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Differences in Cytokine Gene Expression after a Stimulation with *Escherichia* Coli and *Porphyromonas Gingivalis* or Lipopolysaccharides Derived from these Bacteria

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Abstract

Monocytes are important cells in innate immunity. The early stage of the innate immunity is regulated by various cytokines produced by monocytes. We conducted a preliminary study to investigate TNFα expression by stimulating THP-1 cells with several bacterial species. The TNFα mRNA levels significantly varied, with the most potent stimulatory effects observed with *P. gingivalis*. In the present study, we focused on *P. gingivalis* and compared differences in cytokine expression profiles after the stimulation of THP-1 with *E. coli*. Bacterial antigen stimulation increased various cytokine gene expressions in THP-1. *P. gingivalis* had significantly more potent effects on the mRNA expressions of TNFα, IL-1β, and IL-10, but not of IL-12p40, than *E. coli*. This result suggests the potent ability of *P. gingivalis* to induce inflammation. THP-1 stimulated with LPS derived from both bacterial species showed that *E. coli* had significantly more potent effects on the expressions of TNFα, IL-1β, and IL-12p40 than *P. gingivalis*. The differences in the bacterial antigens and the LPS stimulation effects suggest involvements of different receptors, such as TLR-2 and -4, which recognize bacterial components. The present results suggest that the *P. gingivalis* somatic cell antigen stimulates a number of pattern recognition receptors at the same time as the synthesis of bacterial components, except LPS. The potent virulence of *P. gingivalis* and persistence of infection might be affected by differences in cytokine production. Pro-inflammatory responses are dependent not only on the bacterial type, but also bacterial components.

Keywords: Porphyromonas gingivalis (P. gingivalis), Escherichia coli (E. coli), Lipopolysaccharide (LPS), Toll-like receptors (TLR), Cytokines, Inflammation.

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Ethical: This study follows all ethical practices during writing.

Contents

Contribution of this paper to the literature

NY: Device of this study. Instruction of the Experiments. Writing the entire paper. YK: Performance for sixty percentiles of the experiments. MO: Performance for forty percentile of the experiments. WO: Providing and teaching the experimental techniques. SS: Preparation of experimental materials (bacterial cells). Statistical processing. MY: Providing and teaching the experimental techniques. TN: Verification of the content as the corresponding author.

1. Introduction

The innate immune system mostly comprises circulating monocytes that recognize conserved bacteria-associated molecules, such as lipopolysaccharide (LPS) and peptidoglycan (PGN), and then differentiate into macrophages in inflamed tissues. These cells express toll-like receptors (TLR) [1] which play a central role in microbial molecular pattern detection. TLR function in signaling pathways via a common adaptor, MyD88, and in the activation of transcription factors, such as nuclear factor kappa B (NF-κB) [2]. Important pro-inflammatory cytokines secreted by macrophages in response to bacterial products through TLR include tumor necrosis factor α (TNFα), interleukin (IL)-1β, IL-6, and IL-12. TNFα and IL-1β are inducers of local inflammatory responses that help to contain infections [3]. In patients with severe sepsis, the host reaction may be characterized as an excessive inflammatory response caused by a cascade of TNFα and IL-1β [4]. It is important to elucidate cytokine expression profiles in order to identify the pathological condition of infectious disease.

Bacterial infections are mediated in part by the production of a number of inflammatory cytokines; however, the pattern of early cytokine production may be dependent on the specificities of the microbial pathogens [5, 6]. Beran *et al.* [7] suggested higher productivity of cytokines with gram-negative bacteria than with gram-positive bacteria. LPS is a Gram-negative bacterial endotoxin, while PGN is a Gram-positive bacterial cell wall component that mainly exerts its effects by activating monocytes and macrophages [8]. However, the mechanisms that trigger the different activation patterns of cytokine profiles in these cells remain unclear. We hypothesize that the reactivity of innate immune cells might vary with bacterial strains, and might trigger different inflammatory responses that are dependent on the microbial constituents of bacterial cell walls.*Porphyromonas gingivalis* (*P. gingivalis*) is a Gram-negative rod-shaped bacterium and a key periodontal pathogen associated with the etiology of periodontal disease [9]. Periodontal disease has been linked to systemic disorders, such as atherosclerosis and type 2 diabetes [10, 11]. The pathogenic components of *P. gingivalis*-LPS is one of the main factors associated with periodontitis and *P.gingivalis*-LPS would trigger various inflammatory responses [12]. *Escherichia coli* (*E. coli*) is a Gram-negative rod-shaped bacterium with LPS that has the ability to stimulate monocytes and macrophages. In the present study, we compared differences in inflammatory cytokine expression profiles after the stimulation of THP-1 human monocytic cells with *E. coli*, *P. gingivalis*, and LPS derived from these bacteria.

2. Materials and Methods

2.1. Bacterial Strains and LPS

All bacterial strains were transferred from Clinical Laboratory, Chiba Emergency Medical Center, Chiba, Japan. Clinically isolated *P. gingivalis* was grown on pre-reduced Brucella broth agar from Kyokuto Pharmaceutical Industrial (Tokyo, Japan), and incubated at 37℃ for 48-72 h in an anaerobic conditioning chamber. *E. coli* (DX5α), *Klebsiella pneumoniae* (clinically isolated strain), *Haemophilus influenzae* (ATCC 49619), *Staphylococcus aureus* (ATCC 29213), and *Streptococcus pneumoniae* (ATCC 49619) were grown on LB broth from Kyokuto Pharmaceutical Industrial (Tokyo, Japan), and incubated at 37℃ for 48-72 h in an anaerobic incubator. Each bacterial strain was killed in 95℃ heated water and then treated with 0.5% formalin. Fixed bacteria were washed twice with phosphate-buffered saline (PBS). Bacterial concentrations were adjusted by the McFarland standards method for 1.5 unit (OD_{600}: 0.354), namely, approximately 100 μg/mL. Standard LPS from *P. gingivalis* was purchased from Invitrogen (San Diego, CA, USA). Standard LPS from *E. coli O26* was purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Cell Culture and Stimulation

Human monocytic THP-1 cells were obtained from the Cell Engineering Division, RIKEN BioResource Research Center (Tsukuba, Ibaraki, Japan). Cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mM), 1% Antibiotic/Antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA), and maintained at 37℃ in a humidified 5% CO_2 incubator. Cultured cells after 2-3 passages were designated as appropriate for subsequent experiments. Cells were placed into 24-well plates at a concentration of 2×10^5 cells/mL/well in conditioned media. Each regent was added to the culture solution and incubated for the indicated time. Regent concentrations were as follows: *E. coli* and *P. gingivalis*-cell antigen: 10 μg/mL; *E. coli* and *P. gingivalis*-LPS: 1 μg/mL. Cells treated with PBS were basal controls.

2.3. RNA Extraction and Reverse Transcription

Total RNA was isolated from THP-1 cells using the acid guanidinium-phenol-chloroform method regent, ISOGEN (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions and then eluted in RNase-free water. Total RNA was quantified using the spectrophotometer NanoDrop (Thermo Fisher Scientific, MA, USA),

and total RNA (1 μg) was then reverse transcribed using random hexamers in 10 μL reactions with the cDNA synthesis kit TaKaRa Prime Script RT Master Mix (Takara Bio, Kusatsu, Shiga, Japan), according to the manufacturer's instructions.

2.4. Cytokine Gene Expression Assay

Quantitative reverse transcriptase (RT)-PCR was performed using the StepOne Real-Time PCR system (Thermo Fisher Scientific, MA, USA) in at least three separate experiments. Amplification reactions were performed in a final volume of 20 μL containing 10 μL of SYBR Premix Ex *Taq* (Takara Bio, Kusatsu, Shiga, Japan), 1 μL of the cDNA template, and 0.8 μM of each pair of primers. The target cytokine genes to be compared were TNFα, IL-1β, IL-6, IL-10, IL-12p40, and TGF-β. The expression of the endogenous gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The expression level of each cytokine gene was normalized to GAPDH (threshold cycle value: Ct) and fold changes for each gene were calculated by comparing the stimulated test and untreated controls from Ct values according to the Ct approach, the comparative ΔΔCt method. Real-time PCR primer pairs are described in Table 1.

Table-1. Primer pairs for the quantification of gene expression using a real-time reverse transcription-Polymerase Chain Reaction (RTPCR).

Nucleotide sequence of real time PCR primers		
Targets	**Sequence (5'-3')**	**Products**
GAPDH-F	GCACCGTCAAGGCTGAGAAC	138 bp
GAPDH-R	TGGTGAAGACGCCAGTGGA	
TNF-α-F	GAAACCTGGGATTCAGGAATG	246 bp
TNF-α-R	GTCTCAAGGAAGTCTGGAAAC	
IL-1β-F	GACACATGGGATAACGAGGC	248 bp
IL-1β-R	ACGCAGGACAGGTACAGATT	
IL-10-F	ACAGCTCAGCACTGCTCTGT	329 bp
IL-10R	AGTTCACATGCGCCTTGATG	
IL-12p40-F	CTTAGGCTCTGGCAAAACCCT	102 bp
IL-12p40-R	AGGAGCGAATGGCTTAGAACC	
IL-6-F	TCTCCACAAGCGCCTTCG	193 bp
IL-6-R	CTCAGGGCTGAGATGCCG	
TGF-β-F	GCCCTGGACACCAACTATTGC	129 bp
TGF-β-R	GAAGTTGGCATGGTAGCCCTT	

2.5. Statistical Analysis

Data were showed as means ± standard error (SE). A two- or three-way analysis of variance (ANOVA) was used to analyze differences among multiple groups. Differences between groups were considered to be significant when the P value was < 0.05.

3. Results

In the preliminary experiment, changes in the gene expression level of the pro-inflammatory cytokine TNFα were examined with 1 μg/mL of various bacteria somatic cell antigens in THP-1 cells (8-hour stimulation). Figure-1 shows significant differences in TNFα mRNA expression levels according to the species of bacteria ($P< 0.05$). The stimulus with *P. gingivalis* was stronger than those with the other bacteria.

The temporal early inflammatory gene expression pattern was evaluated in THP-1 cells following treatment with formalin treated *E. coli*- or *P. gingivalis*-somatic cell antigen by quantitative RT-PCR. An inflammatory response was indicated by significant increases in the mRNA levels of TNFα, IL-1β, IL-12p40, and IL-10 from the basal value ($P< 0.01$). The results obtained were shown in Figure-2a ~ -2d, and indicated that the gene expression levels of TNFα, IL-1β, and IL-10 in THP-1 cells were significantly higher with *P. gingivalis* than with *E. coli* ($P< 0.01$). Regarding IL-12p40 expression levels in THP-1 cells, the *P. gingivalis* somatic cell antigen stimulus was weaker than that of *E. coli*. No significant changes were observed in the responses of IL-6 and TGF-β levels to both bacteria for at least 6 hours after the stimulation, while a modest change (16-fold) was noted in the *P. gingivalis* (but not *E. coli*)-induced expression of IL-6 24 hours after the stimulation (data not shown). Overall, the ability of *P. gingivalis* to stimulate somatic cells was more potent than that of *E. coli*.

In contrast, differences were observed in the results obtained using the LPS stimulation by *E. coli* or *P. gingivalis* and somatic cell antigen stimulation patterns. The results obtained were shown in Figure-3a ~ -3d and indicated that the up-regulated expression of TNFα, IL-1β, and IL-12p40 was significantly stronger with LPS from *E. coli* than with that from *P. gingivalis* ($P< 0.01$). The stimulatory ability of *E. coli*-LPS was stronger than that of *P. gingivalis*-LPS. However, no significant changes were observed in IL-10 expression levels following either LPS stimulus.

When time courses of gene expression were investigated from *E. coli*- and *P. gingivalis*-somatic cell antigens for 24 hours after the stimulation Figure-4a and -4b, the maximum level of TNFα expression by *E. coli* was noted 4 hours after the stimulation, whereas that by *P. gingivalis* was 2 hours. The maximum expression levels of IL-1β and IL-10 were at the same time point, 8 hours after the stimulation with *E. coli*- and *P. gingivalis*- somatic cell antigens. The approximately 250-fold reduction in IL-10 expression levels after the *E. coli* stimulation persisted for 24 hours, whereas a decrease to approximately 40-fold was noted with the *P. gingivalis* stimulation. The maximum level of IL-12p40 expression by *P. gingivalis* was noted 12 hours after the stimulation, whereas the level of IL-

12p40 by *E. coli* continued to increase even after 24 hours. The increase and decrease patterns and the expression levels were significantly difference between *E. coli* and *P. gingivalis* ($P < 0.01$).

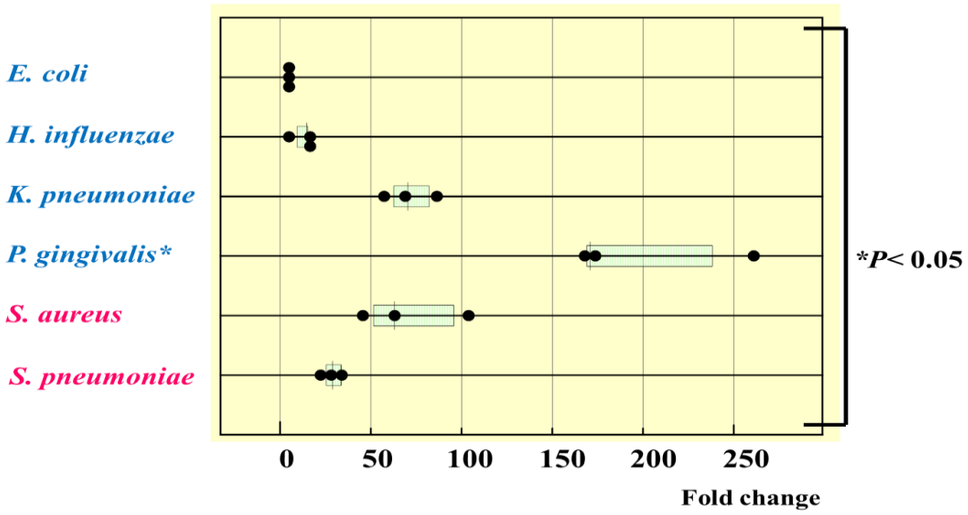

Figure-1. Preliminary analysis for TNFα gene expression changes in THP-1 cells by various bacteria. THP-1 cells were stimulated with 1 μg/mL of the indicated bacteria somatic cell antigen or vehicle (phosphate-buffered saline: PBS) for 8 hours. The results obtained were normalized to GAPDH and calculated as a fold change from basal levels using quantitative real-time RT-PCR. *P.gingivalis* was stronger than those with the other pathogens. Significant differences between various bacteria groups by Dunn method of multiple comparison ($P< 0.05$).

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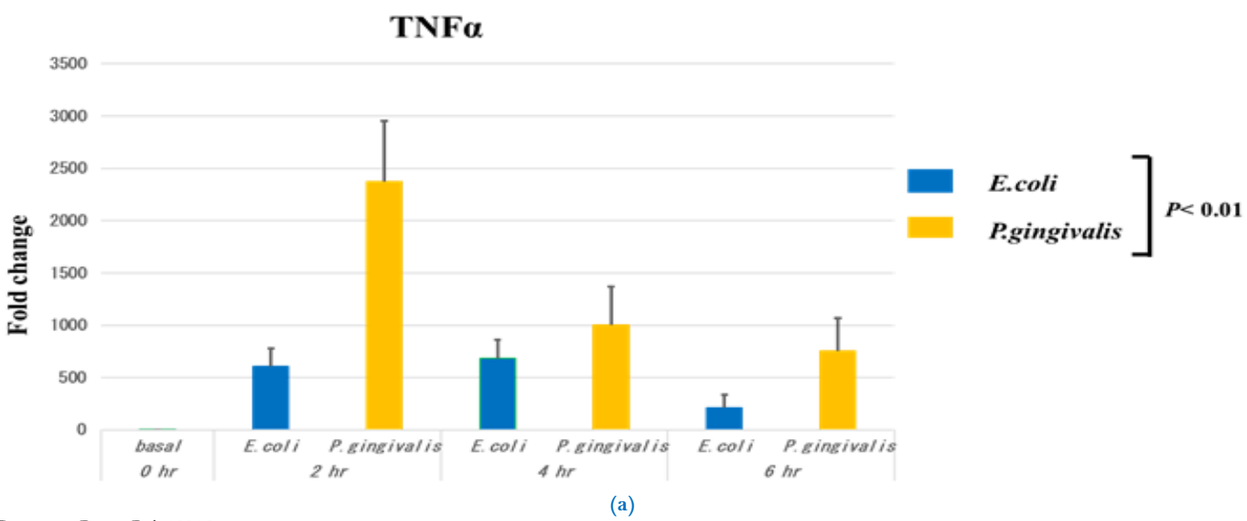

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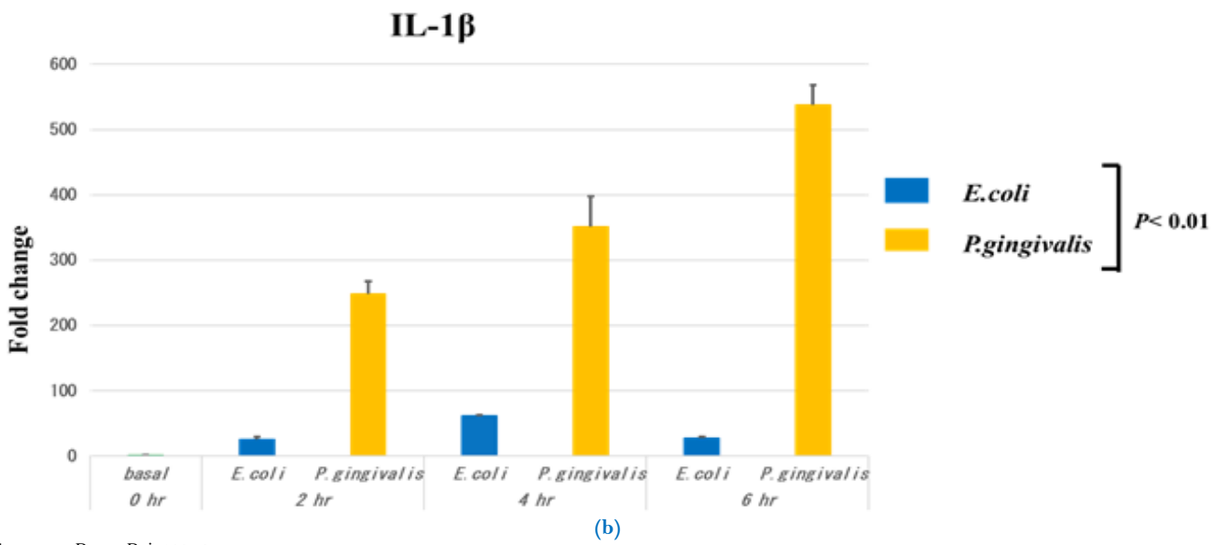

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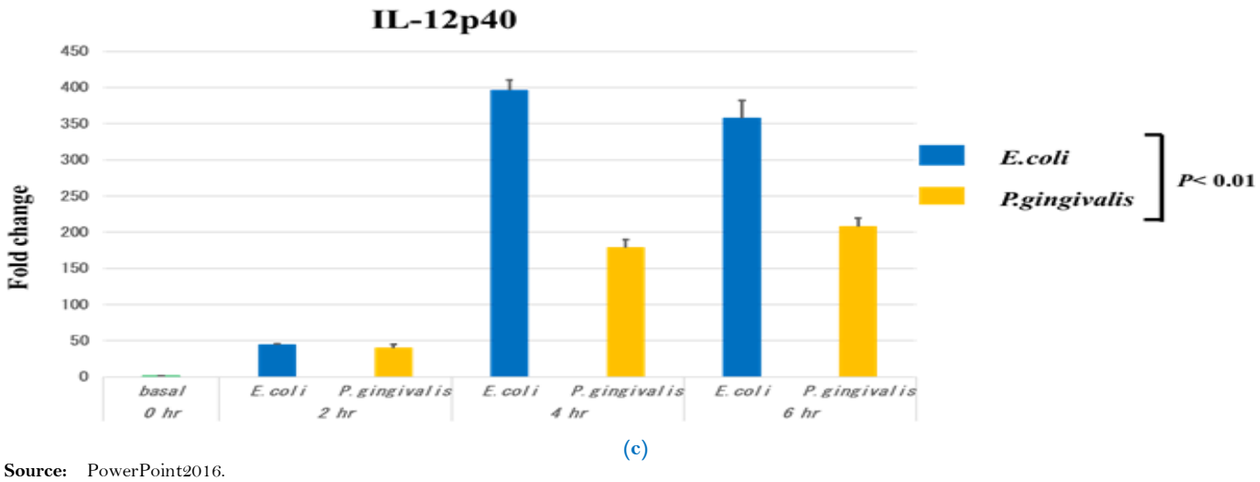

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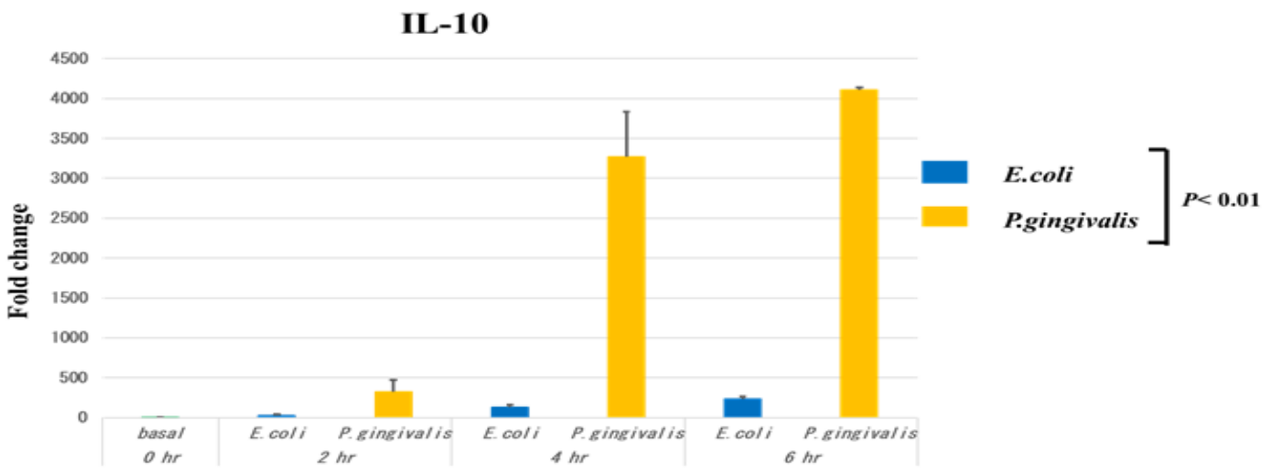
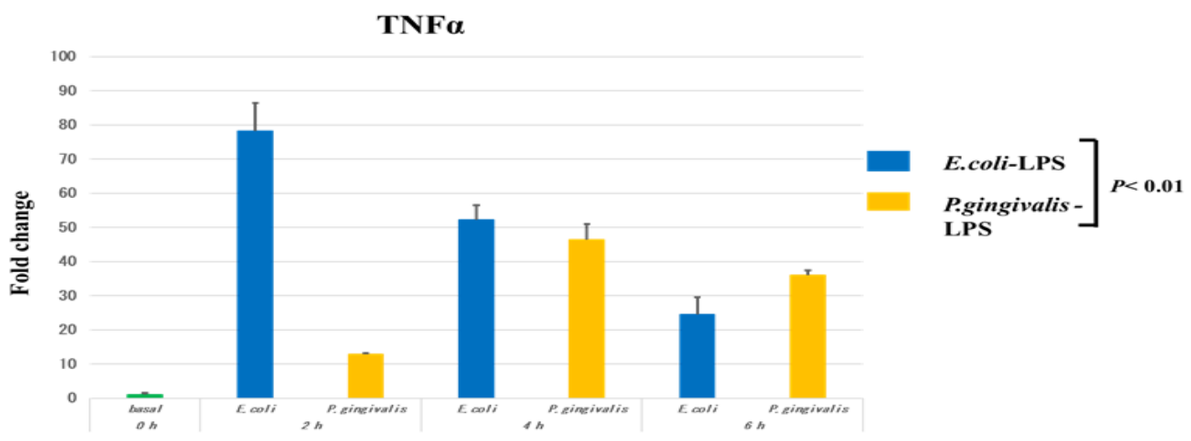


(d)

Figure-2. Cytokine gene expression (mRNA) changes by *E. coli*- and *P. gingivalis*-somatic cell antigens in THP-1 cells 2, 4, and 6 hours after the stimulation. 10 μg/mL *E. coli* and 10 μg/mL *P. gingivalis* up-regulate TNFα (a), IL-1β (b), IL-12p40 (c), and IL-10 (d). Data are expressed as means ± SE. Results were normalized to GAPDH and calculated as a fold change from basal levels using quantitative real-time RT-PCR.

Note: Significant differences between *E. coli*-and *P. gingivalis*-somatic cell antigen groups by a two-way ANOVA ($P< 0.01$).



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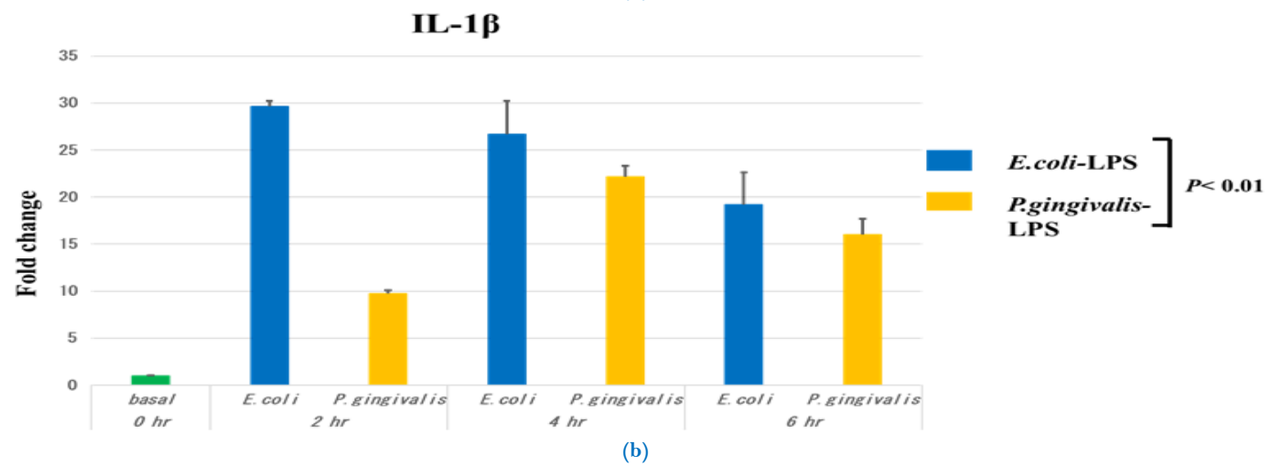

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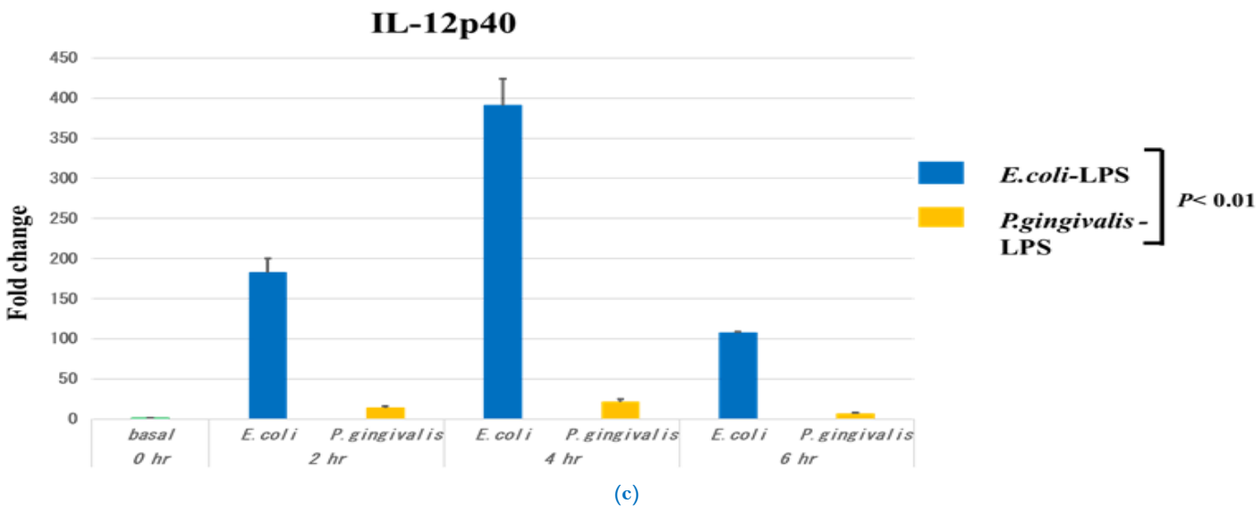

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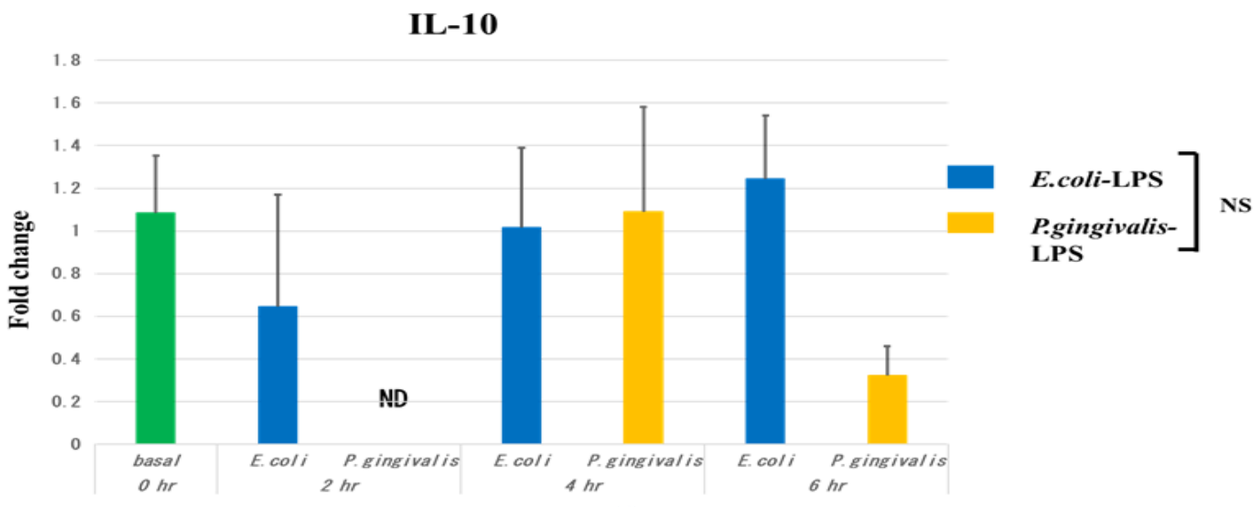


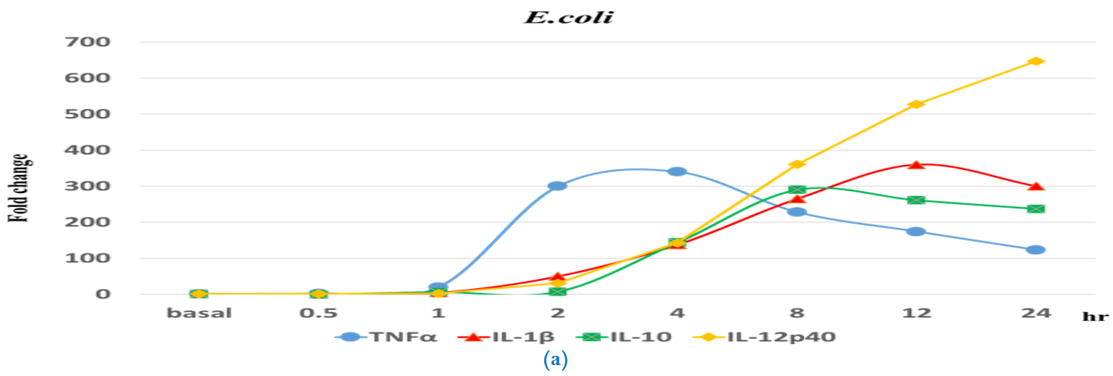
(d)

Figure-3. Cytokine gene expression (mRNA) changes by *E. coli*- and *P. gingivalis*-LPS in THP-1 cells 2, 4, and 6 hours after the stimulation. 1 μg/mL *E. coli*-LPS and 1 μg/mL *P. gingivalis*-LPS up-regulate TNFα (a), IL-1β (b), IL-12p40 (c), and IL-10 (d). Data are expressed as means ± SE. Results were normalized to GAPDH and calculated as a fold change from basal levels using quantitative real-time RT-PCR. Significant differences between *E. coli*- and *P. gingivalis*-LPS groups by a two-way ANOVA ($P< 0.01$) except to IL-10. NS: no significant differences. ND: not detected.

4. Discussion

Innate immunity is a system related to the first biophylaxis to infection by pathogens. Monocytes assume a role that is important for immune responses. They possess pattern recognition receptors that bind pathogen structures, such as TLR, receive pathogen infection information, and activate the initial inflammatory response. Activated monocytes differentiate to macrophages and produce inflammatory cytokines, and also play an important role in acquired immunity. Factors related to inflammatory cytokine production depend on pathogen compositions and interactions with pattern recognition receptors. Differences in the types of pathogens lead to variations in cytokine production and profiles. Our preliminary study showed that differences in TNFα mRNA expression levels according to the species of bacteria were more significant than the distinction of Gram-negative and -positive bacteria. Therefore, we compared the effects of *P. gingivalis* and *E. coli* on TNFα expression levels.

The expression levels of various types of cytokine genes increased in THP-1 cells stimulated with formalin-treated bacteria. Regarding their effects on the mRNA expression of TNFα, IL-1β, and IL-10, *P. gingivalis* was clearly stronger than *E. coli*. This result suggests the potent ability of *P. gingivalis* to induce inflammation, which may be a factor contributing to the progression of periodontal disease and the onset of general clinical conditions. Interestingly, this was not the case for IL-12p40. However, *E. coli* showed significantly more potent and lasting bacterial and LPS stimulation on IL-12 p40 than *P. gingivalis*. IL-12 stimulates interferon (INF)-γ production and the differentiation of Type 1 T-helper cells and functions to induce acquired immunity [13]. *P. gingivalis* may contribute to persistent survival by suppressing the immune mechanisms of the host more weakly than *E. coli*. The expression of anti-inflammatory cytokine IL-10 was significantly reduced 24 hours after stimulation with *P. gingivalis*, unlike with *E. coli*. Differences in the IL-12p40 and IL-10 expression time course may play a role in the sustained viability of *P. gingivalis*. On the other hand, TNFα, IL-1β, and IL-12p40 expression levels were increased by the stimulation with LPS derived from both bacteria. However, the effects of *E. coli*-LPS were stronger than those of *P. gingivalis*-LPS, and this pattern was opposite to that of the bacterial cell stimulation in either case. Moreover, the increased expression of IL-10 was not confirmed at least 6 hours after the stimulation. Barsby *et al.* reported the increased production of TNFα, IL-1β, the IL-18 receptor and IL-6 in THP-1 cells stimulated with *E. coli*-LPA and *P. gingivalis*-LPS [14].



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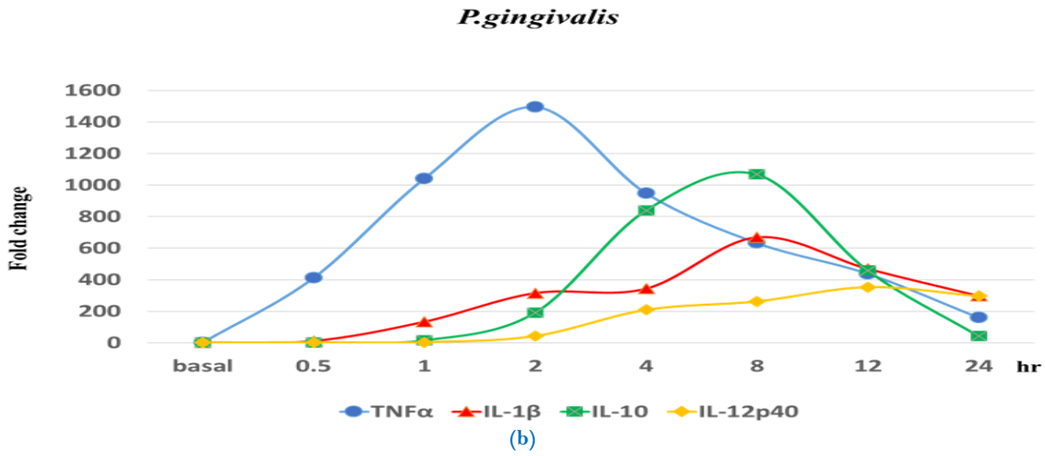

(b)

Figure-4. Time dependence of TNFα, IL-1β, IL-12p40 and IL-10 gene expression (mRNA) changes by *E. coli*- and *P. gingivalis*-somatic cell antigens in THP-1 cells. Fold change points are expressed as average values. THP-1 cells were stimulated with 10 μg/mL *E. coli* (a) and *P. gingivalis* (b) until the indicated time points from 0 to 24 hours in quantitative real-time RT-PCR. Significant differences between *E. coli* and *P. gingivalis* groups by a three-way ANOVA ($P< 0.01$).

As observed in our study, *E. coli*-LPS had more potent effects than *P. gingivalis*-LPS. However, the increased expression of IL-6 was not observed in the present study. Su *et al.* stimulated rat bone marrow-induced dendritic cells with *E. coli*-LPS and *P. gingivalis*-LPS, and reported that the production of IL-12 was enhanced more by *E. coli*-LPS, while that of IL-10 was greater with *P. gingivalis*-LPS [15]. Some cytokine expression profiles were inconsistent between previous findings and the present results, and this may be attributed to differences in the origin strains of LPS used in experiments. In other words, in addition to differences in strains, differences in the molecular structures of bacterial components appear to have subtly influenced innate immune cells. The results obtained in the present study showed that *P. gingivalis* bacterial cells induced immune responses that were stronger than those caused by purified LPS. Barksby *et al.* [14] and Su *et al.* [15] reported that the different effects between *E. coli*- and *P. gingivalis*-LPSs were caused by the involvement of TLR-4 or -2. MyD88 is commonly involved in the signaling pathways of various TLRs. However, there are MyD88-independent pathways, and Toll/IL-1 receptor (TRI) domain-containing adapters, TRIF, is involved in the TLR-4 pathway, but not in the TLR-2 pathway [16]. *E. coli*-LPS activates both pathways of MyD88 and TRIF as an agonist of TLR-4, while *P. gingivalis*-LPS is not involved in the TRIF pathway because it is an agonist of TLR-2. Therefore, *E. coli*-LPS had more potent effects. However, the *P. gingivalis* somatic cell antigen has been suggested to stimulate various types of pattern recognition receptors at the same time as the synthesis of bacterial components, except for LPS, while it causes a strong response.

P. gingivalis is the most important pathogen involved in the onset of chronic periodontal diseases and also plays a role in systemic clinical conditions. Omura *et al.* reported a case of death by sepsis due to *P. gingivalis* [17]. These findings demonstrate that *P. gingivalis* causes strong systemic inflammation as well as periodontal diseases. The present results confirmed that *P. gingivalis* induced inflammation more strongly than other pathogens. Differences in the types of pathogens affect not only various stimulus but also various properties. This might influence the difference in pathogenicity. Sun *et al.* reported inhibited TNFα expression and enhanced IL-10 expression in a study on endotoxin tolerance (the suppression of some inflammatory responses) by repeated exposure to LPS [18]. In their study, the endotoxin tolerance-inducing effect by *P. gingivalis*-LPS was more suppressive than that of *E. coli*-LPS. As described above, we confirmed that the IL-10 expression time course after the *P. gingivalis* bacterial cell stimulation disappeared faster than that after the *E. coli* bacterial cell stimulation. A difference in the expression profile of the anti-inflammatory cytokine IL-10 may contribute to this complex *in vivo* inflammatory response. A number of phenomena have not been clarified in the innate immune system yet. In the future, we will investigate the pathology of endotoxin tolerance associated with treatment resistance.

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