresses the septic shock caused by aLTA or LPS stimulation (Kim et al., 2008a; 2008b). The distinct immunological activities of aLTA and pLTA might contribute to the different physiological effects of *S. aureus* and *L. plantarum* on the human innate immune response.

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Received February 5, 2010; accepted March 11, 2010; published online May 20, 2010

Keywords: 2-DE, gram-positive bacteria, lipoteichoic acid, proteome analysis, THP-1 cells

Toll-like receptor 2 (TLR2) is the cognate pattern-recognition receptor for LTA in the innate immune response to Gram-positive bacteria (Kawai and Akira, 2007). Both aLTA and pLTA activate immune cells via TLR2 signaling, but have different effects on cytokine production and innate immunity (Kim et al., 2007; Schroder et al., 2003; Takeuchi et al., 2000). The different immunological properties of these LTAs are suggested to be mainly due to differences in chemical structure, parti-cularly the D-alanine contents in the glycerophosphate back-bone (Grangette et al., 2005; Velez et al., 2007). How different LTA chemical structures result in different innate immune responses is not known, and further investigation is needed to understand the molecular events triggered by LTA-TLR2 interaction.

Analysis of proteomic differerences between aLTA- or pLTA-treated cells would facilitate the unraveling of the biological processes triggered by aLTA or pLTA stimulation. Proteomics offers a unique means for qualitative and quantitative comparison of protein expression under different conditions (Wu and Wilmouth, 2008). In this study, comparative proteomics was used to investigate the translational changes in human monocytic THP-1 cells treated with either aLTA and pLTA. The data will help elucidate the distinct and similar responses of central immune cells to exposure to the pathogenic *S. aureus* and the probiotic *L. plantarum*, and yield insights into the mechanisms of *S. aureus* pathogenesis and *L. plantarum* health-promotion.

MATERIALS AND METHODS

Cell culture

Human monocytic THP-1 cells were maintained in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified 5% (v/v) CO_2 incubator, and passaged every 3 days.

Cell stimulation and total protein preparation

THP-1 cells were seeded at a density of 5.0×10^5 cells per ml in 75 cm^2 tissue culture flasks and stimulated for 24 h with highly purified and structurally intact aLTA or pLTA (25 μg/ml) prepared as described previously (Kim et al., 2007; 2008a). Untreated cells were used as controls. Three replicates, each consisting of three flasks, were performed for each sample. After stimulation, cells were harvested by centrifugation at 4°C, 1500 × *g* for 10 min, and cell pellets were washed twice with cold phophate-buffered saline. Total protein was extracted with cell lysis solution (50 mM Tris-HCl buffer, pH 7.8, 7 M urea, 2 M thiourea, 4% CHAPS, 12% isopropanol, 2mM β-mercaptoethanol and protease inhibitor cocktail). After 30 min at room temperature, samples were sonicated for 3 min on ice and centrifuged at 18,000 *x g* for 30 min at 10°C, and supernatants collected. Protein concentrations were quantified with a 2D-Quant kit (Bio-Rad, USA). Equal amounts of total protein from each of the three culture flasks were pooled and stored at -80°C.

Two-dimensional gel electrophoresis (2-DE)

First dimension isoelectric focusing (IEF) was performed in a Protean IEF Cell system (Bio-Rad, USA), with 1.5 mg protein in 400 μl rehydration buffer was loaded onto an immobilized pH 3-10 linear gradient ReadyStrip IPG (17 cm, Bio-Rad, USA), and strips were allowed to rehydrate for 16 h at room temperature. IEF was carried out at 19°C, at 0 V for 1 h, 50 V for 10 h, 500 V for 1.5 h, 2,000 V for 1.5 h, 5,000 V for 1.5 h, and 10,000 V for 85,000 Vh. Following IEF separation, focused IPG strips were equilibrated for 2 × 14 min under gentle rocking in equilibration buffer (375 mM Tris-HCl pH 8.8, 6 M urea, 2% w/v SDS, 20% v/v glycerol, and trace bromophenol blue). The first incubation was in equilibration buffer with 2% DTT (w/v), and the second was in equilibration buffer with 2.5% iodoace-tamide (w/v).

Second dimension SDS-PAGE was performed in a Bio-Rad Dodeca gel electrophoresis apparatus. Equilibrated strips were applied to 13% SDS-PAGE gels, and overlaid with melted 1% agarose solution in SDS electrophoresis buffer. For molecular weight markers, 2 mm filter paper squares containing 5 μl of standard proteins were inserted at each end of the focused strips. Electrophoresis was performed at 16°C, 40 mA for 40 min, followed by 60 mA for 6 h until the dye front reached the bottom. Gels were stained with Coomassie Brilliant blue (CBB) (Gelcode blue, Pierce, USA), according to the manufacturer's instructions.

Image acquisition and analysis

Image acquisition of stained gels was with an ImageScanner 2D (Amersham, USA) at 500 dpi. Protein spot detection, quantification, and matching of results were performed using PDQuest Advanced 8.0.1 software (Bio-Rad, USA). Molecular masses were determined using the standard protein markers, and p*I* values were estimated from 2-D gel positions, and confirmed using the immobilized pH gradient strips. A match consisting of 9 images was created, 3 for aLTA-treated cell, 3 for pLTA-treated cell and 3 for control samples. Errors of omission and commission were corrected manually for each gel after careful visual inspection of the computerized gel images. Replicates were combined into master gels to determine spots that were reproducibly present. Master gels for different conditions were compared to find differences in spot intensity that resulted from treatment.

In-gel digestion with trypsin and preparation for peptide solution

Protein spots were punched out from the CBB-stained preparative 2-D gels, cut into pieces, and destained twice with 100 μl 25 mM NH_4HCO_3/50% ACN pH 8.0 at 37°C. Gel pieces were dehydrated with 100% ACN for 15 min and dried in a vacuum centrifuge before reduction with 10 mM dithiothreitol (DTT) in 25 mM NH_4HCO_3 (pH 8.0) for 1 h at 37°C, and alkylation with 100 mM iodoacetamide in 25 mM NH_4HCO_3 (pH 8.0) for 1 h at room temperature in the dark. Gel pieces were washed with 50% ACN in 25 mM NH_4HCO_3, treated with 100% ACN for 10 min and dried in a vacuum centrifuge. For in-gel trypsin digestion, 10 μl of 50 mM NH_4HCO_3 (pH 8.0) containing 10 μg/ml sequence-grade Trypsin (Roche Applied Science) was added for overnight incubation at 37°C. Supernatant was collected, and tryptic peptides were further extracted twice with 50 μl extraction buffer (50% ACN/5% formic acid) at 37°C for 1 h. Extracts were pooled, and evaporated to completeness in a vacuum centrifuge. Dried peptides were redissolved in 0.1% formic acid, purified using a ZipTipC18 microcolumn (Millipore, USA) and eluted either with 5 mg/ml of α-cyano-4-hydyoxycinnamic acid (CHCA) in 50% ACN/0.1% TFA for MALDI-TOF analysis, or with 60% ACN for nanoLC-ESI Q-TOF MS analysis.

MALDI-TOF/TOF MS and protein identification

Purified peptide solution (1 μl) was spotted onto a MALDI target plate and air-dried. Peptide mass fingerprints were obtained using an ABI-4700 MALDI TOF/TOF mass spectrometer (Applied Biosystems, USA), equipped with a nitrogen laser. The analyzer was operated in reflector/delayed extraction mode with manual acquisition control. Positive reflect mode was run with scan range m/z600-m/z5000, accelerating voltage 20 KV, wave length of laser 337 nm, frequency 200 Hz, delayed ex-

tract time 470 ns. Signals from 125 shots were used to create one spectrum, and spectra were processed using the Data Explorer software V2.9 (Applied Biosystems). Trypsin autocleavaged peaks were used as internal mass calibration standards, and all spectra were externally calibrated using a protein standard I kit (Bruker) of Angiotesin_II_[M+H]+_mono, m/z 1046.5418, Angiotesin_I_[M+H]+_mono, m/z 1296.6848, Substance_P, m/z1347.7354, Bombesin, m/z1619.8223, ACTH_clip (1-17), m/z2093.0862, ACTH_clip(18-39), m/z 2465.1983 and Somatostatin(28), m/z 3147.4710.

Generated peak list files were queried against the non-redundant protein database NCBInr 20090131 (www.ncbi.nlm.nih.gov/) of 7783044 sequences using the MASCOT search engine (V2.0/V2.1, Matrix Science, UK) with peptide mass tolerance 0.2 Da, trypsin digest with one missed cleavage, no fixed modification, carboxyamidomethylatoin (Cys) and oxidation (Met) as the variable modifications. Taxonomy was set as *Homo sapiens*. The minimum MOWSE scores for protein identification were 66 for NCBInr, corresponding to a statistical significance level of $p < 0.05$. The identification of a protein was considered reliable if it was identified at this level of significance from at least two parallel gels.

NanoLC-Q-TOF mass spectrometry (MS) and protein identification

Some protein spots were identified using LC-ESI Q-TOF MS/MS. Trypsin peptide analysis was performed on a nanoflow HPLC system, Ultimate 3000 (Dionex Corporation, USA) and a micro-TOF-Q mass spectrometer (Bruker Daltonics) with nanospray ion source Appollo II (Bruker). LC-MS grade solvents were used for all steps (Fisher Scientific, USA), and HPLC solvents were A, 0.1% formic acid; B, 80% ACN/0.1% formic acid.

Purified peptides were dried and redissolved in 0.1% TFA. Peptide samples (8 μl) were desalted on an online reverse phase (RP) C18 trapping column (Symmetry C18, 180 μm inner diameter, id, × 50 mm, 5 μm particle diameter; Waters, USA) for 1 min at 20 μl/min with 80% solvent A. Peptides on the trapping column were separated on an analytical RP C18 nano-column (75 μm id × 180 mm, 5 μm particle diameter; Waters, USA) and eluted directly into the electrospray ionization (ESI)-quadrupole TOF tandem mass spectrometer at 300 nL/min. The linear gradient was 5 min initial isocratic step with 98% A and 2% B, followed by 0 to 60% B in 45 min, 10 min isocratic with 80% B, and 80 to 2% B in 5 min.

The micro-TOF-Q was operated in an automated data-dependent acquisition and positive-ion mode by performing MS/MS scans (1.5 s) for the three most intense peaks from each MS scan (0.5 s) with a mass scan range of m/z 50-3000. Total cycle time range was 3.5 to 5 s. The voltage of non-coated nanoflow spray capillary (Vcap) was -1350 V with flow rate 3 L/min and drying gase temperature 160°C. Glufibrinopeptide was used to calibrate in MS/MS mode. Peak lists were generated using Data Analysis 4.1 software (Bruker Daltonics) and automatically combined into a single mgf file for every LC-MS/MS run.

MS/MS data were deconvoluted and transferred to mgf files for querying against NCBInr 20090131 as described above with parameters above and fragment ion mass tolerance, 0.6 Da. The mass type of the parent and fragment peptides was monoisotopic, peptide charge, +1, +2 and +3. MS data were statistically analyzed using Mascot (www.matrixscience.com). Only proteins with MOWSE score ≥ 38 were regarded as significant ($P < 0.05$), according to the selection criteria defined by the manufacturer.

Total RNA isolation and semi-quantitative reverse-transcription (RT)-PCR analysis

THP-1 cells were seeded at a density of 5×10^5 cells per ml in 6-well tissue culture plates and stimulated for 6 h with highly purified and structurally intact aLTA (25 μg/ml) or pLTA (25 μg/ml). Untreated cells were used as the controls. Total cellular RNA was extracted using RNA Isolation Solvent RNA-Bee (TEL-TEST, USA), according to the manufacturer's protocol. RNA amount and quality were determined by spectrophotometry and assessed by agarose gel electrophoresis. cDNAs from total RNA were prepared with the Improm-II™ reverse transcription system (Promega), according to the manufacturer's instructions. RT-PCR was performed using specific primers for the selected genes, and mRNA expression was normalized to GAPDH. PCR products were analyzed on 1.2% agarose gels visualized with ethidium bromide.

RESULTS AND DISCUSSION

Two-DE analysis

The protein expression profiles of THP-1 cells treated with aLTA or pLTA, along with an untreated control were analyzed by 2-DE (Fig. 1). Total proteins were extracted from three biological replicates per sample, separated in parallel and gel images were quantitated for protein spots that showed significant alteration after aLTA or pLTA treatment. More than 80 spots showed greater than three-fold changes in density, 10 spots were present only in cells exposed to aLTA or pLTA, and one protein showed completely inhibition of expression after pLTA stimulation.

MS identification of differentially expressed proteins

Differentially expressed proteins were isolated from the 2-D gels, trypsin digested and analyzed by MALDI-TOF or LC-ESI Q-TOF MS. We successfully identified 60 differentially expressed proteins, 11 using MS/MS of LC-ESI Q-TOF and 49 using PMF of MALDI-TOF, and found that 25 proteins were responsive only to aLTA, 27 were responsive only to pLTA, and 8 were regulated by both (Tables 1 and 2, Fig. 2), including two proteins (Manganese superoxide dismutase, Mn-SOD and Fumarate hydratase precursor) were upregulated by aLTA but downregulated by pLTA. The different protein expression profiles between cells treated with aLTA or pLTA suggested distinctive stimulatory potentials on immune cells, even though both substances engage a common TLR2 signaling pathway in the innate immune and inflammatory responses (Kim et al., 2008a).

Functional analysis of differentially expressed proteins

Differentially expressed proteins were classified into functional categories of anti-oxidation, chaperones, metabolic processes, RNA processing, signal transduction, and immune or inflammatory response. Several proteins had not previously been associated with LTA-triggered signaling, and might be involved in *S. aureus* sepsis pathogenesis. Specific protein functions are discussed bellow.

Anti-oxidative proteins

Superoxide dismutase (SOD) and catalase are the major enzymes for converting oxygen radicals to water and oxygen. Like LPS, LTA is a strong inducer of reactive oxygen radical bursts from monocytes and phagocytic cells (Ginsburg et al., 1998; Levy et al., 1990). Although oxygen radicals play an important role in inflammation, and the destruction of invading pathogens (Karupiah et al., 2000), they can also lead to oxida-tive host

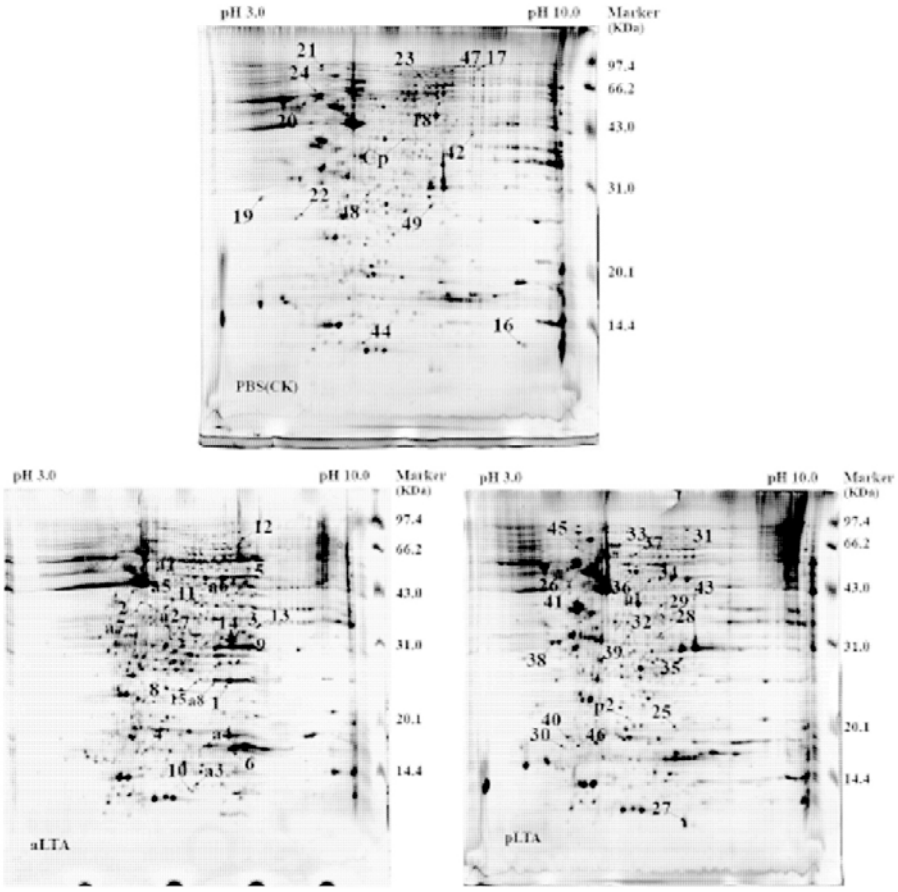

Fig. 1. Protein expression profiles of THP-1 cells incubated with aLTA (25 μg/ml) or pLTA (25 μg/ml) for 24 h (PBS-treated cells were used as the control samples). Total proteins were resolved using a linear pH 3-10 gradient IPG strip in the first dimension and by SDS-PAGE on a 13% acrylamide gel in the second. Differentially expressed proteins significantly affected by aLTA or pLTA stimulation were indicated by arrows and numbers that correspond to the gel numbers given in Tables 1 and 2.

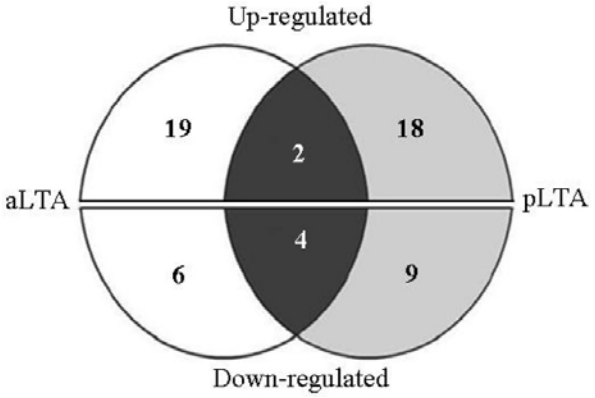

Fig. 2. Venn diagram indicated for the regulatory effects of aLTA and pLTA on the protein expression of THP-1 cells. Twenty-one proteins were up-regulated and 10 proteins were down-regulated by aLTA; while 20 and 13 proteins were up-regulated and down-regulated by pLTA respectively. The protein expression levels had three-fold or more changes in response to aLTA or pLTA stimulation.

tissue damage. Our data demonstrated that aLTA treatment significantly up-regulated the expression of Mn-SOD in THP-1 cells, consistent with the finding that activation of several pattern recognition receptors (TLR2-4, TLR7/8) strongly upregulates Mn-SOD expression (Rakkola et al., 2007). In contrast, pLTA down-regulated Mn-SOD expression. The different effects of aLTA and pLTA on Mn-SOD expression were confirmed by semi-quantitative RT-PCR (Fig. 3). The results suggested that pLTA had a weaker influence than aLTA on both induction of proinflammatory cytokines (Kim et al., 2008a; Ryu et al., 2009), and on the process of anti-oxidation.

D-dopachrome tautomerase (DDT) is another oxidation-related protein that was observed in aLTA-, but not pLTA-treated THP-1 cells. DDT converts D-dopachrome into 5,6-dihydroxyindole and is involved biosynthesis of the anti-oxidant melanin (Heiduschka et al., 2007; Wang et al., 2006). DDT is strongly up-regulated in carbon tetrachloride (CCl_4)-treated rats and provides protection from oxidative stress induced by CCl_4 (Hiyoshi et al., 2009). The tertiary structure of DDT is remar-kably similar to macrophage migration inhibitory factor (MIF), although they have only 38% identity and 49% homology (Sugimoto et al., 1999). MIF is a pro-inflammatory cytokine that is released from activated macrophages and other immune effector cells (Rendon et al., 2007), and may contribute to tumor-associated processes (Howard et al., 2004). DDT is the only known MIF homolog, and its physiological functions are poorly understood. Recent studies presented evidence that MIF and DDT, individually and additively, promote vascular endo-thelial growth factor (VEGF) and IL-8 expression in human lung adenocarcinoma cell lines (Coleman et al., 2008). Since DDT is also an aLTA-responsive protein, it may be a useful biomarker, and its possible participation in the innate immune responses in the pathogenesis of *S. aureus*-caused sepsis should be explored.

Table 1. List of proteins detected only in aLTA- / -pLTA-treated or the control THP-1 monocytes. Protein names and functions have been assigned according to PubMed and Swiss-Prot/TrEMBL. These newly expressed proteins were induced in response to aLTA or pLTA stimulation for 24 h. The spot labels are identical to those given in Fig. 1.

Spot label	Identified protein	Accession number	Mr (kDa) (theor.)	p*I* (theor.)	MOWSE score	Peptide matched	Coverage (%)	Cell-treated
a1	Annexin VII isoform 1	P20073	50.3	6.3	223	5	12	aLTA[a]
a2	Human elongation factor-1-delta	P29692	31.2	5.0	88	2	8	aLTA
a3	D-dopachrome tautomerase	P30046	12.7	6.7	151	4	39	aLTA
a4	R33729_1	Q969H8	11.3	7.0	72	3	21	aLTA
a5	C protein	P07910	31.9	5.0	269	7	16	aLTA
a6	Transaldolase 1	P37837	37.5	6.4	126	5	11	aLTA
a7	C protein	P07910	31.9	5.0	145	5	19	aLTA
a8	Unnamed protein product	BAG53464	19.7	7.8	102	9	64	aLTA
p1	DUT deoxyuridine triphosphatase	P33316	26.7	9.7	133	3	11	pLTA[b]
p2	40S ribosomal protein S12	P25398	14.5	6.3	140	4	21	pLTA
Cp	Inosine monophosphate dehydrogenase 2	P12268	51.6	8.1	141	4	8	CK[c]

[a]aLTA designated proteins present only in the aLTA-treated cellss but not in the pLTA-treated or the control cells
[b]bLTA designated proteins detected only in the pLTA-treated cellss but not in the aLTA-treated or the control cells
[c]This protein was present in the control cells but absent in the pLTA-treated cells

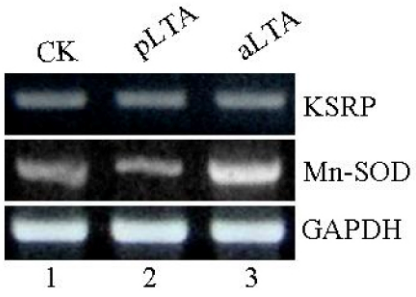

Fig. 3. Expression of Mn-SOD and KSRP were detected by semi-quantitative RT-PCR. THP-1 cells were stimulated with aLTA (25 μg/ml) or pLTA (25 μg/ml) for 6 hrs, and the un-treated cells were used as the control (CK). Total RNAs were extracted and reversely transcripted into cDNAs. Semi-quantitative RT-PCR was performed and innormalized with GAPDH.

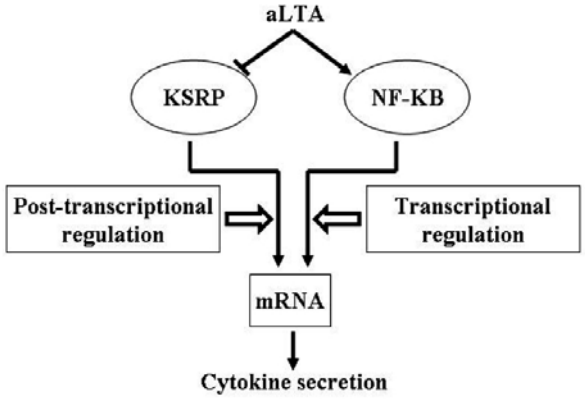

Fig. 4. Schematic diagram of the regulatory mechanism of aLTA-triggered cytokine production. aLTA simulation could activate NF-κB that regulates the gene expression of cytokines on the one hand, and could decrease the activity of KSRP that mediates mRNA stabilization on the other hand. Through the transcriptional and the post-transcriptional regulation, aLTA treatment could increase and stabilize the mRNAs, and consequently elevate cytokine production.

Chaperones

Two types of heat shock proteins (HSPs) were differentially expressed in LTA-treated cells. HSP60 was up-regulated by pLTA but not aLTA, while Gp96, a HSP90 homolog (Sorger and Pelham, 1987), was substantially down-regulated by both aLTA and pLTA. In addition to serving as a chaperone, HSP60 plays important roles in immune responses (Ohashi et al., 2000; Wallin et al., 2002; Zanin-Zhorov et al., 2003; 2005a) and inflammation (Hu et al., 1998; Mor and Cohen, 1992). *In vitro* and *in vivo* studies showed that HSP60 is a negative immunostimulator that up-regulates the expression of suppressor of cytokine signaling (SOCS3) in T-cells via TLR2 and STAT3 activation (Zanin-Zhorov et al., 2005b). pLTA-induced up-regulation of HSP60 might provide clues about the inhibitory effect of pLTA on LPS-induced TNF-α production in THP-1 cells and endotoxic shock in mice (Kim et al., 2008a). Gp96, also known as tumor rejection antigen 1 (*TRA1*), is an essential HSP in both innate and acquired immunity (Schild and Rammensee, 2000; Warger et al., 2006). Hypoxia and CpG-ODN treatment up-regulate Gp96 expression (Kuo et al., 2005; Paris et al., 2005), while *Orientia tsutsugamushi* down-regulates its expression, in a pathogenic immune evasion mechanism that acts through Gp96 expression modulation (Cho et al., 2004). Since CpG-ODN and LTA mediate innate immunity through signaling of TLR9 and TLR2, respectively (Hemmi et al., 2000; Schroder et al., 2003), the difference in expression of Gp96 in CpG-ODN- and LTA-treated cells suggests that Gp96 fulfills different functions in the TLR9- and TLR2-activated immune responses.

Metabolic proteins

Several proteins involved in metabolic pathways were identified as differentially expressed in aLTA- and pLTA-treated cells, including fumarate hydratase (fumarase), carbonic anhydrase II (CAII), prolyl 4-hydroxylase (beta subunit), phosphoglycerate kinase 1 (PGK1), inosine monophosphate dehydrogenase 2

Table 2. List of proteins differentially expressed in aLTA- or pLTA-treated THP-1 monocytes. Protein names and functions have been assigned according to PubMed and Swiss-Prot/TrEMBL. Differentially expressed proteins (ratio ≥ 3) that were up- and down-regulated in response to aLTA or pLTA stimulation for 24 h are presented. The aLTA and pLTA columns correspond to the expression of each protein relative to its expression in control cells. Results are means of three independent experiments performed for each condition. The spot labels are identical to those given in Fig. 1.

Spot label	Identified protein	Accession number	Mr (kDa) (theor.)	p*I* (theor.)	MOWSE score	Peptide matched	Coverage (%)	Fold Change	
								aLTA	pLTA
1	Manganese superoxide dismutase	P04179	22.1	6.9	88	12	75	28.3	-3.3
2	Heterogeneous nuclear ribo-nucleoprotein C isoform b	P07910	32.3	4.9	115	16	52	8.9	2.7
3	Carbonic anhydrase II	P00918	29.1	6.6	82	8	47	4.7	1.2
4	Coactosin-like protein	Q14019	16.3	5.9	99	15	82	4.6	3.9
5	Fumarate hydratase precursor	P07954	52.9	6.7	98	11	28	4.2	-3.7
6	Cyclophilin A	P62937	17.9	7.8	98	17	85	4.0	1.4
7	Endoplasmic reticulum protein 29 isoform 1	P30040	28.9	6.8	84	22	69	3.9	1.3
8	ATP synthase, H^+-transporting, mitochondrial F0 complex, subunit d isoform a	Q75947	18.5	5.2	99	15	83	3.8	3.0
9	Hydroxysteroid (17-beta) dehydrogenase 10, isoform 2	Q99714	25.9	6.7	69	11	53	3.8	-1.8
10	Human leukocyte antigen B27 (HLA-B27)	P01889	11.7	6.1	77	9	86	3.7	1.2
11	Ribosomal protein P0	P05388	34.2	5.7	74	8	38	3.5	2.8
12	Unnamed protein product	Q96AE4	66.1	7.1	148	22	39	3.5	-2.4
13	Methylenetetrahydrofolate dehydrogenase 2 precursor	P13995	35.9	8.7	94	11	46	3.3	2.7
14	Enoyl-CoA hydatase,short chain 1	P30084	31.0	6.1	116	20	63	3.1	2.5
15	Manganese superoxide dismutase	P04179	22.1	6.9	111	16	66	3.1	-1.1
16	FKBP-type peptidyl-prolyl cis-trans isomerase FKBP1A	P62942	11.8	8.1	66	9	87	-20.1	-3.1
17	Unnamed protein product	GI:194379996	49.0	8.3	150	39	65	-7.7	-3.4
18	HSPC117	Q9Y3I0	55.2	7.0	96	23	40	-7.1	0.8
19	ANP32A protein	P39687	27.3	4.0	163	15	45	-5.6	-1.6
20	Calreticulin precursor variant	P27797	46.9	4.3	96	24	44	-4.3	-1.6
21	Heat shock protein gp96 precursor	P14625	92.3	4.8	226	66	68	-4.2	-5.6
22	Heme binding protein 2	Q9Y5Z4	22.9	4.6	108	11	51	-3.5	0.9
23	KH-type splicing regulatory protein	Q92945	73.1	6.9	225	47	54	-3.3	1.5
24	Prolyl 4-hydroxylase, beta subunit precursor	P07237	57.1	4.8	281	51	80	-3.2	-1.8
25	Ubiquitin-fold modifier-conjugating enzyme 1	P61960	19.8	7.1	104	12	45	1.3	9.8
26	RBBP4 retinoblastoma binding protein 4	Q09028	46.1	4.9	80	12	28	1.4	8.9
27	Ubiquitin	P62979	8.4	6.6	165	12	87	2.7	7.7
28	Peroxisomal delta 3,5-delta 2,4-dienoyl CoA isomerase	Q13011	32.7	6.4	71	7	31	1.1	6.3
29	LIM and SH3 protein 1	Q14847	29.7	6.6	94	17	50	2.4	5.8
30	Elongin B isoform a	Q15370	13.1	4.7	78	7	58	0.9	4.5
31	Polypyrimidine tract-binding protein-associated splicing factor	P23246	66.2	9.4	112	35	51	1.7	4.2
32	Gelsolin-like capping protein	GI:55597035	38.5	5.9	86	11	45	-1.4	4.0
33	Unnamed protein product	P38646	72.4	5.7	330	60	71	1.6	3.7
34	Chain A, TapasinERP57 HETERODIMER	P30101	54.2	5.6	301	42	71	1.8	3.6
35	Triosephosphate isomerase	P60174	26.5	6.5	185	22	92	2.8	3.5
36	Heterogeneous nuclear ribonucleoprotein H 1	P31493	49.2	6.9	199	35	64	-1.2	3.4
37	Heat shock 60 KDa protein 1	P10809	61.0	5.7	210	29	56	-2.1	3.4
38	Clathrin, light polypeptide A	P09496	23.6	4.5	103	16	42	1.3	3.4
39	Proteasome activator subunit 1	Q6IBM2	28.6	6.3	80	12	63	1.0	3.2
40	Myosin regulatory light chain MRCL3	P19105	20.4	4.6	77	11	53	-2.3	3.1
41	Ribonuclease/angiogennin inhibitor 1	P13489	49.4	4.7	261	34	81	1.3	-11.1
42	Phosphoglycerate kinase	P00558	44.9	8.3	229	40	71	-1.4	-9.1
43	Enolase 1	P06733	47.1	7.0	323	40	81	-1.6	-7.1
44	Enhancer of rudimentary homolog	P84090	12.3	5.6	112	13	56	-2.0	-4.0
45	UBQLN1 ubiquitin 1	Q9UMX0	62.4	5.0	75	23	30	-3.5	-3.6
46	Huntingtin-interacting protein K	Q9NX55	19.3	5.4	72	17	48	-1.6	-3.2
47	Acontitase 2, mitochondrial	Q99789	85.5	7.6	163	27	37	0.9	-3.1
48	Glyoxalase domain containing 4	Q9HC38	33.2	5.4	121	22	73	1.4	-3.1
49	Lysophospholipase II	Q95372	24.7	6.8	79	14	51	1.2	-3.0

(IMPDH2), enolase 1, transaldolase 1, and triosephosphate isomerase. Fumarase catalyzes the seventh reaction of the tr icarboxylic acid cycle, in which acetyl-CoA from glycolysis pr oduces CO_2, reduced electron carriers (NADH and $FADH_2$) and a small amount of ATP. Fumarase is also a tumor suppressor (King et al., 2006). Our data showed that fumarase expression was up-regulated by aLTA but not pLTA. CAII plays an important role in carbon dioxide metabolism and intracellular pH regulation, and is a tumor-associated antigen (Yoshiura et al., 2005) and an inflammation-inducible serum protein (Yasukawa et al., 2007). Up-regulation of fumarase and CAII by aLTA but not pLTA stimulation suggested that these proteins are involved in immune and inflammatory responses triggered by aLTA.

PGK1 was significantly down-regulated in pLTA-treated cells. In addition to its role as a glycolytic enzyme and a suppressor of proangiogenic factors such as VEGF, PGK1 is also thought to be involved in the onset of malignancy because of its role in regulation of the CXCR4/CXCL12 axis (Wang et al., 2007). Hypoxia and hydrogen peroxide induce PGK1 expression (Jang et al, 2008; Lam et al., 2009), and PGK1 overexpression has been observed in several cancers (Hwang et al., 2006; Zieker et al., 2008; Zhang et al., 2005).

IMPDH2 was the only protein suppressed by pLTA. IMPDH2 is a major therapeutic target because it is up-regulated in some neoplasms, suggesting a role in malignant transformation (Liu et al., 2006). Thus, pLTA might be a promising therapeutic agent for carcinomas, and the inhibitory effect of pLTA on the expression of some tumor-related proteins might contribute to elucidating the mechanism of *L. plantarum* as a health-promoting bacterium that protects against intestinal infection and carcinogenesis (Bloksma et al., 1979; Mohamadzadeh et al., 2005).

Immune and inflammatory response-related molecules

Three proteins in immune and inflammatory responses were observed in aLTA-, but not pLTA-treated cells: R33729_1, cyclophilin A (CyP A) and human leukocyte antigen B27 (HLA-B27). All were up-regulated by aLTA stimulation. R33729_1 is also called c19orf10 (chromosome 19 open reading frame 10) or IL-27 in the NCBI database. The structure and biological function of R33729_1 are unknown, but high expression levels in the synovial fluids of arthropathy patients (Weiler et al., 2007) suggests that it may be a pro-inflammatory factor. R33729_1 is a novel gene and is aLTA-inducible, but its role in aLTA-activated immune and inflammatory responses remains to be determined.

CyP A was elevated in aLTA-treated cells. CyP A belongs to the immunophilin family and is the host cell receptor of the potent immunosuppressive drug cyclosporin A (Liu et al., 1991). Besides its protein folding peptidylprolyl cis-trans-isomerase activity, CyP A is a pro-inflammatory cytokine and potent chemoattractant (Jin et al., 2004; Sherry et al., 1992; Xu et al., 1992). The up-regulation of CyP A by aLTA stimulation, the activation of MAP kinases and NF-κB by recombinant CyP A, and the overexpression of CyP A detected in some carcinomas, suggest that CyP A might be important in the pathogenesis of inflammatory diseases and may serve as a potential therapeutic target of carcinomas (Li et al., 2008).

HLA-B27 is a human leukocyte antigen (HLA) that helps the immune system distinguish between the body's own cells and foreign, harmful substances. TNFα, IFNβ and IFNγ elicit the up-regulation of HLA-B27, which is strongly associated with endoplasmic reticulum (ER) stress and activation of the unfolded protein response, a homeostatic mechanism used by cells to resolve ER stress caused by diverse stimuli (Turner et al., 2005; 2007). The effect of aLTA stimulation on the unfolded protein response is unknown (Schroder and Kaufman, 2005). The up-regulation of HLA-B27 in aLTA-treated THP-1 cells might be an important link between the innate and acquired immune responses.

Post-transcription RNA-binding factors

Several cytokines including TNFα and IL-8 are crucial mediators of the innate and inflammatory responses, so their expression is tightly regulated at both the transcriptional and post-transcriptional levels. The importance of post-transcriptional regulation in immune system homoeostasis and the response to challenge by pathogens, is increasingly being appreciated. Regulation of mRNA stability is central to post-transcriptional modulation of gene expression. Several proteins responsible for post-transcriptional RNA processing were differentially expressed in aLTA- or pLTA-treated cells, including heterogeneous nuclear ribonucleoprotein C (hnRNP-C), heterogeneous nuclear ribonucleoprotein H1 (hnRNP-H1), polypyrimidine tract-binding protein-associated splicing factor (PSF), and KH-type splicing regulatory protein (KSRP).

HnRNP-C is a nuclear pre-mRNA binding protein in vertebrate cell proliferation and differentiation. This H_2O_2-responsive protein is up-regulated in atherosclerosis and in preatherosclerotic intimal hyperplasia in humans, and may be useful as a marker of vascular cell activation (Panchenko et al., 2009). In addition to significant up-regulation in aLTA-treated cells, two new spots for HnRNP-C were observed by 2-DE (Fig. 1). The results revealed that hnRNP-C may have several modifications that reflect a change in its quality or activity. HnRNP-H1, another member of the hnRNP family, was up-regulated by pLTA but not aLTA. The change in hnRNP expression in LTA-treated cells may correlate with LTA-mediated innate immunity.

Most mRNAs encoding cytokines and chemokines are inherently unstable, because of AU-rich elements (AREs) in their 3′-untranslated regions (Bevilacqua et al., 2003; Chen and Shyu, 1995). ARE-binding proteins (ABPs) mediate the degradation of target mRNAs, and determine the stability of mRNAs. PSF and KSRP are ABPs that were differentially expressed in LTA-stimulated cells. PSF was up-regulated by pLTA, and KSRP was down-regulated by aLTA at protein level; however, pLTA and aLTA had no different effects on the expression of KSRP at gene level (Fig. 3), which implies that aLTA might regulate KSRP expression through post-translation. PSF is as a suppressor of gene expression (Mathur et al., 2001), and, with its homolog $p54^{nrb}$, forms a multifunctional heterodimer that participates in the decay of TNFα mRNA and tumorigenesis (Buxade et al., 2008; Shave-Tal and Zipori, 2002). The possibility that up-regulation of PSF by pLTA might confer pLTA tolerance in pLTA-pretreated cells is intriguing (Kim et al., 2008a).

KSRP is a destabilizing factor that causes rapid degradation of ARE-containing mRNAs (Chen et al., 2001; Garneau et al., 2007). Several unstable mRNAs for proteins in innate immunity and inflammation are regulated by KSRP (Winzen et al., 2007). Like LPS, aLTA is potent immunostimulating agent of *S. aureus* and our previous unpublished microarray data demonstrated that many genes encoding cytokines or inflammatory factors are markedly up-regulated by aLTA. Given the inhibitory effect of aLTA stimulation on KSRP expression, two factors might contribute to the molecular mechanism by which aLTA strongly induces cytokine and chemokine secretion (Fig. 4). First, at the transcriptional level, aLTA might induce robust expression of inflammation-related genes through TLR2-mediated activation of NF-κB or MAP kinases (Kawai and Akira, 2007). Second, at

the post-transcriptional level, aLTA might stabilize ARE-containing mRNAs through down-regulation of the critical destabilizing factor KSRP.

CONCLUSION

In this study, comparative proteomic analysis revealed that the proteomes of aLTA- and pLTA-treated THP-1 cells have a few similarities but a greater number of differences. Several immune or inflammatory response proteins were expressed differently in aLTA- and pLTA-stimulated cells. These data provide direct evidence that aLTA and pLTA have different immunomodulatory effects, which may be due to their different chemical structure. The altered proteome pattern helps delineate aLTA-triggered signaling that might contribute to understanding the pathogenesis of *S. aureus* sepsis. The identified proteins are potential biomarkers for further investigation and therapy of septic disease worldwide.

ACKNOWLEDGMENTS

This work was supported by Basic Science Research Program through the National Research Foundation of Korea grant funded by the Korea Government (Ministry of Education, Science and Technology) (KRF-2008-313-F00132) and a grant of the 21C Frontier Microbial Genomics and Applications Center Program, Ministry of Education, Science and Technology (2009K000716), Republic of Korea.

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