Comparison of Cytokine Gene Induction in RAW 264.7 Cells by *Porphyromonas gingivalis* and *Escherichia coli* Lipopolysaccharide

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Porphyromonas gingivalis **lipopolysaccharide (Pg LPS) is an important virulence factor in chronic periodontitis. The aim of this study was to compare the expression of inflammatory cytokine genes in** *Escherichia coli* **LPS (Ec LPS) and Pg LPS-stimulated mouse macrophage RAW 264.7 cells. Cells were treated with Ec LPS and Pg LPS for 18 hours, and the cytokine gene expression profile was assessed using microarrays and confirmed by real-time PCR. Microarray analysis showed that both types of LPS induced a significant increase in the expression of** *IL-17β, IL-2, Ccl4, Cxcl2* **and** *TNFα* **compared with the control. However,** *LT-b* **was up-regulated by Pg LPS but not by Ec LPS. Real-time PCR analysis of these genes showed similar results for** *LT-b, Ccl4, Cxcl2,* **and** *TNF-α* **but found that** *IL-17β* **and** *IL-2* **were upregulated by Pg LPS but not by Ec LPS. These data indicate that Pg LPS stimulates the transcription of** *IL-17β, IL-2, Ccl4, Cxcl2, LT-b,* **and** *TNFα,* **all of which may be involved in the pathogenesis of chronic periodontitis.**

Key words: *Porphyromonas gingivalis,* **cytokine, gene expression, lipopolysaccharide.**

Introduction

Periodontal disease is an infectious process characterized by chronic inflammation affecting the supporting structures of the teeth. Specific oral bacteria, such as *Porphyromonas*

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gingivalis, have been suggested to play a primary role in the initiation and exacerbation of periodontal inflammation (Mangan *et al*., 1993). It has been implicated that the host response to periodontal bacteria plays an important role in the process of periodontitis (Offenbacher *et al*., 1993; Page *et al*., 1991). *P. gingivalis* is an absolutely anaerobic gram-negative bacillus and associated with chronic periodontitis (Lamont *et al*., 1998; Dzink *et al*., 1988). The mononuclear phagocyte is one of the principal cells related to the inflammatory response under chronic infection (Zembala *et al*., 1989). The immune cells including T, B, and phagocytic cells present in the periodontium could contribute to local host responses in response to periodontal bacteria (Williams *et al*., 1990).

LPS, a major component of the outer membrane of gram-negative bacteria such as *P. gingivalis* (Socransky *et al*., 1992) and has the ability to activate host cells, especially mono-nuclear phagocytes (Morrison *et al*., 1987). *P. gingivalis* lipopolysaccharide (Pg LPS) differs from other bacterial LPS in its ability to induce cytokines. Unlike *Escherichia coli* LPS (Ec LPS), which induces Th1 cytokines with high levels of interferon-γ but little or no IL-4, IL-5 and IL-13, Pg LPS induces IL-5, IL-10 and IL-13, and lower levels of interferon-γ (Pulendran *et al*., 2001). Although the exact reason for this is still not known, it has been conjectured that the unique head and branched longer fatty acid tail of the lipid A of Pg LPS may elicit a less endotoxic response (Hamada *et al*., 1990; Nair *et al*., 1983; Ogawa *et al*., 1994).

Real-time PCR with SYBR Green double-stranded DNA (dsDNA) binding dye is a sensitive, efficient, and reliable assay for the quantification of cytokine mRNA. Therefore, in this study, we carried out microarray experiment and the microarray results were confirmed by real-time PCR to investigate the differential expressions of inflammatory genes including cytokines and chemokines induced by Pg LPS and

Ec LPS in RAW 264.7 mouse macrophage cells, in an attempt to search out the specific gene closely related to *P. gingivalis* infection.

Materials and Methods

Bacterial Growth

P. gingivalis strain A7A1-21 was cultured in Tryptic soy broth (Difco, NJ), which contained 5 mg/ml hemin and 0.5 mg/ml of vitamin K at $37^{\circ}C$ in a 5% CO_2 incubator.

LPS purification

P. gingivalis was grown under 5% CO_2 condition and harvested at the end of the logarithmic phase of growth. LPS extraction was achieved by the hot phenol-water method (Westphal *et al.*, 1965). Briefly, the bacterial cell pellet was suspended in pyrogen-free water, and then an equal volume of 90% phenol at $60^{\circ}C$ was added dropwise for 20 min and stirred constantly. The aqueous phase was separated by centrifugation at 7,000 rpm for 15 min at $4^{\circ}C$ and collected. This process was repeated, and the aqueous phase was pooled and dialyzed against deionized water for 3 days at $4^{\circ}C$. The dialyzed LPS preparation was then centrifuged at 40,000 rpm for 1.5 h at $4^{\circ}C$ in a Beckman (Palo Alto, CA) ultracentrifuge. The precipitate was suspended with 30 ml of pyrogen-free water, dialyzed against distilled water for 3 days, lyophilized, and stored at $4^{\circ}C$. LPS samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained for protein with Coomassie blue to confirm the purity of the LPS moieties.

Cell culture

The mouse macrophage cell line RAW 264.7 was purchased from American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum (Life Technologies, Inc., Paisley, Scotland), 100 U/ml of penicillin, and 100 μg/ml of strepto-mycin and were incubated at $37^{\circ}C$ in a humidified atmosphere of 5% CO_2.

RNA preparation and cDNA synthesis

RAW 264.7 cells were treated for 2 h and 18 h with 0.1 μg/ml of Pg LPS. Total RNA from these cells was isolated using TRIzol (Invitrogen, Carlsbad, CA) according to the manu-facturer's instructions. The concentration of the RNA obtained was determined by measuring the absorbance at 260 and 280 nm. 1 μg of RNA was reverse transcribed with AccuPower RT PreMix (Bioneer Co., Daejeon, Korea) in a total volume of 20 μl with random hexamer primers at $37^{\circ}C$ for 60 min.

Microarray

The gene expression array analysis was performed using the mouse Inflammatory cytokines & Receptors Gene Array (SuperArray Inc., Bethesda, MD) according to the manu-facturer's instructions. This array membrane is composed of 96 cytokine and receptor genes associated with inflammatory response, a plasmid pUC18 negative control, and four house-keeping genes including genes encoding glyceraldehydes-3-phosphate dehydrogenase (GAPDH), cyclophilin A, ribosomal protein L13a, and β-actin, each of which is printed with tetra spots format. The biotin-16-dUTP-labeled cDNA probes were synthesized from 5 μg of total RNA. After pre-hybridization with GEAhyb Hybridization Solution (SuperArray Inc.) containing 100 μg of DNA/ml of denatured salmon sperm DNA (Invitrogen) for 2 h at $60^{\circ}C$, the array membrane was hybridized with denatured cDNA probes overnight at $60^{\circ}C$. Following washing the membrane twice with 2x sodium citrate dihydrate (SSC), 1% sodium dodecyl sulfate (SDS) and twice with 0.1x SSC, 0.5% SDS for 15 min at $60^{\circ}C$ each, the membrane was blocked with GEAblocking Solution Q (SuperArray Inc.) for 40 min and incubated with alkaline phosphatase-conjugated streptavidin for 10 min at room temperature. Chemiluminescent detection was performed using CDP-Star chemiluminescent substrate and array image was recorded with X-ray film. The image was scanned with a scanner TouchToss SIS-3800 (Samsung, Seoul, Korea). The resulting scanned image was converted to raw data file using Scanalyse software. GEArray Analyzer software (SuperArray Inc.) was used for data analysis. The relative expression levels of different genes were estimated by comparing its signal intensity with that of internal control of β-actin.

Real-time PCR

Real-time PCR was performed in the system of Light cycler (Roche Applied Science, Indianapolis, IN, USA) for the quantification of the mRNA expression levels. In a LightCycler, 1 ul of first strand cDNA template was added into a 25 ul PCR mix, containing 12.5 ul 2X RT^2 Real-time[TM] SYBR Green PCR master mix, 1 ul RT^2 PCR primer set (SuperArray Inc). The mixture was incubated for an initial denaturation at $95^{\circ}C$ for 15min, followed by 40 PCR cycles. Each cycle consisted of $95^{\circ}C$ for 30s, $55^{\circ}C$ for 30s, and $72^{\circ}C$ 30s. A standard curve was generated for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to enable normalization of each gene to a constant amount of GAPDH.

Results

Kinetics of cytokine mRNA expression as measured by microarray

The genes and spots of microarray experiment were shown in Fig. 1. The microarray results indicating the relative mRNA expression levels induced by Pg LPS and Ec LPS were shown in Table 1. A change of at least 2-fold in gene expression, as compared to the untreated control, was considered to be significant.

	A	B	C	D	E	F	G	H
1	Blr1	Xcr1	Il8rb	Cxcr3	Cxcr4	Ccr1	Ccr2	Ccr3
2	Ccr4	Ccr5	Ccr6	Ccr7	Ccr8	Gpr2	Cx3cr1	Ifng
3	Il10	Il10ra	Il10rb	Il11	Il11ra1	Il12a	Il12b	Il12rb1
4	Il12rb2	Il13	Il13ra1	Il13ra2	Il15	Il15ra	Il16	Il17
5	Il17b	Il17r	Il18	Il1a	Il1b	Il1r1	Il1r2	Il18r1
6	Il2	Il20	Il21	Il25	Il2ra	Il2rb	Il2rg	Il4
7	Il5	Il5ra	Il6	Il6ra	Il6st	Il9	Il9r	Lta
8	Ltb	Ltbr	Mif	Ccl1	Ccl11	Ccl12	Ccl17	Ccl19
9	Ccl2	Ccl20	Ccl21a	Ccl22	Ccl24	Ccl25	Ccl3	Ccl4
10	Ccl5	Ccl6	Ccl7	Ccl8	Ccl9	Cxcl10	Cxcl11	Cxcl13
11	Cxcl14	Cxcl15	Cxcl2	Cxcl5	Cxcl9	Xcl1	Cx3cl1	Cxcl12
12	Sdf2	Tgfa	Tgfb1	Tgfb2	Tgfb3	Tnf	Tnfrsf1a	Tnfrsf1b
13	PUC18	PUC18	PUC18	Blank	Blank	Blank	Gapd	Gapb
14	Ppia	Ppia	Ppia	Ppia	RPl13a	RPl13a	Actb	Actb

Fig. 1. Cytokine gene table of the microarray used in the experiment.

Table 1. Mouse Inflammatory Cytokines & Receptors

Functional Gene Grouping	Symbol	Gene Name	Description	Ec LPS	Pg LPS
	Il10	IL-10	Interleukin 10	0.94	1.06
	Il10ra	IL-10Ra	Interleukin 10 receptor, alpha	1.00	1.18
	Il10rb	IL-10Rb	Interleukin 10 receptor	0.84	1.11
	Il11	IL-11	Interleukin 11	0.94	1.24
	Il11ra1	IL-11Ra1	Interleukin 11 receptor, alpha chain 1	0.94	1.22
	Il12a	IL-12A	Interleukin 12A	0.84	1.00
	Il12b	IL-12B	Interleukin 12B	0.80	0.90
	Il12rb1	IL-12R[b]	Interleukin 12 receptor, bata 1	0.80	0.90
	Il12rb2	IL-12RB2	Interleukin 12 receptor, bata 2	1.06	1.18
	Il13	IL-13	Interleukin 13	1.06	1.17
	Il13ra 1	IL-13 Ra1	Interleukin 13 receptor, alpha 1	0.94	1.11
	Il13ra 2	IL-13 RA2	Interleukin 13 receptor, alpha 2	1.00	1.06
	Il15	IL-15	Interleukin 15	0.85	1.00
Interleukins & Receptors	Il15ra	IL-15ra	Interleukin 15 receptor, alpha chain	0.81	1.00
	Il16	IL-16	Interleukin 16	0.65	0.77
	Il17	IL-17	Interleukin 17	0.77	0.82
	Il17b	IL-17B	Interleukin 17B	3.70	4.26
	Il17r	IL-17R	Interleukin 17 receptor	0.91	1.32
	Il18	IL-18	Interleukin 18	0.81	1.10
	Il1a	IL-1a	Interleukin 1 alpha	0.90	0.95
	Il1b	IL-1b	Interleukin 1 beta	0.95	1.05
	Il1r1	IL-1R1	Interleukin 1 receptor, type I	0.74	0.91
	Il1r2	IL-1R2	Interleukin 1 receptor, type II	0.71	0.83
	Il18r1	IL1rrp	Interleukin 18 receptor 1	0.75	0.79
	Il2	IL-2	Interleukin 2	2.07	3.30
	Il20	IL-20	Interleukin 20	0.89	1.21
	Il21	IL-21	Interleukin 21	0.76	1.00
	Il25	IL-25	Interleukin 25	0.79	1.14

Table 1. (continued) Mouse Inflammatory Cytokines & Receptors

Functional Gene Grouping	Symbol	Gene Name	Description	Ec LPS	Pg LPS
Interleukins & Receptors	Il2ra	CD25	Interleukin 2 receptor, alpha chain	0.81	0.95
	Il2rb	IL-2 Rβ	Interleukin 2 receptor, beta chain	0.78	0.96
	Il2rg	IL-2 Rγ	Interleukin 2 receptor, gamma chain	0.73	0.81
	Il4	IL-4	Interleukin 4	0.75	0.79
	Il5	IL-5	Interleukin 5	0.89	1.32
	Il5ra	IL-5R	Interleukin 5 receptor, alpha	0.84	1.16
	Il6	IL-6	Interleukin 6	0.81	1.00
	Il6ra	IL-6R	Interleukin 6 receptor, alpha	0.78	1.04
	Il6st	GP130	Interleukin 6 signal transducer	0.85	1.10
	Il9	IL-9	Interleukin 9	0.73	0.86
	Il9r	IL-9R	Interleukin 9 receptor	0.67	0.78
Chemokine receptor	Blr1	CXCR5	Burkitt lymphoma receptor 1		
	Xcr1	Ccxcr1	Chemokine (C-motif) receptor 1	0.88	1.00
	Il8rb	CXCR2/Cmkar2	Interleukin 8 receptor, beta	0.94	1.06
	Cxcr3	Cmkar3	Chemokine (C-X-C motif) receptor 3	0.89	1.06
	Cxcr4	Cmkar4	Chemokine (C-X-C motif) receptor 4	0.89	0.94
	Ccr1	Cmkbr1	Chemokine (C -C motif) receptor 1	0.79	0.95
	Ccr2	Cmkbr2/MIP-1α	Chemokine (C -C motif) receptor 2	0.79	0.95
	Ccr3	Cmkbr3	Chemokine (C -C motif) receptor 3	0.84	0.89
	Ccr4	Cmkbr4	Chemokine (C -C motif) receptor 4	0.94	1.06
	Ccr5	Cmkbr5	Chemokine (C -C motif) receptor 5	0.88	0.94
	Ccr6	Cmkbr6	Chemokine (C -C motif) receptor 6	0.88	1.00
	Ccr7	Cmkbr7	Chemokine (C -C motif) receptor 7	1.22	1.61
	Ccr8	CCR-8/Cmkbr8	Chemokine (C -C motif) receptor 8	0.89	1.00
	Gpr2	Cmkbr9/CCR10	G protein-coupled receptor 2	1.16	1.79
	Cx3cr1	CX3CR-1	Chemokine (C-X3-C) receptor 1	0.65	0.94
Subfamily A (Cys-Cys)	Ccl1	Scya1/TCA-3	Chemokine (C -C motif) ligand 1	0.85	1.05
	Ccl11	Scya11/eotaxin	Small Chemokine (C -C motif) ligand 11	0.85	0.95
	Ccl12	Scya12/MCP-5	Chemokine (C -C motif) ligand 12	0.74	0.87
	Ccl17	Scya17/TARC	Chemokine (C -C motif) ligand 17	0.67	0.81
Subfamily A (Cys-Cys)	Ccl19	Scya19	Chemokine (C -C motif) ligand 19	0.80	0.88
	Ccl2	Scya2	Chemokine (C -C motif) ligand 2	0.94	1.41
	Ccl20	MIP3A/Scya20	Chemokine (C -C motif) ligand 20	0.85	1.15
	Ccl21a	Scya21a	Chemokine (C -C motif) ligand 21a(serine)	0.76	1.19
	Ccl22	Scya22/MDC	Chemokine (C -C motif) ligand 22	0.73	1.00
	Ccl24	Eotaxin-2(Scya24)	Chemokine (C -C motif) ligand 24	0.76	1.00
	Ccl25	TECK/Scya25	Chemokine (C -C motif) ligand 25	0.72	0.96
	Ccl3	MIP-1a/Scya3	Chemokine (C -C motif) ligand 3	1.10	1.10
	Ccl4	MIP-1b/Scya4	Chemokine (C -C motif) ligand 4	2.56	2.47
	Ccl5	RANTES/Scya5	Chemokine (C -C motif) ligand 5	0.94	1.33
	Cc6	Scya6	Chemokine (C -C motif) ligand 6	1.00	1.63
	Ccl7	MCP-3/Scya7	Chemokine (C -C motif) ligand 7	0.81	1.24
	Ccl8	MCP-2	Chemokine (C -C motif) ligand 8	0.76	1.14
	Ccl9	MIP-1gamma/Scya9	Chemokine (C -C motif) ligand 9	0.89	1.54

Table 1. (continued) Mouse Inflammatory Cytokines & Receptors

Functional Gene Grouping	Symbol	Gene Name	Description	Ec LPS	Pg LPS
Chemokine Subfamily B (Cys-X-Cys)	Cxcl10	IP-10/Scyb10	Chemokine (C-X-C motif) ligand 10	1.50	1.17
	Cxcl11	Scyb11	Chemokine (C-X-C motif) ligand 11	0.75	0.92
	Cxcl13	BLC/BCA-1/Scyb13	Chemokine (C-X-C motif) ligand 13	0.73	0.77
	Cxcl14	BRAK (MIP-2gamma)	Chemokine (C-X-C motif) ligand 14	0.89	1.33
	Cxcl15	Scyb15	Chemokine (C-X-C motif) ligand 15	0.94	1.33
Subfamily B (Cys-X-Cys)	Cxcl2	MIP-2/Scyb2	Chemokine (C-X-C motif) ligand 2	4.75	4.95
	Cxcl5	Scyb5/LIX	Chemokine (C-X-C motif) ligand 5	0.80	1.80
	Cxcl9	MIG/Scyb9	Chemokine (C-X-C motif) ligand 9	0.85	1.20
Other Subfamily Members	Xcl1	Lymphotactin (Scyc1)	Chemokine (C motif) ligand 1	0.81	1.24
	Cx3cl1	Fractalkine (Scyd1)	Chemokine (C-X3-C motif) ligand 1	0.78	1.00
	Cxcl12	SDF-1a	Chemokine (C-X-C motif) ligand 12	0.74	0.87
	Sdf2	SDF-2	Stromal cell derived factor 2	0.94	1.44
TGF Ligands	Tgfa	TGF-a	Transforming growth factor alpha	0.89	1.26
	Tgfb1	TGF-b1	Transforming growth factor,beta 1	1.05	1.89
	Tgfb2	TGF-b2	Transforming growth factor,beta 2	0.85	2.00
	Tgfb3	TGF-b3	Transforming growth factor,beta 3	0.90	1.45
TNF Ligands and receptors	Lta	TNF-b	Lymphotoxin A	0.60	0.70
	Ltb	LT-b	Lymphotoxin B	1.38	2.33
	Ltbr	LtbR	Lymphotoxin B receptor	0.83	1.46
	Tnf	TNFa	Tumor necrosis factor	2.38	2.56
	Tnfrsr1a	TNFR2	Tumor necrosis factor receptor superfamily, member 1a	0.79	1.38
	Tnfrsr1b	TNFR1	Tumor necrosis factor receptor superfamily, member 1b	0.77	1.00
Other Related Genes	Ifng	IFN r	Interferon gamma	0.80	0.85
	Mif	MIF	Macrophage migration inhibitory factor	0.82	1.21

Table 2. Gene expressed specifically by both Pg LPS and Ec LPS measured by microarray

Gene Name	Description	Ec LPS	Pg LPS	Pg LPS/Ec LPSx100(%)
IL-17β	Interleukin 17B	3.70	4.26	115
IL-2	Interleukin 2	2.07	3.30	159
LTβ	Lymphotoxin B	1.38	2.33	168
CCL4	Chemokine (C -C motif) ligand 4	2.56	2.47	96
CXCL2	Chemokine (C-X-C motif) ligand 2	4.75	4.95	104
TNF-α	Tumor necrosis factor	2.38	2.56	107

Pg LPS induced a significant increase in the expression of IL-17β, IL-2 in Interleukines & receptors group, CCL4 in subfamily A (Cys-Cys) group, CXCL2 in subfamily B (Cys-X-Cys) group, LTβ and TNFα in TNF Ligands and receptors group, as compared to control not treated with either LPS. Except for LTβ, Ec LPS up-regulated the same genes as Pg LPS. LTβ was significantly up-regulated by Pg LPS not by Ec LPS. These results are summarized in Table 2.

Kinetics of cytokine mRNA expression as measured by real-time PCR

In order to confirm the results of microarray, real-time PCR was carried out using SYBR Green I as a fluorescent indicator of double stranded DNA production. After relative quantification and normalization, the expression levels of six genes were calculated. In similar with the results of microarray experiments except for IL-17β and IL-2, both LPS enhanced the mRNA expression of LTβ, CCL4, CXCL2, and TNF-α compared with the control (Fig. 2). The expressions of IL-17β and IL-2 mRNA were more increased only by Pg LPS treatment not by Ec LPS.

Discussion

In this study, we demonstrated that Pg LPS induced marked
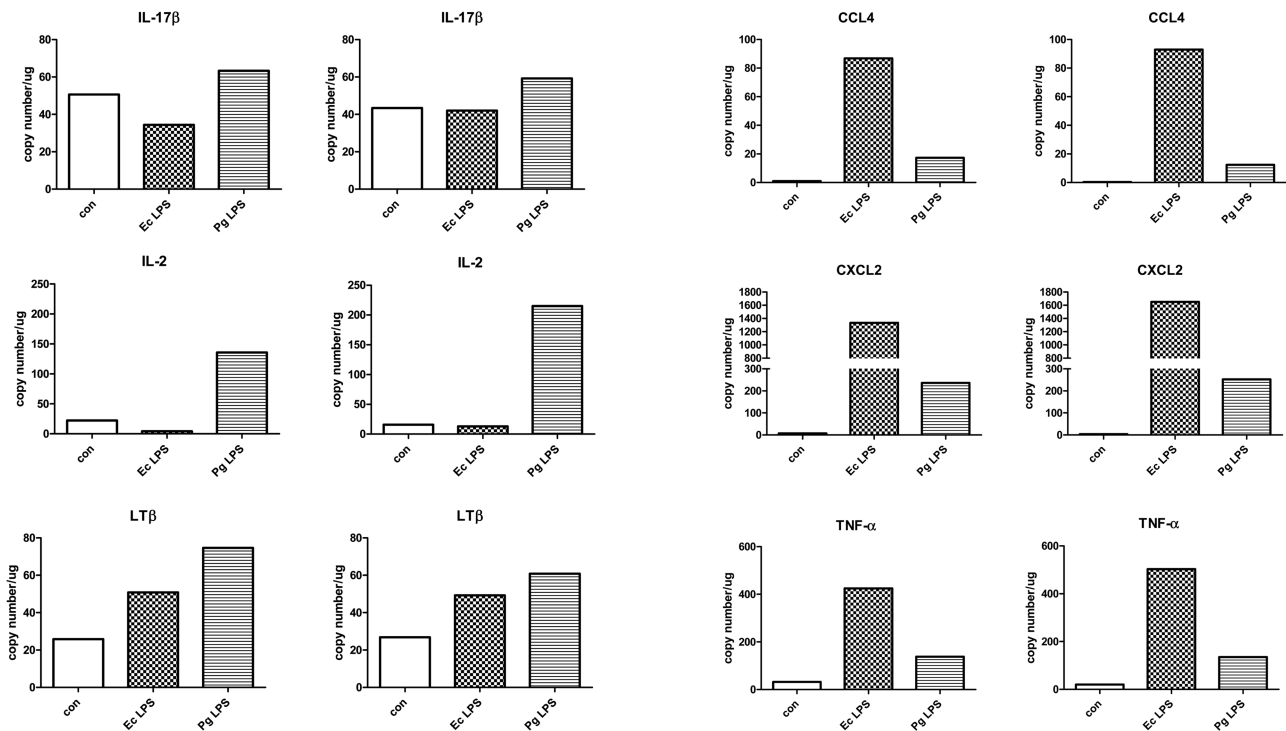
Fig. 2. Normalized relative quantification of mRNA expression by real-time PCR for IL-17β, IL-2, LTβ, CCL4, CXCL2, and TNF-α. They are two representatives of each experiment for each cytokine.

mRNA expressions of IL-17β, IL-2, CCL4, CXCL2, LTβ, and TNFα in mouse macrophage RAW 264.7 cells.

Cytokines are a family of intercellular signaling proteins which are produced and secreted to defend against microbial challenges by host immune system. Some cytokines, such as IL-1, IL-6, IL-8, interferon-γ, and TNF-α, promote inflammation. Other cytokines, such as TGF-β, and IL-10, play a role in down-regulating inflammatory responses (Feng *et al*., 2006). The levels of the pro- and anti-inflammatory cytokines in specific sites and at various time points, along with other factors, i.e. eicosanoids, nitric oxide and soluble cytokine receptors, affect the control of initiation, progression and inhibition of inflammation (Feng *et al*., 2006). *P. gingivalis* fimbriae have been reported to elicit the production of several proinflammatory cytokines, such as IL-1β, IL-6, IL-8 and TNF-α in human peripheral blood monocytes and macrophages (Ogawa *et al*., 1994).

Chemokines, a family of chemotactic cytokine, are small heparin-binding proteins that direct the movement of circulating leukocytes to site of inflammation and injury (Zembala *et al*., 1989). Chemokines affect cell by activating surface receptors that are seven-transmembrane-domain G-protein-coupled receptors: leukocyte responses to particular chemokines are determined by their complement of chemokine receptors (Charo *et al*., 2006). Chemokine binding to the receptor activates signaling cascades that culminate in the rearrangement, change of shape, and cell movement (Charo *et al*., 2006). Some chemokines including Interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and

regulated-upon-activation normal T-cell expressed and secreted (RANTES) have been reported in associated with human periodontal disease (Tonetti *et al*., 1994; Gamonal *et al*., 2001). However, total analysis for the gene expressions of chemokine and chemokine receptors induced by periodontopathic bacteria, especially Pg LPS was not reported. In present study, we focused on periodontopathogenic bacteria and their unique properties of modulating host cytokines/chemokines.

LPS is a major component of the outer membrane of gram-negative bacteria (Socransky *et al*., 1992). LPS is known to be able to induce the response of local cells to secrete high level of several cytokines that lead to periodontal tissue destruction. *P. gingivalis* has been believed to be an important periodontopathic bacterium because it is isolated at a high frequency from subgingival site of advancing lesion in adult periodontitis patients (Slots *et al*., 1988). Furthermore, many investigators have indicated that Pg LPS may lead to destruction of periodontal tissue by a direct action or indirectly by inducing inflammatory reaction (Birkedal-Hansen *et al*., 1998). Previously, it has been reported that Pg LPS stimulates the induction of IL-1α, IL-1β, IL-6, and IL-8 in human gingival fibroblasts (Ohmori *et al*., 1987). It has been also reported that Pg LPS leads to periodontal destruction and alveolar bone resorption through IL-6 and IL-8 released from human periodontal ligament fibroblasts (Yamaji *et al*., 1995). In human peripheral blood monocytes, Pg LPS can activate this cells for proliferation, cytokine production, and CD14 (LPS surface receptor) expression (Roberts *et al*., 1997).

DNA microarray technology is a new and powerful tool which allows for the rapid and efficient simultaneous analyses of a large number of nucleic acid hybridizations. This technology has proven extraordinarily useful in terms of our knowledge regarding the interrelationships of gene expression involved in the functioning of immune systems. In the present microarray experiment, the activation of the genes in the groups of Interleukins and receptors, chemokines and receptors, TGF ligands, and TNF ligands and receptors were analyzed. As summarized in Table 2, when a change of at least 2-fold in gene expression was considered as significant, six genes including IL-17β, IL-2, CCL4, CXCL2, LTβ, and TNFα were up-regulated by Pg LPS treatment. With the exception of LT-β, the expressions of the rest five genes were also up-regulated in response to Ec LPS treatment.

Real-time quantitative PCR has proved to be accurate, less labor-intensive, and powerful for studying gene expression in mammalian cells than end point reverse transcription-PCR (RT-PCR). The technique is based on fluorescence-kinetic RT-PCR enabling quantification of the PCR product in "real-time" and measures PCR product accumulation during the exponential phase of the reaction (Giulietti *et al.*, 2001). Real-time PCR is designed to provide information as rapidly as the amplification process itself, thus requiring no post PCR manipulations.

In the present study, we carried out real-time PCR by use of SYBR Green I, a DNA-binding dye that incorporates into dsDNA. It has an undetectable fluorescence when it is in its free form, but once bound to the dsDNA it stars to emit fluorescence (Giulietti *et al.*, 2001). In accordance with the microarray results, Pg LPS treatment up-regulated the expressions of six genes, when compared to the untreated control. Ec LPS treatment up-regulated the gene expressions of CCL4, CXCL2, LTβ, and TNFα, IL-17β and IL-2 genes were down-regulated by Ec LPS treatment when compared to the control. Especially, Pg LPS more up-regulated IL-17β, IL-2, and LTβ gene expressions than Ec LPS, while it profoundly increased the mRNA expressions of IL-2, LTβ and CXCL2 as compared to the control.

IL-17β has been reported to be involved with migration of neutrophil. IL-2 is a well known T cell growth factor, so it increases the production of other cytokines such as IL-4 and IFN-γ. Lymphotoxin-b (TNF-β) exerts locally as recruitment and activator of neutrophils, so it mediates acute inflammatory response. The principal producing-cell of TNF-α is mono-nuclear phagocyte. Production of TNF-α by macrophage is most effectively stimulated by LPS. The major physiologic function of TNF-α is to stimulate the recruitment of neutrophil and monocyte, so it is an essential mediator of acute inflammatory response at gram-negative bacterial infection. It works on innate immune system locally and generally. CXCL2 (Groβ, MIP2-α) are chemoattractant for neutrophils whereas CCL4 (MIP-1β) generally attract monocytes, lymphocytes, basophils, and eosinophils. These chemokines are released locally at sites of inflammation, attract immune cells, and play a central role in the inflammatory process and the final outcome of immune response.

In summary, this study indicates that Pg LPS stimulates the mRNA expressions of IL-17β, IL-2, CCL4, CXCL2, LTβ, and TNF-α in mouse macrophage RAW 264.7 cells. These kinds of cytokines/chemokines might be involved the pathologic process of periodontitis by directing the pro-liferation of T cell and the movement of immune cells such as neutrophils, monocytes, lymphocytes to the inflammatory site. Furthermore, in contrast to Ec LPS, Pg LPS is likely to have more ability to evoke T cell response by strong expression of IL-2 gene contributing to the process of periodontal disease.

Acknowledgements

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