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Original Article

# Role of nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 6 in activation of inflammation in human umbilical vein endothelial cells stimulated by *Porphyromonas gingivalis*-an *in vitro* study

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**KEYWORDS**
Atherosclerosis;
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NLRP6;
*Porphyromonas gingivalis*

**Abstract** *Background/purpose: Porphyromonas gingivalis (P. gingivalis)* could induce the activation of vascular endothelial cells and promote the formation of atherosclerosis. Nucleotide-binding oligomerization domain-like receptor family pyrin domain containing (NLRP) 6 could recognize *P. gingivalis*, but its role in atherosclerosis was unknown. The purpose of this study is to investigate the role of NLRP6 in the activation of inflammation in human umbilical vein endothelial cells (HUVECs) stimulated by *P. gingivalis*.
*Materials and methods:* The expression level of NLRP6 in HUVECs with or without *P. gingivalis*-challenge was observed. Down-regulating the expression of NLRP6 in HUVECs, the expression levels of interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein (MCP)-1 were detected. Then, the HUVECs with NLRP6-overexpressed were stimulated by *P. gingivalis*, the levels of inflammatory cytokines above were examined and compared with those in HUVECs triggered by *P. gingivalis* only. To evaluate the effect of NLRP6 on bacterial immune escape, the NLRP6 was overexpressed, and the colonies of *P. gingivalis* that survived in HUVECs were calculated.

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*Results:* NLRP6 was expressed in HUVECs and decreased after *P. gingivalis* stimulation. Down-regulation of NLRP6 decreased the expression levels of IL-1β, IL-6, IL-8, TNF-α and MCP-1 in HUVECs. Those cytokines above in NLRP6-overexpressed HUVECs with *P. gingivalis*-stimulation significantly increased than in the cells with *P. gingivalis*-stimulation only. Furthermore, over-expression of NLRP6 decreased the colonies of *P. gingivalis* survival in HUVECs.
*Conclusion:* NLRP6 regulated the activation of inflammation in HUVECs triggered by *P. gingivalis* and played an important role in *P. gingivalis* survival in endothelial cells.


## Introduction

Cardiovascular disease (CVDs) is the leading cause of death worldwide,[1] atherosclerosis is by far the most frequent underlying cause of CVDs.[2] Endothelial injury is regarded as an early key event of atherosclerosis. Numerous studies demonstrated an association between periodontitis and CVDs, and there is increasing evidence that periodontal disease has negative cardiovascular effects.[3,4] Severe periodontitis was proved to be associated with an increased incidence of coronary heart disease during a 13-year follow-up study.[5] And the severe form of treated periodontitis was associated with an increased risk of major adverse cardiovascular events among older Taiwanese patients.[6] *Porphyromonas gingivalis (P. gingivalis)*, a major periodontal pathogenic bacteria, has been found in atherosclerotic plaques.[7,8] *P. gingivalis* could invade artery walls and persist inside cardiovascular endothelial cells,[9,10] and has been shown to play an important role in the process of atherosclerosis formation. It could induce aortic and coronary lesions, and accelerate atherosclerosis.[11] In addition, *P. gingivalis* could lead to the inflammatory response in the endothelial cells through pattern recognition receptors (PRRs)-mediated activation of inflammatory signaling pathways, which is manifested by increased expression of chemokines, and inflammatory factors, as well as damaged endothelial barrier and increased permeability.[12]

Nucleotide-binding oligomerization domain-like receptor family pyrin domain containing (NLRP) 6 is a newly identified PRR in the nucleotide-binding oligomerization domain-like receptors (NLRs) family and is considered a key immune response receptor molecule in innate immunity. NLRP6 has been shown to participate in inflammasome signaling,[13] and play critical roles in host defense against infection, flora homeostasis and tumorigenesis.[14—17] In the pathogenesis of periodontitis, NLRP6 overexpression triggers pyroptosis, which promotes periodontal inflammatory destruction.[18] But the presence and function of NLRP6 in the pathogenesis of atherosclerosis are currently still unknown. The aims of this study were to determine whether NLRP6 was expressed in endothelial cells as well as the impact of *P. gingivalis* on NLRP6 expression. Moreover, the effect of NLRP6 on the activation of inflammation in endothelial cells will be explored.

## Materials and methods

### Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell (San Diego, CA, USA). HUVECs were cultured in endothelial cell medium (ScienCell), supplemented with 5% fetal bovine serum (ScienCell) and 1% endothelial cell growth supplements (ScienCell) at 37 °C with 5% $CO_2$. HUVECs in passages 3—6 were used for all experiments.

### Immunocytofluorescence

HUVECs monolayers were seeded on coverslips. After 24 h, HUVECs monolayers were washed twice in phosphate-buffered saline (PBS), then fixed (4% formaldehyde in PBS) for 10 min and permeabilized for 5 min at room temperature. Cell monolayers were blocked with 5% bovine serum albumin in PBS for 1 h at room temperature, and incubated at 4 °C overnight with primary antibodies rabbit polyclonal anti-NLRP6 (ABclonal, Wuhan, China), followed by secondary antibody incubation (ABclonal)) and DAPI (ZSbio, Wuxi, China) counterstaining. Fluorescence was analyzed by a fluorescence microscope (Olympus, Tokyo, Japan).

### Bacterial culture and challenge

*P. gingivalis* strain W83 was cultured as described before.[19] After growth for 24 h, bacteria were washed 3 times with PBS, measured at 600 nm, and then adjusted to an optical density of 0.5, corresponding to a concentration of $10^8$ colony-forming units (CFU)/mL. Bacterial suspensions were added to confluent HUVECs at the multiplicity of infection (MOI) of 50:1 or 100:1 for 4 h, or otherwise at MOI of 100:1 for 2 h or 4 h.

### RNA interference

NLRP6 small-interfering (si) RNA and control siRNA were obtained from Sangon (Beijing, China). siRNA transfection was performed using Rfect (Baidai, Changzhou, China), according to the manufacturer's instructions. siRNA was

transfected into HUVECs for 48 h, and then the efficiency of the transfection was detected. Meanwhile, the relative expression levels of interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, and monocyte chemoattractant protein (MCP)-1 were measured.

### Infection with adenovirus carrying NLRP6 expression cassette

Adenovirus type 4 containing full-length NLRP6 (adv4-NLRP6) and control adv4 vectors were produced by GenePharma Biotech (Suzhou, China). HUVECs were seeded in 60-mm dishes and grown to 90% confluence following infection with adenovirus at MOI of 50:1 in the presence of polybrene (5 μg/mL, GenePharma) for 0—14 h. The expression levels of NLRP6 were measured. The NLRP6-overexpression HUVECs following were challenged with *P. gingivalis* at MOI 100:1 for 2 h. And then the *P. gingivalis* was removed, the expression levels of IL-1β, IL-6, TNF-α, IL-8, and MCP-1 were detected 6 h later.

### RNA isolation and real-time polymerase chain reaction (PCR)

Total RNA was isolated from cells using TRIzol reagent (ThermoFisher, Waltham, MA, USA) as described previously.[20] Reverse transcription was performed using a PrimeScript™ (Takara, Osaka, Japan). Real-time PCR was performed in triplicate using an SYBR Green Reagent (Roche, Basel, Switzerland). Primers were bought from Sangon and listed in Table 1. Expression levels of target genes were analyzed by a comparative $2^{-\Delta\Delta Ct}$ method after normalizing to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### Western blot analysis

The protein expression of NLRP6 was measured by western blot, using the protocol as described previously.[20] Immunodetection was performed using antibodies against GAPDH (ZSbio; cat. SC-17790), NLRP6 (ABclonal; cat. A15628), at 4 °C overnight. After incubating with peroxidase-linked secondary antibodies, enhanced chemiluminescence (ECL) reagent (ThermoFisher) was used to visualize immunoreactive proteins. The protein expression analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Table 1** Sequences of primers used for real-time PCR.

| Genes | Primer sequences (5′→3′) |
|---|---|
| GAPDH | Forward: CATGTACGTTGCTATCCAGGC |
| | Reverse: CTCCTTAATGTCACGCACGAT |
| IL-β | Forward: AGCTCGCCAGTGAAATGATG |
| | Reverse: GCCCTTGCTGTAGTGGTGGT |
| IL-6 | Forward: GATTCAATGAGGAGACTTGCC |
| | Reverse: TGTTCTGGAGGTACTCTAGGT |
| TNF-α | Forward: CCTCTCTCTAATCAGCCCTCTG |
| | Reverse: GAGGACCTGGGAGTAGATGAG |
| IL-8 | Forward: CTCTTGGCAGCCTTCCTGATTT |
| | Reverse: TGGGGTGGAAAGGTTTGGAGTA |
| MCP-1 | Forward: TGAAAGTCTCTGCCGCCCTT |
| | Reverse: TTGATTGCATCTGGCTGAGCG |

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, Interleukin; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemoattractant protein-1.

### Enzyme-linked immunosorbent assay (ELISA)

Protein levels of IL-1β, IL-6, TNF-α, IL-8 and MCP-1 were measured by ELISA kits (Meimian, Jiangsu China), according to the manufacturer's instructions. Then a microtiter plate reader was used to read the plate at a 450 nm wavelength.

### Invasion assay

HUVECs with NLRP6-overexpressed were seeded at a density of $10^6$ cells/well in a 6-well plate, and then challenged by *P. gingivalis* at MOI of 100:1 for 2 h. Then the medium was removed and the cells were washed with PBS three times. Medium containing gentamicin (300 μg/mL) and metronidazole (200 μg/mL) (Sigma, St. Louis, MO, USA) was added to the wells and then incubated for 60 min at 37 °C to kill all the extracellular bacteria. Finally, the cells were washed with PBS three times and lysed with 500 μl of sterile distilled water during a 20-min incubation at 37 °C under aerobic conditions. Dilutions of the lysates of cells infected with *P. gingivalis* were plated in triplicate on BHI agar plates supplemented with 5.0% sheep blood by easySpiral (Interscience, Mourjou, France). Plates were cultured anaerobically, and the CFU/mL of invasive bacteria was counted according to the manufacturer's instructions.

### Statistical analysis

Unpaired two-tailed Student's t-test and one-way analysis of variance (ANOVA) followed by Tukey's test were carried out to assess the group difference. Statistical analyses were performed with SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Results are presented as the mean ± standard deviation from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ was considered to be statistically significant.

## Results

### NLRP6 was expressed in HUVECs

We used immunofluorescence labeling for NLRP6 in cultivated HUVECs cells, and the results showed that NLRP6 was expressed in the cytoplasm of HUVECs cells (Fig. 1).

### The expression of NLRP6 in HUVECs is decreased after *P. gingivalis*-stimulation

The expression of NLRP6 in HUVECs was significantly reduced by *P. gingivalis* stimulation at MOI of 50:1 and 100:1 for 4 h, and the expression of NLRP6 was further decreased in the higher MOI group (Fig. 2A and B).

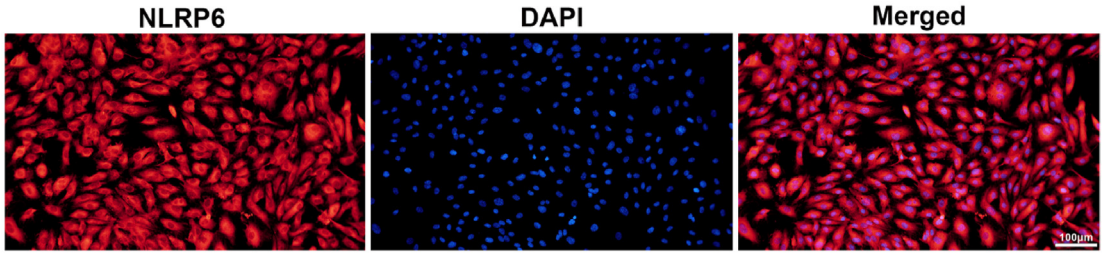


**Figure 1** NLRP6 expression in HUVECs. (Original magnification × 40, White scale:100 μm). NLRP6, nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 6; HUVECs, human umbilical vein endothelial cells; DAPI, 4′,6-diamidino-2-phenylindole.

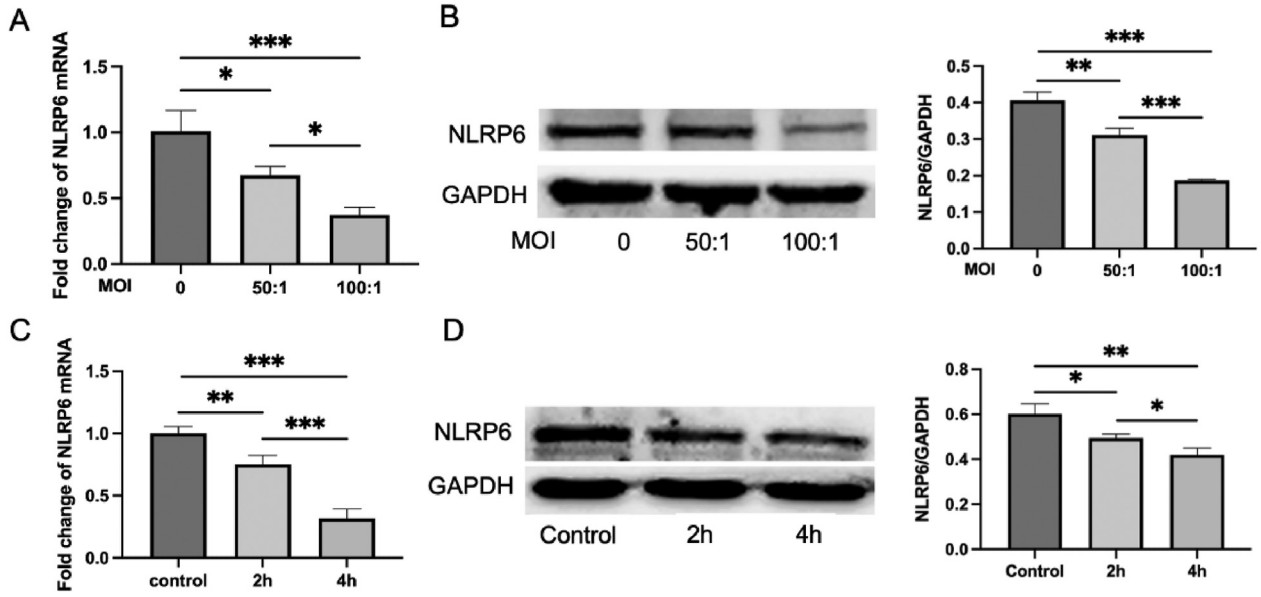


**Figure 2** *Porphyromonas gingivalis* decreases NLRP6 expression in HUVECs. The mRNA and protein expression levels of NLRP6 in HUVECs after challenged by *P. gingivalis* at MOI of 50:1 or 100:1 for 4 h (A, B) or at MOI of 100:1 for 0–4 h (C, D).*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. NLRP6, nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 6; MOI, multiplicity of infection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Meanwhile, when HUVECs were challenged by *P. gingivalis* at MOI of 100:1 for 2 h or 4 h, the expression of NLRP6 also decreased, which declined further with increasing stimulation time (Fig. 2C and D).

## NLRP6 promotes the *P. gingivalis*-induced expression of inflammatory factors and chemokines in HUVECs

siRNA-mediated gene silencing significantly reduced the expression level of NLRP6 in HUVECs (Fig. 3A). Meanwhile, the expressions of inflammatory factors IL-1β, IL-6 and TNF-α, chemokines IL-8 and MCP-1 in HUVECs were significantly decreased at both mRNA and protein levels (Fig. 3C and D). When HUVECs were infected with Adv4-NLRP6 for over 10 h, the expression of NLRP6 was greatly elevated in HUVECs (Fig. 3B). The expression levels of these inflammatory cytokines and chemokines above in HUVECs increased when *P. gingivalis*-treated, while significantly further increased in *P. gingivalis*-treated HUVECs with NLRP6-overexpressed (Fig. 3E and F). These results showed that NLRP6 could promote the expressions of the inflammatory cytokines and chemokines in HUVECs challenged by *P. gingivalis*.

## NLRP6 inhibits *P. gingivalis* invasion and survival in HUVECs

The effect of NLRP6 on *P. gingivalis* invasion and survival in HUVECs was explored. Compared with the control group, the number of *P. gingivalis* colonies in NLRP6-overexpressed HUVECs decreased apparently (Fig. 4A). The amount of *P. gingivalis* that invaded and survived in the control group was $(7.7 \pm 0.6) \times 10^4$ CFU/mL, whereas the number of *P. gingivalis* in the NLRP6-overexpressed group was $(3.3 \pm 0.4) \times 10^4$ CFU/mL, the difference was statistically significant ($P < 0.001$, Fig. 4B). The present findings suggested that activation of NLRP6 could inhibit the survival of *P. gingivalis* in HUVECs.

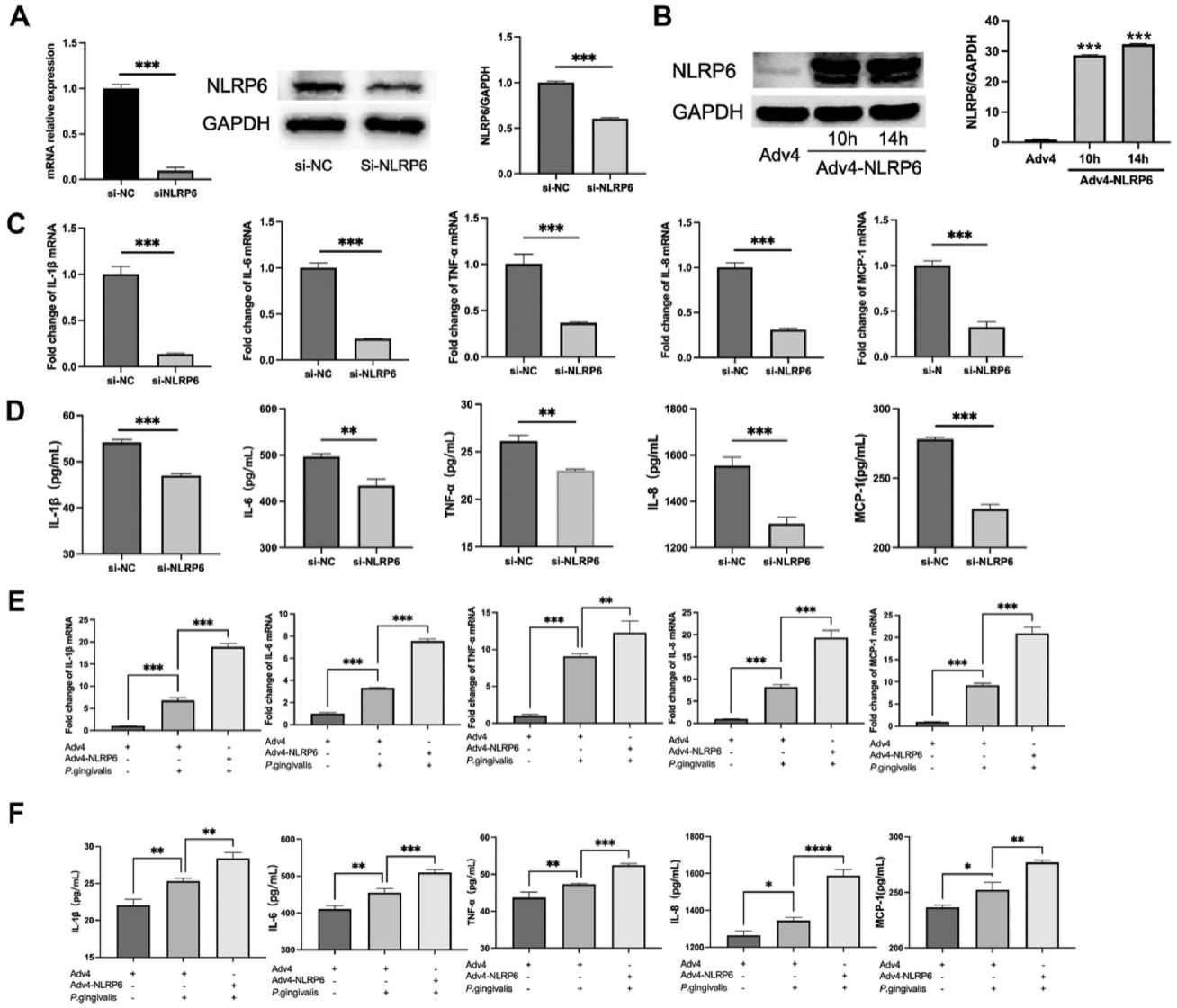


**Figure 3** NLRP6 promotes the expression of *P. gingivalis*-induced inflammatory factors and chemokines in HUVECs. The expression of NLRP6 in HUVECs was significantly decreased by interference with the si-NLRP6 (A) or increased by infection with Adv4-NLRP6 (B). The mRNA and protein levels of IL-1β, IL-6, TNF-α, IL-8, MCP-1 were inhibited in HUVECs with NLRP6-silenced (C, D). But when compared with HUVECs with *P. gingivalis*-treated only, the mRNA and protein levels of IL-1β, IL-6, TNF-, IL-8 and MCP-1 were further promoted in NLRP6-overexpressed HUVECs followed by *P. gingivalis* treated. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. NLRP6, nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 6; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Adv4, Adenovirus type 4; *P. gingivalis*, *Porphyromonas gingivalis*; IL, Interleukin; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemoattractant protein-1.

## Discussion

In the present study, the expression of NLRP6 in HUVECs was reported for the first time. NLRP6 has been shown to express in a number of tissues and cells, including immunological cells such as cluster of differentiation (CD) 4 T cells, CD8 T cells, eosinophils, granulocytes, monocytes, B cells, and dendritic cells, and plays a central role in the inflammatory response.[21,22] It's also found in the gut, intestine and colon, liver, gastric tissues and other tissues,[14,22] where it modulates flora homeostasis, intestinal inflammation, hepatitis, and gastric cancer.[17,23,24] However, it's the first time to identify that NLRP6 was expressed in HUVECs by our study. And the expression of NLRP6 in endothelial cells was decreased by *P. gingivalis*-treated. These findings further expand our knowledge of NLRP6.

In this study, we found that downregulation of NLRP6 decreased the inflammatory cytokines IL-1β, IL-6, and TNF-α, the chemokines molecules IL-8 and MCP-1 in HUVECs. While NLRP6-overexpression promotes the *P. gingivalis*-induced expression of inflammatory factors and chemokines in HUVECs. These factors play an important role in the process of atherosclerosis formation. IL-1β, IL-6, and TNF-α could activate inflammatory signaling pathways, which in turn regulate the high expression levels of cell adhesion

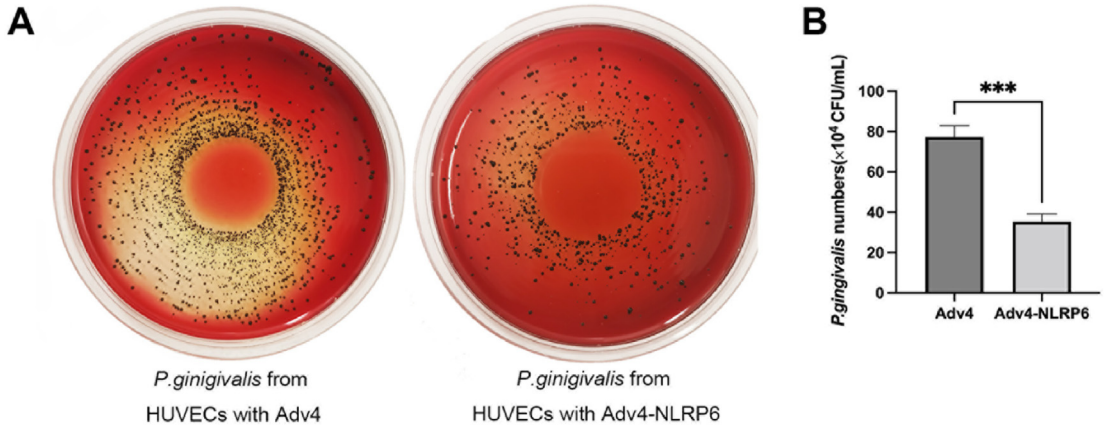


**Figure 4** NLRP6 inhibits *P. gingivalis* invasion and survival in HUVECs. The *P. gingivalis* colony from the lysate from NLRP6-overexpressed or control HUVECs lived on the BHI agar plates (A). The number of *P. gingivalis* in two groups (B). ***: $P < 0.001$. NLRP6, nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 6; HUVECs, human umbilical vein endothelial cells; Adv4, Adenovirus type 4; *P. gingivalis, Porphyromonas gingivalis*.

molecules in endothelial cells, thus promoting the proliferation and migration of vascular smooth muscle cells and the transformation of macrophages into foam cells.[25–28] In addition, chemokines IL-8 and MCP-1 play an important role in the recruitment of monocytes and neutrophils to the endothelium and infected site.[29] These immune cells could recognize, phagocytosis, and kill the infected bacteria with the stimulation of pro-inflammation molecules.[30] Therefore, NLRP6 may take part in the process of atherogenesis by participating in the regulation of cytokines and chemokines in endothelial cells.

NLRP6, as one of pattern recognition, was reported to play an important role in many inflammatory conditions and diseases. However, its role is complicated. In some studies, it was demonstrated to participate in the inflammasome pathway, induce the release of IL-1β and IL-18 and lead to pyroptosis and the burst of inflammation.[31] Thus, the increased expression of NLRP6 was shown in gingival fibroblasts triggered by *P. gingivalis*,[18] in the bone-marrow derived neutrophils and macrophages triggered by *Klebsiella pneumoniae*.[32] Reversely, in some conditions or diseases, NLRP6 could also suppress inflammation by inhibiting the nuclear factor κB (NF-κB) signaling pathway.[14] Therefore, the effects of NLRP6 may be completely opposite in different cells. In this study, upregulation of NLRP6 promoted the expressions of inflammatory mediators and chemokines in *P. gingivalis*-treated HUVECs, indicating this receptor functioned as a pro-inflammatory factor in vascular endothelial cells like other PRRs.

Interestingly, we found that NLRP6 was suppressed after *Porphyromonas gingival* stimulation, unlike other pro-inflammatory PRRs such as toll-like receptor 2 (TLR2) or nucleotide-binding oligomerization domain-like receptors 1 (NOD1) were activated after *P. gingivalis*-infected. It was implied that NLRP6 might have another effect on *P. gingivalis*-stimulated HUVECs. Therefore, this study detected the role of NLRP6 in the immune escape of *P. gingivalis*. Our results demonstrated that NLRP6 could regulate the survival of *P. gingivalis* in the vascular endothelial cells. Previous studies have proved that *P. gingivalis* was able to adhere to endothelial cells' surfaces, subsequently invade the cells and present in the cytoplasmic vacuole.[33] The invasion and survival of *P. gingivalis* could not only directly cause the injury of vascular endothelium, resulting in various functional disorders, in addition, achieving immune escape of *P. gingivalis* play an important role in its pathogenic mechanism. Li et al.[34] found that when *P. gingivalis* invaded the epithelial cells, it could exit the initially infected host cells and then infect deeper cells, exacerbating the inflammatory response and the tissue destruction. This study found that *P. gingivalis* invaded and survived in endothelial cells by reducing the expression of NLRP6, which demonstrated for the first time that NLRP6 can regulate the process of *P. gingivalis* invasion and survival in endothelial cells, thus playing a critical role in the immune escape of *P. gingivalis*. In addition, down-regulating the expression of NLRP6 in HUVECs by *P. gingivalis* could maintain the expression levels of inflammatory cytokines at a certain high level without too excessive, in order to avoid recruiting a large number of immune cells into the focus and killing the infected bacteria.[30,35] The results above expand our understanding of the role of NLRP6 in the innate immune response, which can help us further recognize the pathogenicity of periodontal pathogens in atherosclerosis to provide new strategies for future treatment.

In this study, all data and results of this study were based on *in vitro* research. These findings should be validated *in vivo* in subsequent studies. The underlying mechanism of how NLRP6 regulated these cytokine secretions and immune response to *P. gingivalis* infection in vascular endothelium is also required to be elucidated. And it will be intriguing to explore further functions of NLRP6 in the promotion of atherosclerosis formation by *P. gingivalis* in future research.

In conclusion, the results of the present study demonstrated that NLRP6 existed in HUVECs and decreased triggered by *P. gingivalis*. NLRP6 participated in the inflammation regulation of vascular endothelial cells and limited the survival of *P. gingivalis* in HUEVCs,

which suggested NLRP6 might play an important role in the pathogenesis of atherosclerosis promoted by *P. gingivalis*.

## Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

## Acknowledgments

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