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Global Transcriptional Analysis Reveals Upregulation of NF-κB-responsive and Interferon-stimulated Genes in Monocytes by *Treponema lecithinolyticum* Major Surface Protein

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MspTL is the major surface protein of *Treponema lecithinolyticum* associated with periodontitis and endodontic infections. Our recent investigation revealed that MspTL induces proinflammatory cytokines and intercellular adhesion molecule 1 in THP-1 cells and periodontal ligament cells. In this study we conducted oligonucleotide microarray analysis to investigate the global transcriptional regulation in THP-1 cells stimulated with purified recombinant MspTL. MspTL upregulated the expression of 90 genes in THP-1 cells at least four fold, and the functions of these genes were categorized into adhesion, apoptosis/antiapoptosis, cell cycle/growth/differentiation, chemotaxis, cytoskeleton organization, immune response, molecular metabolism, proteolysis, signaling, and transcription. The majority of the modified genes are known to be NF-κB-responsive and interferon-stimulated genes (ISGs). The expression of 12 selected genes was confirmed by real-time RT-PCR. Because prostaglandin E_2 (PGE_2) is an important inflammatory mediator and Cox-2 was found to be induced by MspTL in the microarray analysis, we determined the level of PGE_2 in the culture supernatants of MspTL-treated cells and found that MspTL significantly increased PGE_2. Our results provide insight into the gene regulation of host cells in response to MspTL, and may contribute to the understanding of the molecular mechanism in periodontitis.

Key words: *T. lecithinolyticum*, Surface protein, Oligonulceotide microarray

Introduction

Treponema lecithinolyticum is associated with periodontitis and endodontic infections (Siqueira *et al*. 2003; Wyss *et al*. 1999). This bacterium induces MMP-2 activation in human gingival fibroblasts and periodontal ligament (PDL) cells (Choi *et al*. 2001a) and osteoclastogenesis in cocultures of mouse calvaria and bone marrow cells (Choi *et al*. 2001b), suggesting an involvement in the destruction of soft and hard tissues in the periodontium. In order to elucidate the molecular pathogenesis, we recently analyzed the function of the major surface protein of *T. lecithinolyticum*, MspTL, which induced the expression of molecules involved in inflammation including IL-1β, IL-6, IL-8, and intercellular adhesion molecule-1 (ICAM-1) in THP-1 and PDL cells (Lee *et al*. 2005).

The studies of interaction between host and pathogen provide insights into host defenses and pathogen-specific modification of those defenses, which may have practical applications in the development of therapeutic drugs and vaccines. Bacterial outer membrane is the outmost barrier to the host cells and outer membrane proteins (OMPs) elicit diverse activities like adhesion, cytotoxicity, antigenicity, and other cell stimulating activities. Since multiple bacteria species are known to be associated with periodontitis, functional elucidation of OMPs from various periodontopathogens is required to understand common molecular pathogenesis, which may provide the strategy to control the multi-complex microbial infection.

It has been useful to study the regulation of several genes

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chosen in host cells by bacterial virulence factors. However, information on the global gene or protein regulation in host cells will facilitate the elucidation of the functional role of virulence factors and thereby the understanding of the molecular pathogenesis (Nau *et al*. 2002; Voyich *et al*. 2005). Transcriptional and proteomic analysis can be used to identify genes and gene products in host cells that are differentially expressed by bacterial virulence factors. Oligonucleotide microarray analysis has been shown to be a reproducible and powerful method for analyzing global gene expression profiles (Barnes *et al*. 2005; Cohen *et al*., 2000; Jin *et al*., 2010).

The purpose of this study was to analyze the global gene regulation of MspTL in order to understand its molecular pathogenesis. We demonstrated that the major surface protein of *T. lecithinolyticum*, MspTL, modulated the expression of numerous genes in THP-1 cells by oligonucleotide microarray analysis. Many of the genes upregulated are involved in inflammation and immune response, and most of them are known to be NF-κB-responsive and interferon-stimulated genes (ISGs). We confirmed the reliability of our microarray data by comparing it with our previous results, and with data analyzed by real-time RT-PCR, and ELISA.

Materials and Methods

Preparation of recombinant MspTL

Treponema lecithinolyticum ATCC 700332 was cultured anaerobically by using OMIZ-Pat medium as described previously (Wyss *et al*. 1999). The gene encoding MspTL of *T. lecithinolyticum* was cloned in *Escherichia coli*, using the expression vector pQE-30, and recombinant MspTL (rMspTL) preparation and endotoxin decontamination were performed as described previously (Lee *et al*. 2005). The endotoxin activity in the rMspTL preparation was determined by the Limulus amebocyte lysate (LAL) assay using a LAL Endochrome kit (Charles River Endosafe, Charleston, S.C.) according to the manufacturer's protocol. The endotoxin activity of purified rMspTL was less than 0.015 EU/μg which was about 1/10000 of that in *E. coli* LPS. For a mock control, *E. coli* transformed with pQE-30 without the insert DNA was cultured and the extract was prepared as described previously for the control of endotoxon contamination during rMspTL preparation (Lee *et al*. 2005).

Treatment of THP-1 cells with rMspTL

THP-1 cells were purchased from ATCC and maintained in RPMI 1640 medium supplemented with L-glutamine (Invitrogen, Carlsbad, CA), 10% heat-inactivated fetal bovine serum (FBS), and antibiotics (100 U of penicillin per ml and 100 μg of streptomycin sulfate per ml). THP-1 cells were cultured overnight and plated in 35-mm-diameter cell culture dishes at a concentration of 1×10^6 cells/ml in serum free medium. The cells were then stimulated with 10 μg/ml of rMspTL for 12 and 24 h. The cells were harvested by centrifugation and used for RNA isolation to perform oligonucleotide microarray analysis and real-time RT-PCR. The conditioned culture medium was collected and stored at −70°C for the measurement of prostaglandin E_2 (PGE_2). Nontreated THP-1 cells were used as a negative control.

Since we used rMspTL that was cloned and expressed in *E. coli*, we rigorously examined the cell stimulating activity of rMspTL to confirm that the activity did not result from *E. coli* endotoxin contamination. THP-1 cells were treated with heat-inactivated rMspTL (95°C for 30 min) or mock extract, and the cellular activity was analyzed by ICAM-1 expression as described previously (Lee *et al*. 2005). Also, THP-1 cells were treated with rMspTL (10 μg/ml) or *E. coli* LPS (10 μg/ml) in the presence of an endotoxin inhibitor, polymyxin B (50 μg/ml), for 12 h and then assessed for ICAM-1 expression.

RNA preparation and oligonucleotide microarray

Total RNA was extracted from rMspTL-treated or non-treated THP-1 cells using TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY) according to the manufacturer's protocol and purified with a RNeasy kit (Qiagen, Valencia, CA). RNA integrity was verified by agarose gel electrophoresis and the ratio of absorbance at 260 and 280 nm (1.9~2.1) was checked to determine RNA quality. RNA was quantitated by measuring the absorbance at 260 nm. Microarray analysis was performed according to the GeneChip manufacturer's instructions (Affymetrix Inc., Santa Clara, CA) at the Affymetrix facility (Seoulin Science, Seoul, Korea). Briefly, the total RNA (20 μg) was reverse-transcribed into double-stranded cDNA using a T7-linked oilgo (dT) primer and SuperScript II reverse transcriptase (Life Technologies, Inc. Rockville, MD). Biotin-labeled cRNA was synthesized from the double-stranded cDNA using a Transcript Labeling kit (Enzo Life Sciences, Farmingdale, NY) and was purified on microspin columns. Purified biotin-labeled cRNA was fragmented and hybridized against the Affymetrix Human Genome U133A array (HG_U133A, Affymetrix Inc., Santa Clara, CA) at 45°C for 16 h. After washing, the GeneChip was stained with streptavidin-phycoerythrin conjugate (Molecular Probes, Eugene, OR). The chip signal was scanned and the image files were automatically acquired by an Affymetrix GeneChip Fluidics Station 4500 System. Using Affymetrix Microarray Suite software (MAS, 5.0), gene expression was evaluated and the expression fold change after MspTL treatment was determined. Background and noise signal was controlled at the standard range. The array used was comprised of 22,283 total probe sets and analyzed 18,400 transcripts of 14,285 human genes. A complete list of the genes present on HG_U133A can be found in the Affymetrix database at http://www.affymetrix.com/analysis/index.affx. The microarray experiments were performed twice using RNA prepared from the THP-1 cells, which were independently treated with rMspTL. Each experimental set was composed of 12 h-control, 12 h-treated cells,

24 h-control, and 24 h-treated cells. The two criteria for selection of upregulated genes were an average signal difference equal to or more than 60 after MspTL stimulation, and at least a four-fold change, compared to the nontreated control cells. Genes were considered downregulated if at least a three-fold decrease in expression was observed. Genes and gene functions were designated according to the Gene Ontology (GO) biological process annotations from Affymetrix using the Netaffx Gene Ontology Mining Tool (https://www.affymetrix.com/analysis/netaffx).

Real-time RT-PCR

To verify the results of microarray analysis, reverse transcription and subsequently real-time PCR of 12 selected genes were performed. RNA from THP-1 cells treated with rMspTL (10 μg/ml) for 12 and 24 h was isolated using TRIzol reagent (Invitrogen Life Technology). cDNA was synthesized by mixing RNA (1 μg) and Maxime RT premix (iNtRON, Korea) in a 20 μl reaction volume and incubating the mixture at 42°C for 1 h. After heating, the cDNA (1 μl) was mixed with 10 μl of SYBR Premix Ex Taq (Takara Bio Inc. Japan) and primer pairs (each 4 pmol) in a 20 μl reaction volume, followed by PCR for 40 cycles with 95°C denaturation for 15 sec, 58°C annealing for 15 sec, and 72°C extension for 33 sec in an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The PCR products were subjected to a melting curve analysis to verify a single amplification product. PCR without RT was performed as a negative control. The housekeeping gene encoding B-actin was used as reference in order to normalize expression levels and to quantitate changes in gene expressions between nontreated control and MspTL-treated cells. The expression fold change of each gene was determined from the difference in cycle numbers to reach a threshold value between control and MspTL-treated cells and was expressed in a $\log_2$ ratio. The selected genes for real-time RT-PCR were chemokine (C-C motif) ligand 2 (*CCL2*/*MCP-1*), *CCL4*/*MIP-1B*, *CCL8*/*MCP-2*, chemokine (C-X-C motif) ligand 10 (*CXCL10*/*IP-10*), Epstein-Barr virus induced gene 3 (*EBI3*), insulin-like growth factor binding protein 3 (*IGFBP3*), interleukin 7 receptor (*IL7R*), interferon-stimulated exonuclease gene 20 kDa (*ISG20*), Kreisler (mouse) maf-related leucine zipper homolog (*KRML*), serum/glucocorticoid regulated kinase (*SGK*), superoxide dismutase 2 (*SOD2*), and tryptophan 2,3-dioxygenase (*TDO2*). The primer sequences of the genes are shown in Table 1.

ELISA for PGE_2

The culture supernatants of THP-1 cells treated with rMspTL (10 μg/ml) for 24 h were assayed to determine PGE_2 level using ELISA kits from R&D Systems (Minneapolis, Minn). Culture supernatants of THP-1 cells treated with *E. coli* LPS (10 μg/ml) for 24 h were used as a positive control. For the inhibition assay, THP-1 cells were preincubated with 10 μM NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide), a cox-2 inhibitor, for 30 min, and then treated with rMspTL (10 μg/ml) or *E. coli* LPS (10 μg/ml) for 24 h. PGE_2 level in the culture supernatants was analyzed by ELISA. The statistical significance of the difference of PGE_2 level between non-treated and rMspTL-treated or between rMspTL-treated and inhibitor-treated cells was evaluated by a one-way analysis of variance. A P value of < 0.05 was considered significant.

Results

Identification of genes modulated by MspTL in THP-1 cells

To investigate the global host cell response to MspTL and to identify genes modulated by the protein, we performed oligonucleotide microarray analysis of total RNA from THP-1 cells treated with rMspTL for 12 and 24 h. In order to confirm that the cell stimulating activity of rMspTL was not

Table 1. Primers for real-time RT-PCR

Gene	Primer sequence (5' to 3')	
CCL2	sense: CTCGCTCAGCCAGATGCAA	antisense: AGCTGCAGATTCTTGGGTTG
CCL4	sense: TTTCTTACACCGCGAGGAAG	antisense: GTATGGAGGAGATGTGTCTC
CCL8	sense: AACTCTATCTGTCATACATCCTA	antisense: AACAACAGCAGTACAAAGCAC
CXCL10	sense: TTCTTAGTGGATGTTCTGACC	antisense: GTGTTTGGAATTGTATGTAGGT
EBI3	sense: TGGGCAAGTAGCAAGGGCT	antisense: CCTGCACTGTGGTAAAGGTA
IGFBP3	sense: GGAGCACGTAGAGATTCACC	antisense: AGCCCAGTCTCTGGGCGT
IL7R	sense: AGAATGGGCCTCATGTGTAC	antisense: GCTCACGGTAAGTTCAGTCT
ISG20	sense: GACCTGAAGACAGACTTCC	antisense: CTCTGGGAGATTTGATAGAG
KRLM	sense: GACCAATGCATTGCGTTTCTT	antisense: AGCGCCAGCAATTTCAAATGG
SGK	sense: TTATGGACCAATGCCCCAGTT	antisense: GAGGGAAGGATTGTACGTATT
SOD2	sense: AAATGCAGTATTTCAGCCTGAT	antisense: AATACTGAAGAGAAAGGTTCC
TDO2	sense: GGATACCGAAGATGAACCCA	antisense: CAGAGCATCGTGGTGCTGA
B-actin	sense: ATTGCCGACAGGATGCAGAAG	antisense: TTGCTGATCCACATCTGCTGG

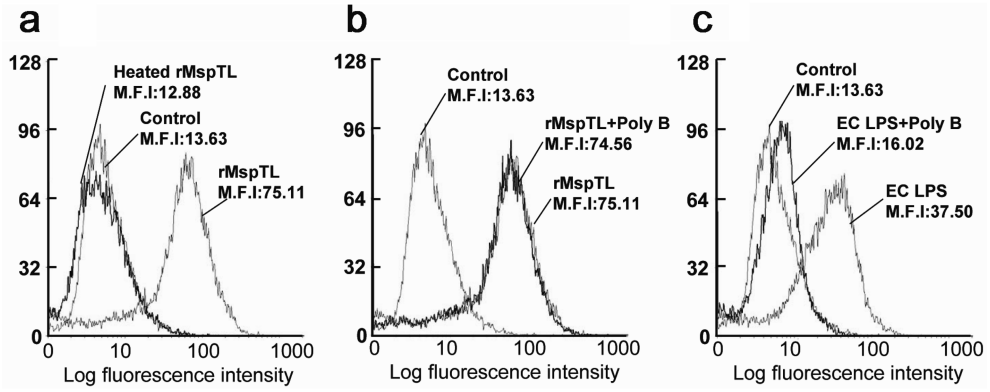

Fig. 1. ICAM-1 expression by rMspTL, not by endotoxin contamination. THP-1 cells (1×10^6 cells/ml) were treated with rMspTL (10 μg/ml) or heated rMspTL (30 min at 95°C, 10 μg/ml) (a). THP-1 cells were treated with rMspTL (10 μg/ml) or *E. coli* LPS (EC LPS, 10 μg/ml) for 12 h in the presence or absence of polymyxin B (Poly B, b and c). ICAM-1 expression was analyzed by flow cytometry using anti-human ICAM-1 mAb and FITC-labeled IgG. Nonterated cells were used as a control. The y axis represents the cell count. M.F.I: mean fluorescence intensity.

caused by endotoxin contamination, we examined the ability of rMspTL to induce ICAM-1 in various contexts. First, heat-inactivated rMspTL did not stimulate THP-1 cells to express ICAM-1, whereas rMspTL significantly induced ICAM-1 expression (Fig. 1a). The mock extract did not induce ICAM-1 expression, either (data not shown). Second, polymyxin B, an endotoxin inhibitor, did not affect ICAM-1 induction by rMspTL (Fig. 1b), whereas ICAM-1 induction by *E. coli* LPS was inhibited by polymyxin B (Fig. 1c). Third, in this study, we used serum free medium for the cell stimulating experiments with rMspTL, and we observed that at an endotoxin concentration equivalent to 1 ng/ml of *E. coli* LPS did not induce ICAM-1 in the absence of serum in our previous study (Lee *et al*. 2006). The endotoxin activity of rMspTL was about 1/10000 of *E. coli* LPS and 10 μg/ml of rMspTL was used for cell stimulation). Taken together, we were certain that the cell stimulating activity of rMspTL was not caused by *E. coli* endotoxin contamination.

Although a vast number of genes were significantly modulated by MspTL, we selected the genes that showed at least a four-fold increase or three-fold decrease in hybridization signals in two independent experiments either by the 12 h- or 24 h-treatment, compared to nontreated control cells. Genes with an intensity value of less than 60 after MspTL treatment were discarded because of a high distribution discrepancy between the two experiments. According to the criteria, 90 genes were upregulated after MspTL treatment (Table 2), whereas only five genes were downregulated (Table 3). The expression fold change of most upregulated genes (80 genes) was higher at the 24 h- than the 12 h-treatment. Of the five downregulated genes, three genes showed a greater expression change at the 24 h- than the 12 h-treatment. The

Table 2. Genes upregulated in THP-1 cells by MspTL

Gene accession no.	Gene name (gene abbreviation)	Fold change
	Adhesion	
NM_021181	SLAM family member 7 (*SLAMF7*)	14.9
NM_007115	Tumor necrosis factor, alpha-induced protein 6 (*TNFAIP6*)	7.0
NM_004288	Pleckstrin homology, Sec7 and coiled-coil domains, binding protein (*PSCDBP*)	7.0
NM_000201	Intercellular adhesion molecule 1 (*ICAM1*)	6.5
NM_004385	Chondroitin sulfate proteoglycan 2 (*CSPG2*)	6.5
NM_022570	C-type lectin domain family 7, member A (*CLEC7A*)	5.7
NM_005103	Fasciculation and elongation protein zeta 1 (zygin I) (*FEZ1*)	5.7
NM_001078	Vascular cell adhesion molecule 1 (*VCAM1*)	4.9
NM_023068	Sialoadhesin (*SN*)	4.9
NM_000582	Secreted phophoprotein 1 (*SPP1*)	4.0
	Apoptosis and antiapoptosis	
NM_003897	Immediate early response 3 (*IER3*)	8.6
NM_004049	BCL2-related protein A1 (*BCL2A1*)	5.6
NM_002462	Myxovirus resistance 1 (*MX1*)	4.9

Table 2. (Continued) Genes upregulated in THP-1 cells by MspTL

Gene accession no.	Gene name (gene abbreviation)	Fold change
	Cell cycle, growth, and proliferation	
NM_000598	Insulin-like growth factor binding protein 3 (*IGFBP3*)	18.4
NM_016441	Cysteine rich transmembrane BMP regulator 1 (chordin-like) (*CRIM1*)	5.3*
NM_003641	Interferon induced transmembrane protein 1 (9-27) (*IFITM1*)	5.0
NM_002607	Platelet-derived growth factor alpha polypeptide (*PDGFA*)	5.0
	Chemotaxis	
NM_000648	Chemokine (C-C motif) ligand 2 (*CCL2*) / *MCP-1*	29.9
NM_005623	Chemokine (C-C motif) ligand 8 (*CCL8*) / *MCP-2*	24.3
NM_006419	Chemokine (C-X-C motif) ligand 13 (*CXCL13*) / *BLC*	24.3
NM_002984	Chemokine (C-C motif) ligand 4 (*CCL4*) / *MIP-1B*	13.0
NM_001565	Chemokine (C-X-C motif) ligand 10 (*CXCL10*) / *IP-10*	13.0
NM_000952	Platelet-activating factor receptor (*PTAFR*)	10.6
NM_002983	Chemokine (C-C motif) ligand 3 (*CCL3*) / *MIP-1α*	9.2
NM_005409	Chemokine (C-X-C motif) ligand 11 (*CXCL11*) / *I-TAC*	8.0*
NM_005408	Chemokine (C-C motif) ligand 13 (*CCL13*) / *MIP-1α*	5.7
NM_006273	Chemokine (C-C motif) ligand 7 (*CCL7*) / *MCP-3*	5.7*
NM_000584	Chemokine (C-X-C motif) ligand (*CXCL8*) / interleukin 8 (*IL8*)	5.3
NM_001736	Complement component 5 receptor 1 (*C5R1*)	4.9
NM_002089	Chemokine (C-X-C motif) ligand 2 (*CXCL2*)/GROβ	4.0
NM_004054	Complement component 3a receptor 1 (*C3AR1*)	4.0*
	Cytoskeleton organization, and cell motility	
NM_002356	Myristoylated alanine-rich protein kinase C substrate (*MARCKS*)	5.7
NM_012307	Erythrocyte membrane protein band 4.1-like 3 (*EPB41L3*)	5.3
	Immune response	
NM_000576	Interleukin 1, beta (*IL1B*)	17.1
NM_005755	Epstein-Barr virus induced gene 3 (*EBI3*)	14.9
NM_002185	Interleukin 7 receptor (*IL7R*)	9.2*
NM_000591	CD14 antigen (*CD14*)	8.0
NM_001547	Interferon-induced protein with tetratricopeptide repeats 1 (*IFIT2*)	7.5
NM_001549	Interferon-induced protein with tetratricopeptide repeats 3 (*IFIT3*)	6.1
NM_002053	Guanylate binding protein 1, interferon-inducible, 67kDa (*GBP1*)	5.7
NM_002038	Interferon, alpha-inducible protein (clone IFI-6-16) (*G1P3*)	5.3
NM_005532	Interferon, alpha-inducible protein 27 (*IFI27*)	5.3
NM_014314	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (*DDX58*)	5.3
NM_003733	2'-5'-oligoadenylate synthetase-like (*OASL*)	4.9
NM_004120	Guanylate binding protein 2 (*GBP2*)	4.9
NM_012420	Interferon-induced protein with tetratricopeptide repeats 5 (*IFIT5*)	4.3
NM_005533	Interferon-induced protein 35 (*IFI35*)	4.0
	Lipid metabolism	
NM_005084	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase) plasma) (*PLA2G7*)	9.2*
NM_007283	Monoglyceride lipase (*MGLL*)	5.7
NM_001995	Acyl-CoA synthetase long-chain family member 1 (*ACSL1*)	4.0
	Nucleic acid metabolism	
NM_002201	Interferon stimulated exonuclease gene 20kDa (*ISG20*)	19.7
NM_002534	2',5'-oligoadenylate synthetase 1, 40/46kDa (*OAS1*)	4.9
NM_006187	2'-5'-oligoadenylate synthetase 3, 100kDa (*OAS3*)	
NM_000480	Adenosine monophosphate deaminase (isoform E) (*AMPD3*)	4.0

Table 2. (Continued) Genes upregulated in THP-1 cells by MspTL

Gene accession no.	Gene name (gene abbreviation)	Fold change
	Amino acid metabolism	
NM_005651	Tryptophan 2,3-dioxygenase (*TDO2*)	22.6
NM_003937	Kynureninase (L-kynurenine hydrolase) (*KYNU*)	4.3*
	Proteolysis	
NM_002421	Matrix metalloproteinase 1 (*MMP1*)	9.2
NM_021616	Tripartite motif-containing 34 (*TRIM34*)	7.5
NM_014479	ADAM-like, decysin 1 (*ADAMDEC1*)	7.0
NM_004994	Matrix metalloproteinase 9 (*MMP9*)	5.7
NM_017414	Ubiquitin specific protease 18 (*USP18*)	5.3
NM_006216	Serpin peptidase inhibitor, clade E, member 2 (*SERPINE2*)	4.9
NM_014265	A disintegrin and metalloproteinase domain 28 (*ADAM28*)	4.6
NM_004223	Ubiquitin-conjugating enzyme E2L 6 (*UBE2L6*)	4.3
NM_002133	Heme oxygenase (decycling) 1 (*HMOX1*)	4.3*
	Signaling	
NM_018993	Ras and Rab interactor 2 (*RIN2*)	8.6
NM_005781	Tyrosine kinase, non-receptor, 2 (*TNK2*)	7.0
NM_015149	Ral guanine nucleotide dissociation stimulator-like 1 (*RGL1*)	5.7
NM_005168	Rho family GTPase 3 (*RND3*)	4.9
NM_003263	Toll-like receptor 1 (*TLR1*)	4.0
NM_006209	Ectonucleotide pyrophosphatase/phosphodiesterase 2 (*ENPP2*)	4.0
	Superoxide metabolism	
NM_000636	Superoxide dismutase 2, mitochondrial (*SOD2*)	9.8
NM_000265	Neutrophil cytosolic factor 1 (47kDa) (*NCF1*)	4.6
NM_000397	Cytochrome b-245, beta polypeptide (*CYBB*)	4.0
	Transcription	
NM_005461	Kreisler (mouse) maf-related leucine zipper homolog (*KRML*)	9.2
NM_005531	Interferon, gamma-inducible protein 16 (*IFI16*)	7.5
NM_000594	Tumor necrosis factor (TNF superfamily, member 2) (*TNF*)	7.5
NM_006074	Tripartite motif-containing 22 (*TRIM22*)	6.5
NM_001706	B-cell CLL/lymphoma 6 (*BCL6*)	5.3
NM_004926	Zinc finger protein 36, C3H type-like 1 (*ZFP36L1*)	4.9
NM_005098	Musculin (activated B-cell factor-1) (*MSC*)	4.6
	Miscellaneous	
NM_005627	Serum/glucocorticoid regulated kinase (*SGK*)	14.9
NM_025079	MCP-1 treatment-induced protein (*MCPIP*)	7.0
NM_014705	Dedicator of cytokinesis 4 (*DOCK4*)	4.9
NM_000617	Solute carrier family 11 (*SLC11A2*)	4.3*
	Unknown	
NM_024873	TNFAIP3 interacting protein 3 (*TNIP3*)	13.0*
NM_006820	Interferon-induced protein 44-like (*IFI44L*)	9.8
NM_016644	Mesenchymal stem cell protein DSC54 (*LOC51334*)	9.8
BF575213	Hypothetical protein MGC5618 (*MGC5618*)	8.0
NM_007350	Pleckstrin homology-like domain, family A, member 1 (*PHLDA1*)	4.3

Fold change: the average fold change from two experiments in gene expression after rMspTL (10 μg/ml) treatment compared to non-treated control cells. *Greater fold change in gene expression observed at 12 h-treatment with rMspTL than at 24 h-treatment
NOTE. MCP-1, monocyte chemoattractant protein-1; BLC, B lymphocyte chemoattractant; MIP-1α, macrophage inflammatory protein-1alpha; IP-10, IFN-gamma inducible protein; I-TAC, interferon-inducible T-cell alpha chemoattractant; GROβ, growth regulatory oncogene beta

Table 3. Genes downregulated in THP-1 cells by MspTL

Gene accession no.	Gene name (gene abbreviation)	Gene function	Fold change
NM_002575	Serine proteinase inhibitor, cladeB, member 2 (*SERPINB2*)	antiapoptosis	−14.9
NM_000222	v-Kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*KIT*)	signaling	−6.1*
NM_003877	STAT induced STAT inhibitor-2 (*STATI2*)	cell growth	−3.5
NM_000349	Steroidogenic acute regulatory protein (*STAR*)	steroid hormone biosynthesis	−3.0
NM_007181	Mitogen-activated protein kinase kinase kinase kinase 1 (*MAP4K1*)	signaling	−3.0*

Fold change: the average fold change in gene expression from two experiments after rMspTL (10 μg/ml) treatment compared to non-treated control cells
*Greater fold change in gene expression by 12 h-treatment with rMspTL than by 24 h-treatment

tendency to increase or decrease the selected gene expression was not changed and there was minimal discrepancy in the expression fold change between two independent experiments. The top ten genes upregulated by MspTL were chemokines (*CCL2, CCL8,* and *CXCL13*), tryptophan 2,3-dioxygenase (*TDO2*), interferon-stimulated exonuclease gene 20kDa (*ISG20*), insulin-like growth factor binding protein 3 (*IGFBP3*), *IL-1B*, Epstein-Barr virus induced gene 3 (*EBI3*), SLAM family member 7 (*SLAMF7*), and serum/glucocorticoid regulated kinase (*SGK*). We compared the gene expression shown in the microarray analysis with that observed in our previous study, which demonstrated an upregulation of ICAM-1 and proinflammatory cytokines including IL-1β, IL-6, and IL-8 by rMspTL via NF-κB activation, when analyzed by RT-PCR, flow cytometry, and ELISA (15). All but IL-6 belonged to the gene list shown in Table 2. IL-6 was also significantly upregulated (data not shown), but it was not included in the list because its expression level was altered less than four-fold.

The modulated genes were grouped in diverse functional categories. MspTL induced the expression of genes that encode proteins involved in adhesion, apoptosis/antiapoptosis, cell cycle/growth/proliferation, chemotaxis, cytoskeleton organization, immune response, metabolism, proteolysis, signaling, and transcription (Table 2). The function of several of the modulated genes is unknown. Genes downregulated by MspTL typically function in cell growth, steroid hormone biosynthesis, antiapoptosis, and signaling (Table 3).

In order to confirm some new genes that were found to be induced by MspTL, real-time RT-PCR was performed on 12 selected genes whose expression was strongly upregulated by MspTL. These included *CCL2*/*MCP-1*, *CCL4*/*MIP-1B*, *CCL8*/*MCP-2*, *CXCL10*/*IP-10*, *EBI3*, *IGFBP3*, interleukin 7 receptor (*IL7R*), *ISG20*, Kreisler (mouse) maf-related leucine zipper homolog (*KRML*), *SGK*, *SOD2*, and *TDO2*. Experiments were performed twice and the fold change values were determined after normalization of each gene to B-actin by using a comparative threshold method. As shown in Fig. 2, all of the genes analyzed were upregulated as shown in the microarray analysis. The trend of expression change from 12 h and 24 h-treatment was fairly consistent between the two techniques, while in general, the expression change shown in real-time RT-PCR was higher than that observed in the microarray analysis.

MspTL increased the gene expression of 11 chemokines belonging to the CXC and CC subfamilies. The CXC chemokines detected were *CXCL2*/*GROB*, *CXCL8*/*IL-8*, *CXCL10*/*IP-10*, *CXCL11*/*I-TAC* and *CXCL13*/*BLC*. The CC chemokines included *CCL2*/*MCP-1*, *CCL3*/*MIP-1α*, *CCL4*/*MIP-1B*, *CCL7*/*MCP-3*, *CCL8*/*MCP-2*, and *CCL13*/*MCP-4*. Adhesion

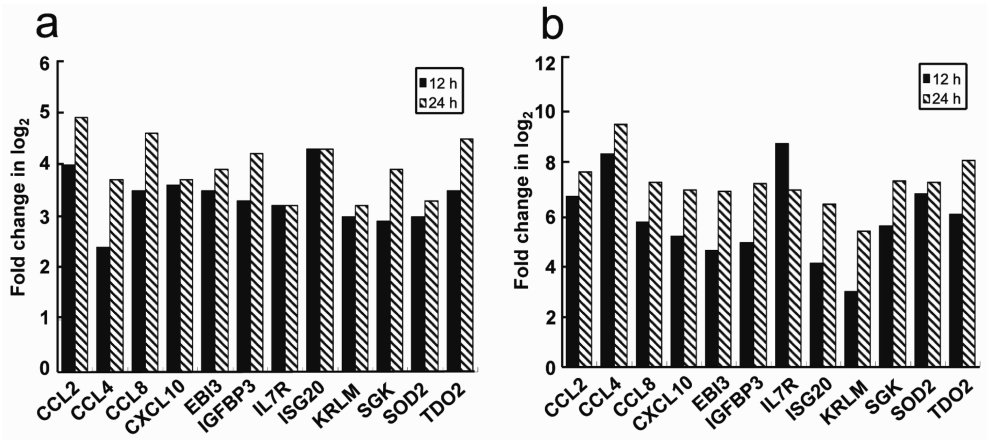

Fig. 2. Expression change of 12 selected genes after rMspTL treatment (12 h and 24 h-treatment) detected by microarray analysis (a) and real-time RT-PCR (b). Each experiment was performed twice and the average fold change in $\log_2$ scale was calculated from the two independent experiments at 12 h- and 24 h-treatments compared to nontreated control cells after normalization of each gene to B-actin.

molecules that are involved in cell to cell or cell to matrix contacts were also significantly increased. And genes encoding tissue degrading or remodeling enzymes like MMP1, MMP9, a disintegrin and metalloproteinase domain 28 (*ADAM28*), and ADAM-like, decysin 1 (*ADAMDEC1*) were observed to be upregulated. Genes involved in superoxide metabolism were found to be upregulated. Cytochrome b-245 beta peptide (*CYBB*) and neutrophil cytosolic factor 1 (*NCF1*) are the primary components of the microbicidal NADPH oxidase system that is the key enzyme for the production of oxygen radicals by human neutrophils. However, superoxide dismutase 2 (*SOD2*), an antioxidative enzyme, was induced by MspTL at the same time.

An interesting finding is that MspTL induced many interferon-stimulated genes (ISGs). These genes included *CCL2*/*MCP-1*, *CXCL10*/*IP-10*, *EBI3*, guanylate binding protein 1 (*GBP1*), *GBP2*, interferon alpha-inducible protein (*G1P3*), immediate early response 3 (*IER3*), interferon-induced protein with tetratricopeptide repeats 2 (*IFIT2*), *IFIT3*, *IFIT5*, interferon alpha-inducible protein 27 (*IFI27*), interferon gamma-inducible protein 16 (*IFI16*), interferon-induced protein 35 (*IFI35*), IFN induced transmembrane protein 1 (*IFITM1*), *ISG20*, myxovirus resistance 1 (*MX1*), *MMP9*, 2',5'-oligoadenylate synthetase 1 (*OAS1*), *OAS3*, 2'-5'-oligoadenylate synthetase-like (*OASL*), and platelet-derived growth factor alpha polypeptide (*PDGFA*). Most of these genes are known to be induced by type I interferons (IFNα, β, and ω) and regulated by interferon regulatory factors (IRFs) (de Veer *et al*. 2001). However, the induction of IFNs by MspTL was not observed. TLR1 and CD14, members of microbial pattern recognition receptor, were also upregulated.

PGE_2 synthesis by MspTL

Because we were interested in the effect of MspTL on PGE_2 synthesis, attention was paid to the regulation of the enzymes involved in prostaglandin synthesis. The gene of prostaglandin-endoperoxide synthase 2 (PTGS2), also known as Cox-2, was significantly induced by MspTL in the microarray analysis, although by less than four fold (3.2 fold by 24 h treatment) threshold (data not shown). To see the coordinated relationship of the gene and protein expression, we measured the PGE_2 level in the conditioned medium of THP-1 cells after 24 h treatment with rMspTL. This experiment was performed independent of the microarray analysis. As shown in Fig. 3, MspTL significantly induced PGE_2 secretion. Pretreatment of the cells with the selective Cox-2 inhibitor NS-398 suppressed the PGE_2 synthesis upregulated by MspTL or *E. coli* LPS.

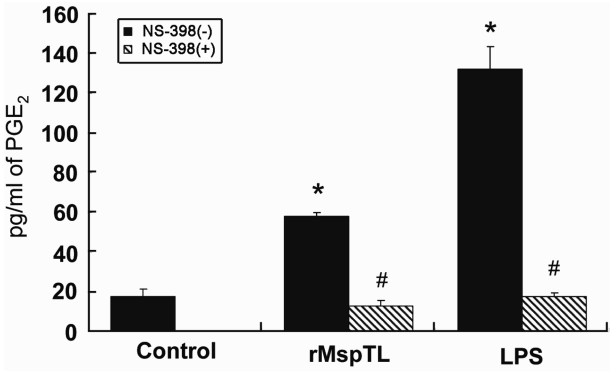

Fig. 3. PGE_2 production by THP-1 cells treated with rMspTL. THP-1 cells (1×10^6 cells/ml) were treated with rMspTL (10 μg/ml) in serum free RPMI 1640 medium for 24 h. For the inhibition assay, THP-1 cells were preincubated with 10 μM NS-398, a Cox-2 inhibitor for 30 min and then treated with rMspTL (10 μg/ml) for 24 h. The conditioned medium was harvested and analyzed for PGE_2 level by ELISA. The experiments were performed twice in triplicates for each assay, and the representative means and standard deviations for triplicate values are shown. *E. coli* LPS (10 μg/ml) was used as appositive control. The asterisks indicate a significant difference ($P < 0.05$) compared with the nontreated control values. The pound signs indicate significant differences ($P < 0.05$) compared with the values of NS-398-treated cells.

Discussion

In this study, we demonstrated that a major bacterial surface protein resulted in extensive gene regulation using the human monocytic cell line THP-1 as naive monocyte cells and oligonucleotide microarray technology. This has the advantage of monitoring the expression of a large number of genes simultaneously. MspTL, a major surface protein of *T. lecithinolyticum* associated with periodontitis and endodontic infections, upregulated 90 genes at least four fold and downregulated five genes at least three fold. The majority of the modified genes are known to be NF-κB-dependent or interferon-stimulated.

The reliability of the microarray data obtained in this study was confirmed by comparing data from the microarray analysis with our previous study, which demonstrated the regulation of several genes and their proteins (Lee *et al*. 2005). Further comparisons were made with those of real-time RT-PCR and ELISA in this study. In our previous study, we observed that MspTL stimulated the expression of ICAM-1 and proinflammatory cytokines like IL-1β, IL-6, and IL-8 both at the gene and protein levels via activation of the transcription factor, NF-κB. In the present study, our microarray data revealed that all these factors were significantly upregulated by MspTL treatment. However, although IL-1β belonged to the top ten genes upregulated by MspTL in the microarray analysis, only a marginal increase of IL-1β level was observed by ELISA in our previous study (Lee *et al*. 2005). In the microarray analysis, two probe sets detected IL-1β gene expression with a similar fold increase in two independent experiments. This large difference between mRNA and protein expression could be in part attributed to the high expression of IL-1 receptor antagonist (IL-1ra) by MspTL, which antagonizes IL-1. MspTL upregulated the IL-1ra gene three fold by 12-h and 3.5 fold by 24-h stimulation (data not shown). For further validation of microarray data, 12 genes

whose expression was most profoundly upregulated by MspTL were selected and their upregulation was verified by real-time RT-PCR. All the genes showed a higher expression level after MspTL treatment compared to the non-treated control cells, as shown in the microarray analysis. In most cases, a higher gene expression level was observed in real-time RT-PCR than in the microarray analysis. The difference of expression fold change between the two techniques could be explained by the nature of the probes or primers used and the target sites. The large expression difference between the two methods was also observed by Galindo *et al*. (Galindo *et al*. 2005).

Recently, we reported that PGE_2 is a main mediator in osteoclastogenesis induced by periodontopathogens including *Porphyromonas gingivalis*, *Treponema denticola*, and *Treponema socranskii* (Choi *et al*. 2005). In the microarray analysis, we observed that the genes of Cox-2, the PGE_2 synthesizing enzyme, was significantly upregulated by MspTL, and PGE_2 upregulation was detected by ELISA. Since *T. lecithinolyticum* whole cell lysates induced osteoclastogenesis (Choi *et al*. 2001b), it remains to be elucidated whether MspTL is an active component for this function. Taken together, these results indicate that the regulation of the representative genes detected in our microarray analysis was consistent with that detected by other methods.

The microarray data shows that MspTL induced an upregulation of various chemokine genes. Chemokines are structurally related molecules with molecular weights of up to 14 kDa. A recent classification of chemokines represents 4 subfamilies, CXC, CC, XC, and CX3C, based on the position of the two amino terminal cysteine residues that participate in disulfide bonding (Zlotnik and Yoshie 2000). They are mediators of chemotaxis, cell adhesion, inflammation and immune response, calcium ion homeostasis, and apoptosis. In humans, there are currently about 40 chemokines identified. MspTL increased five CXC chemokine genes and six CC chemokine genes. The chemokines of the CXC subfamily act predominantly on neutrophils as mediators of acute inflammation and those of the CC subfamily act predominantly on T cells, monocytes, eosinophils, and basophils. *CCL2/MCP-1* was the most profoundly increased chemokine by MspTL. *CCL2/MCP-1* was detected at a significantly higher level in gingival crevicular fluid of generalized aggressive periodontitis patients than in healthy subjects, and its level was positively correlated with both probing depth and clinical attachment loss (Emingil *et al*. 2004). *P. gingivalis* is known to induce CCL2/MCP-1 and ICAM-1 in endothelial cells and monocytes (Choi *et al.* 2009; Kuramitsu *et al*. 2003; Walter *et al*. 2004). CCL2/MCP-1 recruits circulating monocytes to the endothelium and then ICAM-1 binds to monocytes, which then translocate into subendothelial space where they mature into macrophages. Macrophages uptake oxidized low density lipid (oxLDL) to form foam cells, which participate in the formation of atherosclerotic plaques. Reactive oxygen species (ROS) are capable of oxidizing LDL to oxLDL. Kuramitsu *et al*. (2003) observed the foam cell formation by *P. gingivalis* and suggested the implication of this bacterium in cardiovascular disease. Since MspTL induced components of NADPH oxidase as well as CCL2/MCP-1 and ICAM-1, the same scenario related to foam cell formation as observed with *P. gingivalis* could be applied, if these factors are also induced in endothelial cells by MspTL. Kabashima *et al*. (Kabashima *et al*. 2002) detected cells that produce CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1B, CXCL10/IP-10, and CCL5/RANTES in inflamed human gingival tissues, using immunohistochemical methods.

It is also interesting to note that MspTL stimulation resulted in a widespread expression of various type I IFN-stimulated genes (ISGs) in addition to proinflammatory cytokines. Interferons are the first known members of the cytokine family of proteins to induce an antiviral state. They also have various functions like cell growth, cell motility, and other cell functions by inducing IFN-stimulated genes (ISGs). Activation of interferon-regulatory factors (IRFs) required for the transcription of ISGs can be induced by IFN-independent as well as IFN-dependent pathways (Hemmi *et al*. 2004; Sarkar *et al*. 2004). Since IFN induction was not observed by MspTL treatment, MspTL-mediated ISG induction is likely IFN-independent.

Based on the microarray data, we searched for a molecule that may be involved in ISG induction via the IRF pathway. Inducible IkappaB kinase (IKK-i) and tank binding kinase 1 (TBK-1) are recently identified kinases related to IKK-α and IKK-β that are involved in NF-κB activation by phosphorylation of IκB, and they can activate both NF-κB and IRF3 (Hemmi *et al*. 2004; Ishii *et al*. 2005; Sankar *et al*. 2006). IKK-i is inducible in response to ligands of TLR3 or TLR4, whereas TBK-1 is constitutively expressed. The overexpression, but not the phosphorylation of IKK-i, activates both IRF3 and NF-κB (Nomura *et al*. 2000). Sanker *et al*. (2006) showed that siRNA to IKK-β did not block IKK-i dependent IP-10 production, suggesting that IKK-i acts upstream of IKK-α and IKK-β. Indeed, MspTL induced IKK-i gene expression 1.7 fold by 12 h and 2.5 fold by 24 h stimulation. IKK-i, along with TBK-1, could be the molecule involved in ISG expression by MspTL as well as the linking molecule between the IRF and NF-κB pathway, which might be activated upon MspTL stimulation.

MspTL induced NADPH oxidase component genes like CYBB and NCF1 (p47-phox). This enzyme is involved in oxygen-dependent phagocytic killing. MspTL also upregulated SGK that mediates cell survival in response to diverse environmental stresses like hyperosmotic stress, heat, UV, oxidative stress, growth factors, or hormones (Leong *et al*. 2003). All these factors are related to the host defense by a bacterial challenge. MspTL also induced SOD2, an antioxidant enzyme, which converts superoxide to hydrogen peroxide. This suggests that MspTL is able to regulate the oxidative

stress response of host cells.

MspTL induced two enzymes in the kynurenine pathway, tryptophan 2,3 dioxygenase (TDO2) and kynureninase (KYNU). Although TDO2 is known to be localized to the liver, its induction was observed in THP-1 cells treated with bacteria (Ren *et al*. 2003). Tryptophan is an essential amino acid, and is required for the biosynthesis of proteins and for several biologically important compounds. Upregulated TDO2 can lead to tryptophan deprivation in host cells by tryptophan degradation which has detrimental consequences to the host. LPS induces indoleamine 2,3-dioxygenase (IDO2) which has a similar function as TDO2 in a variety cells of extrahepatic tissues in both an IFN-γ-dependent and -independent manner (Fujigaki *et al*. 2001). IDO2 was not expressed in either MspTL-treated or nontreated control THP-1 cells used in this study.

MspTL induced the genes for matrix metalloproteinases (MMP1, MMP9) and disintegrin metalloproteinases (ADAM28, ADAMDEC1). MMPs are involved in tissue degradation, which is a characteristic of periodontitis, and the ADAM family functions as adhesion proteins and endopepitdase. The upregulation of all these factors will facilitates an inflammatory response and can be linked to the pathogenesis of periodontitis.

In summary, this study examined global gene regulation in THP-1 cells in response to the major surface protein MspTL of *T. lecithinolyticum*, a periodontopathogen. THP-1 cells may not be the best model for the study of host cell response to bacterial virulence factors. Furthermore, one limitation of our study was that we observed the gene regulation at time points after an extended stimulation (12 and 24 h) with MspTL, therefore, we may have missed genes whose expressions were altered at an early time after MspTL treatment. Despite of these limitations, we provide insights into the host cell response to this protein, detecting a broad range of factors involved in the inflammatory and immune reactions. Interpretation of our microarray data suggests that MspTL induces at least two categories of genes, NF-κB-responsive and IFN-stimulated genes (ISGs). Although we used only THP-1 cells in this study, our data may serve as a reference for the response of other cell types including periodontal tissue cells and endothelial cells. Our data on gene regulation by MspTL may contribute to the elucidation and collection of the functional roles of OMPs from periodontopathogens, which is necessary to elucidate common pathogenesis and to identify new therapeutic targets.

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