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# *Porphyromonas gingivalis* lipopolysaccharide induces miR-146a without altering the production of inflammatory cytokines

Tomoyuki Honda, Naoki Takahashi, Sayuri Miyauchi, Kazuhisa Yamazaki *

*Center for Transdisciplinary Research, Niigata University, Niigata, Japan*
*Laboratory of Periodontology and Immunology, Department of Oral Health and Welfare, Niigata University, Faculty of Dentistry, Niigata, Japan*

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ABSTRACT

Lipopolysaccharide (LPS) from *Porphyromonas gingivalis*, an oral Gram-negative bacterium, acts as a virulence factor for periodontal disease. Although *P. gingivalis* LPS does not induce proinflammatory cytokines as strongly as *Escherichia coli* LPS, it is still able to exploit negative Toll-like receptor (TLR) regulatory pathways and facilitate pathogen persistence. Recent reports suggest that microRNAs (miRNAs) are also involved in the regulation of TLR signaling. Here, we demonstrate that *P. gingivalis* LPS strongly induces miRNA-146a expression in THP-1 cells and THP-1-derived macrophages. However, the inhibition or overexpression of miR-146a, through the transfection of a specific inhibitor or precursor, respectively, had little effect on cytokine production in macrophages stimulated with *P. gingivalis* LPS. Moreover, the expression of interleukin-1 associated-kinase-1 (IRAK-1) and tumor-necrosis factor (TNF) receptor-associated factor-6 (TRAF6), potential target molecules of miR-146a, were not affected by the stimulation with *P. gingivalis* LPS. Because TLR signaling induces various negative regulators, these results call into question the role of miR-146a in cells stimulated with TLR ligands.



## 1. Introduction

The inflammatory response is central to effective host defense against invading pathogens that are primarily recognized by Toll-like receptors (TLRs). TLRs activate signaling pathways that induce the expression of immune and proinflammatory genes. However, an uncontrolled inflammatory response resulting in the inappropriate production of proinflammatory cytokines is detrimental to the host cells. Therefore, TLR signaling is tightly regulated at multiple levels by various negative regulators, such as interleukin-1 associated-kinase-M (IRAK-M) and suppressor of cytokine signaling-1 (SOCS1) [1,2].

In a previous study, we demonstrated that lipopolysaccharide (LPS) from *Porphyromonas gingivalis*, a common periodontopathic bacterium, specifically induced IRAK-M, which then suppressed the dissociation of IRAK-1 from its receptor complex, resulting in escape from subsequent degradation [3]. During chronic infection with this bacterium in periodontal tissue, this activity may serve as a mechanism of escape from immune surveillance. In addition, *P. gingivalis* can instigate subversive crosstalk between TLR2 and other innate receptors to blunt the antimicrobial response mediated by TLR2 [4]. These findings suggest that *P. gingivalis* has evolved to evade host immune systems.

MicroRNAs (miRNAs) are a newly identified class of endogenous small (18–25 nt long) noncoding RNAs that post-transcriptionally regulate gene expression by forming imperfect base pairs with sequences in the 3′ UTR of target mRNAs to prevent protein synthesis by repressing translation or inducing mRNA degradation [5,6]. More than 1000 miRNAs have been identified in mammals (miRBase v.18) [7], and they have been found to be associated with diverse biological processes, such as cell differentiation, metabolism, tumorigenesis, and immunity [8–10]. During innate immune response activation in response to enterobacterial LPS, changes in the expression of selected miRNAs, namely miR-146a [11], miR-155 [12], and miR-9 [13], have been observed in monocytic cell lines and mouse macrophages. These events could be involved in the negative regulation of TLR-signaling pathways, which is followed by the suppression of the inflammatory response. However, only limited data are available that describe the effects of *P. gingivalis* on the expression of miRNAs, particularly miR-146a. Nahid et al. demonstrated that *P. gingivalis* LPS continuously amplified the miR-146a level, which is inversely correlated to TNF-α production in THP-1 monocytes [14]. The same group showed that oral inoculation with a combination of *P. gingivalis*, *Treponema denticola* and *Tannerella forsythia* induced elevated expression of miR-146a in the maxillae and spleens of infected mice, and the expression of miR-146a was negatively correlated with that of TNF-α [15].

* Corresponding author. Address: Laboratory of Periodontology and Immunology, Department of Oral Health and Welfare, Niigata University, Faculty of Dentistry, 5274 Gakkocho 2-ban-cho, Chu-o-ku, Niigata 951-8514, Japan. Fax: +81 25 227 0744.
*E-mail address:* kaz@dent.niigata-u.ac.jp (K. Yamazaki).

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To further clarify the role of miR-146a in periodontitis, the effect of *P. gingivalis* LPS on the expression of miR-146a was examined in THP-1 monocytes, and the subsequent effect of miR-146a on proinflammatory cytokine expression was analyzed by loss- and gain-of-function experiments. Interestingly, although *P. gingivalis* LPS upregulated the expression of miR-146a in THP-1 monocytes, the induction of miR-146a did not strongly suppress the production of proinflammatory cytokines.

## 2. Materials and methods

### 2.1. Reagents and antibodies

LPS from *P. gingivalis* 381 was kindly provided by Hidefumi Kumada and Toshio Umemoto (Department of Microbiology, Kanagawa Dental University, Yokosuka, Japan), and the LPS from *Escherichia coli* O111:B4 and phorbol myristic acetate (PMA) were purchased from Sigma–Aldrich (St. Louis, MO). $Pam_3CysSerLys4$ ($Pam_3CSK4$) was purchased from EMC microcollections (Tübingen, Germany). Anti-miR miRNA Inhibitors and Pre-miR miRNA Precursor Molecules were purchased from Ambion (Austin, TX). Anti-human IRAK-1 (H-273), anti-human TRAF6 (H-274, Santa Cruz Biotechnology, Santa Cruz, CA), anti-human GAPDH (ab8245; Abcam, Cambridge, UK), and the ECL Plus Western Blotting Reagent Pack (GE Healthcare, Buckinghamshire, UK) were used for Western blotting.

### 2.2. Cell preparation and culture

The monocytic cell line THP-1, maintained as described previously [3], was incubated in a 24-well culture plate (TPP, Trasadingen, Switzerland) at a concentration of $1 \times 10^6$ cells/ml in medium without FCS. After 12 h of incubation, the cells were stimulated with various doses of *P. gingivalis* LPS (0.001–10 μg/ml) for 4, 8 and 12 h or other TLR ligands (1.0 μg/ml) for 8 h.

For miRNA loss- and gain-of-function experiments, THP-1 cells were incubated for 48 h in a 24-well culture plate at a concentration of $5 \times 10^5$ cells/ml in medium supplemented with 10 ng/ml PMA to induce differentiation into macrophage-like cells (hereafter referred to as "macrophages") prior to stimulation.

### 2.3. miRNA microarray analysis

Total RNA was isolated from unstimulated and stimulated THP-1 cells using TRIzol reagent (Invitrogen, Carlsbad, CA). The RNA samples were labeled and hybridized using a commercial kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. Hybridization was carried out on Agilent human miRNA microarrays (Release 12.0), which were subsequently scanned with an Agilent DNA Microarray Scanner (model G2565CA).

### 2.4. Quantification of miRNA and mRNA expression

For quantitative analysis of miRNA expression, real-time RT-PCR was performed using the TaqMan MicroRNA Reverse Transcription Kit, the TaqMan microRNA Assay (primer/probe sets for miRNAs) and the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols.

For mRNA analysis, cDNA was synthesized as described previously [3], and amplified using TaqMan Gene Expression Assay primer/probe sets for mRNAs (Applied Biosystems) and EagleTaq Master Mix (Roche Molecular Systems, Branchburg, NJ) according to the manufacturers' instructions.

PCR reactions were conducted using the ABI PRISM 7900 HT sequence detection system (Applied Biosystems) and ABI PRISM SDS 2.0 software (Applied Biosystems). The relative expression level of each miRNA and mRNA was normalized to that of the appropriate internal control (RNU44 for miRNA, GAPDH for mRNA) using the $2^{-\Delta\Delta}C_t$ cycle threshold method [16].

### 2.5. Transfection with miRNA inhibitor and precursor

Macrophages were washed extensively with RPMI 1640 and cultured further in medium without FCS and antibiotics for 12 h. The cells were then transfected with 50 nM inhibitor for 8 h or 50 nM precursor for 24 h using siPORT NeoFX Transfection Agent (Ambion) according to the manufacturer's instructions, followed by exposure to stimulants for various lengths of time at a concentration of 0.1 μg/ml. Random-sequence inhibitors or precursors were transfected as matched controls. The doses of the inhibitor and precursor were selected based on preliminary experiments.

### 2.6. Western blotting

The cells were washed twice with ice-cold PBS, and the protein was extracted using M-PER Mammalian Protein Extraction Reagent supplemented with Halt Protease Inhibitor Cocktail and Halt Phosphatase Inhibitor Cocktail (Pierce Biotechnology, Rockford, IL, USA). The protein concentration was determined using the Pierce BCA Protein Assay Kit (Pierce Biotechnology).

Ten micrograms of each sample was solubilized by SDS sample buffer, separated by SDS–PAGE, transferred to PVDF membranes (Millipore Co., Bedford, MA), and Western blotted with each antibody. The proteins were detected using ECL Plus Western blotting detection reagents (GE Healthcare) and a LumiVision PRO 400EX (Aisin Seiki, Aichi, Japan).

### 2.7. Cytokine assay

The levels of TNF-α, IL-1β and IL-6 in the supernatants of each macrophage culture were determined using commercially available ELISA kits (Pierce Biotechnology) according to the manufacturer's instructions.

### 2.8. Statistical analysis

All experiments were performed in triplicate or quadruplicate wells for each set of conditions and were repeated at least twice. The results were expressed as the mean ± standard deviation (SD). When two groups were compared, an unpaired *t*-test was used. Multiple comparisons were performed by ANOVA-Williams test. Probability values of $<0.05$ were considered statistically significant.

## 3. Results

### 3.1. miRNA expression profile in P. gingivalis LPS-stimulated THP-1 cells

Microarray analysis demonstrated that eight miRNAs were upregulated, and two miRNAs were downregulated, with a greater than 2-fold change compared with unstimulated controls. Among the upregulated miRNAs, miR-146a was substantially and significantly induced by stimulation with *P. gingivalis* LPS ($p = 0.00137$, unpaired *t*-test). Although the expression level of miR-155 was relatively high relative to that of other miRNAs, the stimulatory effect of *P. gingivalis* LPS was very weak (Supplementary Fig. 1).

Consistent with the microarray findings, quantitative RT-PCR demonstrated that the expression of miR-146a was increased by *P. gingivalis* LPS in a dose- and time-dependent manner, with the