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Activation of nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 6 by *Porphyromonas gingivalis* regulates programmed cell death in epithelium

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Abstract *Background/purpose:* Gingival epithelial cells form a physiological barrier against bacterial invasion. Programmed cell death (PCD) regulated by pathogen precognition receptors (PRRs) lead to tissue destruction and is closely related to inflammatory diseases. The purpose of this study was to investigate whether nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 6 (NLRP6) expresses in periodontal epithelium and induces PCD of epithelial cells infected by *Porphyromonas gingivalis* (*P. gingivalis*), therefore involves in periodontitis.

Material and methods: The expression of NLRP6 was detected in periodontal epithelium from human gingival sections and HaCaT cells stimulated by *P. gingivalis*. NLRP6 was over-expressed by adenovirus infection in HaCaT or knocked down by siRNA in *P. gingivalis* infected HaCaT, and

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the cell death was observed by transmission electron microscopy and flow cytometry analysis. In addition, qPCR and Western blot were performed to determine the expression of NLRP6 and the pyroptosis excutors, caspase-1 and gasdermin D. Enzyme-linked immunosorbent assay were performed to detect the secretion of IL-1 β and IL-18.

Results: NLRP6 was up-regulated in both gingival epithelium of patients with periodontitis and *P. gingivalis* infected HaCaT. Over-expression of NLRP6 in HaCaT led to caspase-1 dependent pyroptosis. Interestingly, knockdown of NLRP6 with siRNA followed by *P. gingivalis* stimulation inhibited pyroptosis and induced apoptosis.

Conclusion: Up-regulation of NLRP6 by *P. gingivalis* in HaCaT led to pyroptosis, while knocking down NLRP6 inhibited pyroptosis and induced apoptosis, which indicated this PRR may play a crucial role in periodontitis by regulating PCD in periodontal epithelium.

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Introduction

Periodontitis is a common oral inflammation-mediated destructive disease of periodontium. There are hundreds of bacteria species in oral microenvironment. Among them, *Porphyromonas gingivalis* (*P. gingivalis*), *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia* and etc. have been identified closely related to periodontitis.^{1,2} They formed the microbial plaque to challenge the gingiva and started the inflammatory events.

Gingival epithelial cells are the forefront exposed to these bacteria plaque. These cells not only act as a physiological barrier, but also produce inflammatory cytokines such as interleukin (IL)-1 β in response to these bacteria.³ The damage of the epithelial layer by infecting organisms is an important step in the pathogenesis of periodontitis because loosening of the epithelial tissue may facilitate the bacterial penetration. However, the mechanisms of how these pathogens destroy the integrity of periodontium remain largely unknown.

Programmed cell death (PCD) is an umbrella term of several types of active cell death.^{4,5} Different from the passive cell death, PCD is a process of active death for better adaptation to the environment. It is mediated by a series of gene expression events and plays an important role in elimination of injured cells.⁶ Pathogen recognition receptors (PRRs) are a protein family which could respond to pathogen-associated molecular patterns (PAMPs) expressed by pathogen, and trigger a series of immune response including PCD.^{7,8} It has been reported that PRR family member nucleotide-binding oligomerization domain-like receptor (NOD-like receptor) family pyrin domain containing 3 (NLRP3), NOD-like receptor caspase recruitment domain containing 4 (NLRC4) and Absent in melanoma 2 (AIM-2) could trigger different types of PCD including apoptosis, pyroptosis and necroptosis while periodontal pathogen infection.^{9–11} However, the full extent of PCD's role in periodontitis and the relationship between PCD and PRRs need further explore.

Nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 6 (NLRP6) is a cytosolic PRR member which contributes to regulate host defense against microbe, inflammation and tumorigenesis.^{12,13}

NLRP6 recruits an adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) that functions as a scaffold of downstream pathway. Co-expression of NLRP6 and ASC activate caspase-1, a key protein of canonical inflammasome.¹⁴ Recent studies reported a certain type of PCD called pyroptosis, which is dependent on caspase-1 and GSDMD activation, and leads to cleavage and secretion of IL-1 β and IL-18.^{15,16} However, till now it is not clear whether NLRP6 mediates pyroptosis of periodontal epithelium cells, and therefore play a role in periodontal micro-organisms-caused epithelial destruction.

This study aimed to detect the expression of NLRP6 in periodontal epithelium and determine whether NLRP6 mediated the pyroptosis of gingival epithelial cells induced by the infection of periodontal pathogen *P. gingivalis*, thus play a role in inflammation and periodontium breakdown. We examined the changes in the expression levels of NLRP6 in both gingiva tissue from periodontitis patients and a human keratinocyte cell line, HaCaT infected by *P. gingivalis*. NLRP6 knockdown by small interference RNA and NLRP6 overexpression by adenovirus carrying NLRP6 expression cassette were both introduced to investigate the role of NLRP6 in the periodontal micro-organisms-caused epithelial destruction in periodontitis.

Materials and methods

The detection of NLRP6 expression in gingival epithelium

Gingival tissue specimens ($n = 18$) were obtained from age matched (32–62 years) healthy subject ($n = 9$) and patients with chronic periodontitis ($n = 9$). The inclusion criteria included the healthy subject (i) healthy and intact periodontium without attachment loss; (ii) crown lengthening for aesthetic needs. The inflammatory specimen was collected during periodontal surgery and the inclusion criteria were (i) chronic periodontitis patients with clinical attachment loss; (ii) probing depth ≥ 5 mm after initial therapy. The exclusion criteria for both group were (i) smoking history or systemic diseases that may affect periodontal tissue; (ii) antimicrobial or medicinal treatments in

the last 6 mon. Protocols for collecting gingival biopsies and immunostaining were conducted as previously described.¹⁷ To detect NLRP6, a tyramide signal amplification [TSA] technique was applied to the tissue sections. Briefly, gingival tissues were fixed by 10% formalin, embedded in wax, sectioned, and incubated with NLRP6 antibody (Abcepta, Suzhou, China) at working solution (1:100), 4°C overnight. Sections were then incubated with secondary antibody followed by peroxidase for 2 h at RT. TSA reagent (Biodragon, Suzhou, China) was used for visualization under laser scanning confocal microscopy. This study was approved by the Medical Ethics Committee at Peking University School of Stomatology (Ethics Approval No. PKUS-IRB-201522049).

HaCaT cells culture

HaCaT cells, spontaneously immortalized human keratinocyte line, were obtained from ATCC, and cultured in 5% CO₂ at 37 °C in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, Logan, UT, USA). The complete medium was supplemented with 10% fetal bovine serum (FBS) (Gibco, Paisley, UK.) 1% penicillin/streptomycin.

Bacterial culture and infection

P. gingivalis W83 was grown anaerobically (5% CO₂, 10% H₂, 85% N₂) for 5–7 d at 37 °C in brain heart infusion broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10 mg/ml hemin, 0.75 mg/ml cysteine, and 1 mg/ml menadione obtained from Sigma. The cultures were then inoculated into fresh BHI broth (supplemented with 5 mg/ml hemin and 0.4 mg/ml menadione) and grown until the optical density at 600 nm reached 1.0. Bacteria were centrifuged, washed, and resuspended in DMEM with 10% FBS at a final concentration of 10⁸ cells/mL. Bacterial suspensions were added to confluent fibroblast monolayer at a multiplicity of infection (MOI) of 1:100. Untreated cultures served as negative controls.

Lipopolysaccharide from *Porphyromonas gingivalis* (*P. gingivalis*-LPS) were purchased from Invivogen (San Diego, CA, USA) and added at a concentration of 100 ng/mL for 24 h.

NLRP6 knock down by small-interfering RNA (siRNA) transfection

NLRP6 siRNA, and scramble siRNA were obtained from RiboBio Technology (Guangzhou, China). Transfection of siRNA was performed by using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. For knockdown of NLRP6, HaCaT cells were seeded in 6-well plates and transfected with 100 pM siRNA for 48h. The control group was treated with transfection reagent plus scrambled siRNA. The efficiency of the transfection and viability were also detected using Cy3-labelled transfection control. Both control and test groups were infected with *Porphyromonas gingivalis* for additional 8 h and then harvested for the subsequent experiments.

Overexpression of NLRP6 by adenovirus infection

Adenovirus type 4 containing full-length NLRP6 (adv4-NLRP6) and adenovirus containing control vectors (adv4) were produced by GenePharma Co. (Shanghai, China). Cells were placed in 60 mm dishes, and grown to 70%–80% confluence. Adenoviral infection was carried out at a multiplicity of infection (MOI) of 10:1 in the presence of polybrene (5 µg/mL) for 12 h.

Analysis of cell death by flow cytometry

HaCaT cells were either double stained with annexin V-FITC and propidium iodide (PI) from a apoptosis detection kit (BD Biosciences, San Jose, CA, USA), or double stained with caspase-1-FITC and PI from the FAM-FLICA caspase assay kits (Immunochemistry Technologies, Bloomington, IL, USA). The positive rates were analyzed using an Epics XL Flow Cytometer (Beckman Coulter, Brea, CA, USA).

RNA isolation and quantitative real-time polymerase chain reaction (qPCR)

Total RNA from both gingival samples and HaCaT cells was extracted from triplicate independent control- and infected HaCaT cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The concentration and purity of RNA was determined by measuring the optical density. Total RNA (1 µg) from each sample was transcribed to cDNA with oligo(dT) primers using a GoScript™ reverse transcription system (Promega, Madison, WI, USA) according to the manufacturer's protocol.

qPCR analysis of NLRP6 and caspase-1/-3/-4 was performed in triplicate with an SYBR Green Reagent (Roche, Indianapolis, IN, USA) and specific primers on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Life Technologies, Warrington, UK). GAPDH was used as an endogenous control. The primers were as follows: GAPDH, forward: 5'-GAAGGTGAAGGTCGGAGT-3', reverse: 3'-GAAGATGGTGATGGGATTTC-5'; NLRP6, forward: 5'-ATGGACGTGGCTGTTCTGAG-3', reverse: 3'-GGAGGCTGGCAGTTGTTTTG-5'; caspase-1, forward: 5'-CGTTCCATGGGTGAAGGTACA-3', reverse: 3'-TGCCCCCTTCGGAATAACGG-5'; caspase-3, forward: 5'-GGAAUAUCCCUCCUGGACAACATT-3', reverse: 3'-UGUUGUCCAGGGGAUUAUCCAG-5'; caspase-4, forward: 5'-TCACCTGCCTGCAAGGAATG-3', reverse: 3'-TCACCTGCCTGCAAGGAATG-5'.

Western blot analysis

Cells were collected and lysed in 100 µl RIPA buffer (Applygen, Beijing, China) with proteinase and phosphatase inhibitors for protein extraction. The protein concentration were measured by a BCA Kit (Thermo, Rockford, IL, USA) Equal amount of protein lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, blocked with 5% non-fat dry milk in TBST (Tris-buffered saline plus Tween-20) for 1 h. Immunodetection was performed using with antibodies against β-actin (ZSbio, Beijing, China; cat. SC-17790), NLRP6 (Abcam, Cambridge, MA,

USA; cat. ab116007), caspase-1 (Cell Signaling Technology, Danvers, MA, USA; cat. 22255).

Transmission electron microscope (TEM) observation

HaCaT cells were cultured in 60-mm dishes and treated as described for adv4-NLRP6 infection experiments. Cells were collected and fixed with 2.5% glutaraldehyde. Morphological changes of HaCaT cells were observed by JEM1400PLUS transmission electron microscope.

Determination of interleukin-1 β and interleukin-18 levels

The amount of IL-1 β and IL-18 in the culture media was measured using an ELISA kit were from Neobioscience (Shenzhen, China; cat. EHC002b, EHC127) according to the manufacturer's instruction.

Statistical analysis

All numerical data and error bars were provided as the mean \pm standard deviation. Statistical significance of the differences among groups was analyzed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Multi-group comparisons of the mean data were performed using one-way analysis of variance with the Student-Newman-Keuls test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Up-regulation of NLRP6 expression in gingival epithelial tissue of periodontitis samples and HaCaT cells challenged by *Porphyromonas gingivalis*

Immunofluorescence results showed that, compared with control group, the number of NLRP6-positive epithelial cells was significantly increased in periodontitis group (Fig. 1A). qPCR results showed NLRP6 expression at mRNA level in periodontitis group was significantly higher than in control group (Fig. 1B). Meanwhile, the activation of NLRP6 was assessed by qPCR and Western blot in *P. gingivalis*-challenged HaCaT cells. The results showed that at 2, 4 and 8 h post-*P. gingivalis* challenge, NLRP6 expression in HaCaT cells was up-regulated at both mRNA and protein levels (Fig. 1C and D). However, NLRP6 expression remains unchanged while stimulated by *P. gingivalis*-LPS. This indicated that *P. gingivalis*-LPS was not equivalent to *P. gingivalis* bacteria at inducing NLRP6 expression aspect.

NLRP6 induces pyroptotic-like morphological changes of HaCaT cells

To further investigate the function of NLRP6, adenovirus carrying NLRP6 overexpression cassette (adv4-NLRP6) was used to infect HaCaT cells. After 12h infection, significant

cell death were observed under optical microscope (Fig. 2A). Under the observation of transmission electronic microscope, HaCaT shows the characteristic of pyroptosis: swelling of cell and nuclear, disruption of membrane and condensation of chromatin (Fig. 2B).

NLRP6 activates caspase-1-mediated pyroptosis in HaCaT cells

To distinguish the type of cell death induced by NLRP6 overexpression, HaCaT cells were stained by annexin V-FITC & PI before flow cytometry analysis. Apoptotic cells will exhibit annexin V(+)/PI(-) at early phase or annexin V(+)/PI(+) at later period.¹⁸ The results showed that compared with control group (infected with adv4), annexin V(+)/PI(+) cells were significantly increased by adv4-NLRP6 infection, however, few annexin V(+)/PI(-) cells were detected (Fig. 3A). qPCR showed that caspase-1, instead of caspase -3 or -4 is significantly increased at mRNA level (Fig. 3B). Therefore, we assumed while NLRP6 over-expression, caspase-1 were activated, and in turn to activate a canonical inflammasome pathway, resulting in caspase-1 induced pyroptosis. Then we used a caspase-1 inhibitor, YVAD to verify this hypothesis. After YVAD addition, the number of cell death detected by flow cytometry was significantly decreased (Fig. 3A). GSDMD, the newly discovered switch of pyroptosis, as well as the downstream of inflammasome, IL-1 β and IL-18, were both up-regulated by adv4-NLRP6 and the up-regulation could be reversed by YVAD according to the results of ELISA and western blot, respectively (Fig. 3C and D).

NLRP6 knockdown inhibits pyroptosis but increases apoptosis in HaCaT cells

To investigate the function of NLRP6 during *P. gingivalis* challenge, a small interference RNA targeting on NLRP6 was transfected into HaCaTs cells. In order to detect whether *P. gingivalis* could induce pyroptosis, HaCaT cells were monitored by caspase-1-FITC & PI agents during the infection in order to be prepared for flow cytometry analysis. After 8 h infection with *P. gingivalis* at MOI 1:50, caspase-1-FITC and PI positive cells are significantly increased (Fig. 4A). When NLRP6 siRNA was transfected before infection, this increase is partially but significantly inhibited (Fig. 4A). Cells were also stained with annexin-V-FITC & PI. Interestingly, apoptotic cells [annexin-V(+)/PI(-)] induced by *P. gingivalis* were significantly increased by NLRP6 siRNA (Fig. 4B). Results of ELISA showed the increase of IL-1 β and IL-18 by *P. gingivalis* infection was also inhibited by NLRP6 siRNA (Fig. 4C). Knockdown efficiency of siNLRP6 was evaluated by qPCR (Fig. 4D).

Discussion

As apoptosis was recognized as the first well known type of programmed cell death, many new forms of PCD have been found in past decades and reclassified by Nomenclature Committee on Cell Death in 2018.¹⁹ Numerous studies have confirmed that different types of PCD are widely involved in

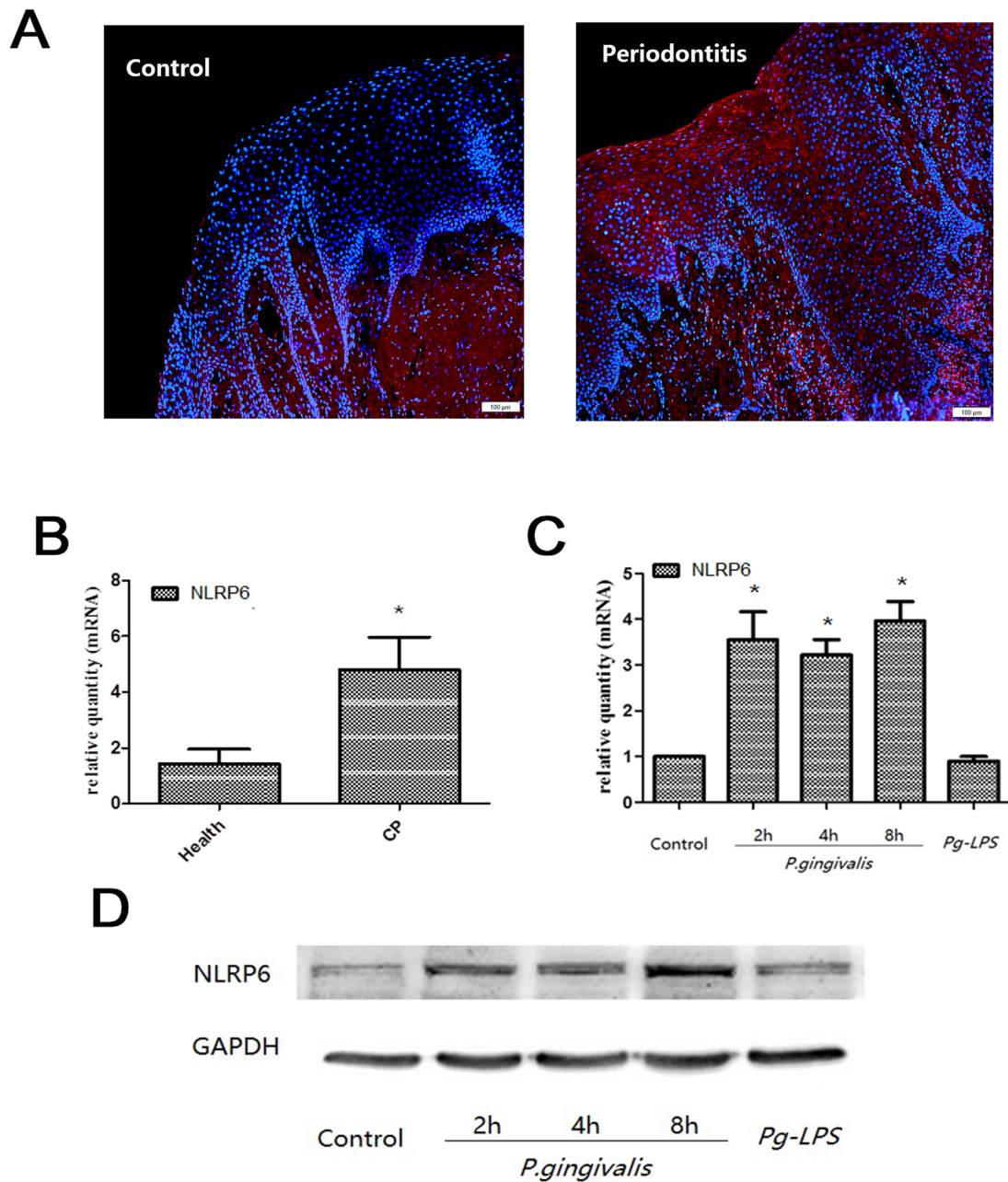


Figure 1 NLRP6 expression in gingival epithelium and HaCaT cells. (A), Immunofluorescence of NLRP6 in gingival section from healthy specimens (Control) or chronic periodontitis specimens (Periodontitis). (B), qPCR analysis of NLRP6 expression in Health or CP group. (C), qPCR analysis of NLRP6 expression in HaCaT cells with *P. gingivalis* or *P. gingivalis*-LPS infection at indicated time point. (D), Western blot analysis of NLRP6 expression in the same cells. *: $P < 0.05$.

a variety of pathological processes although the mechanism in certain diseases remains largely unknown. It was reported that PRRs activated by PAMPs or DAMPs resulted in triggering diverse inflammatory responses, losing control over single or mixed types of cell death, thus play a role in immune diseases.²⁰ In this study, we firstly identified that the expression of NLRP6 increased in human keratinocyte cell line HaCaT stimulated by a typical periodontal pathogen, *P. gingivalis*. By adv4-NLRP6 infection, we further confirmed that overexpression of NLRP6 in HaCaT cells

induced pyroptosis. Pyroptosis is a newly discovered PCD dependent on a canonical inflammasome protein, caspase-1, and a pore formation effector, GSDMD.²¹ This PCD is predicted to be proinflammatory due to facilitating the release of intracellular proinflammatory molecules, including IL-1 β and IL-18.²² Recent studies have suggested that pyroptosis is closely related to periodontitis. LPS from *E. coli* and *P. gingivalis* could activate NLRP3 inflammasome and pyroptosis in periodontal ligament cells.^{23,24} Cheng et al. reported that *P. gingivalis* LPS promoted pyroptosis in

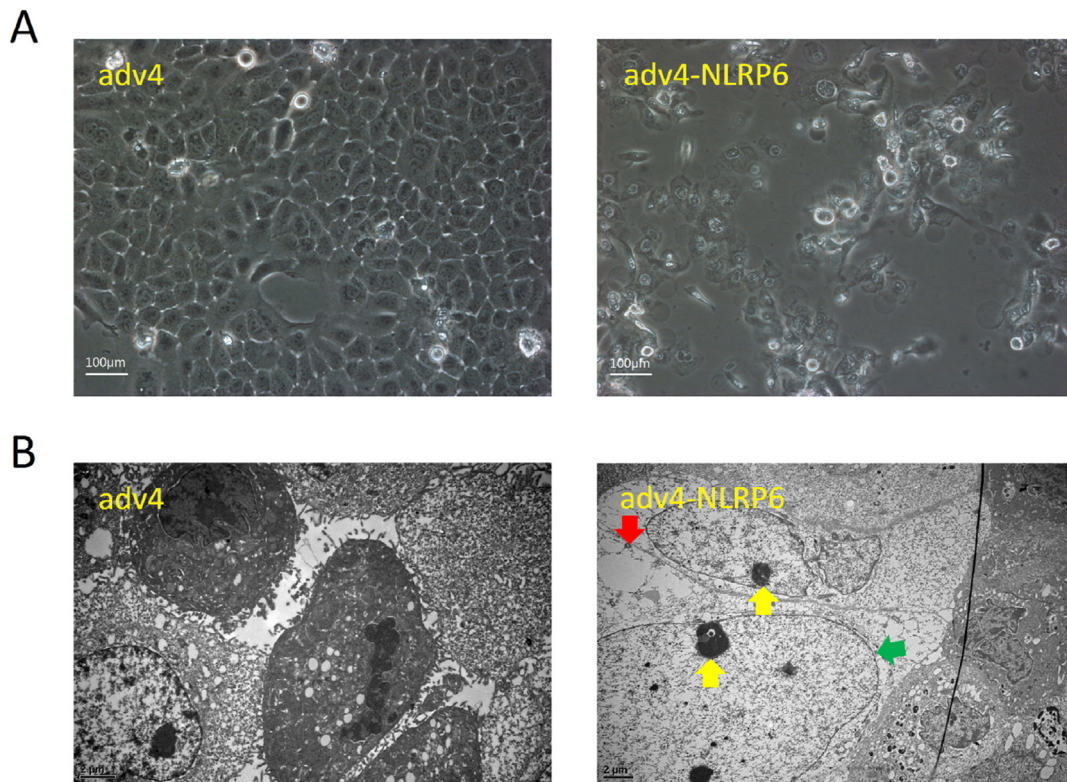


Figure 2 Morphological observation of adv4 or adv4-NLRP6 infected HaCaT cells. (A), Optical microscope observation. (B), Transmission electronic microscope observation. Pyroptotic cells show membrane rupture (red arrow) as well as chromatin margination and condensation (yellow arrow); nuclei (green arrow) remain intact but exhibit swelling.

human gingival fibroblasts (hGFs) under hypoxia.²⁵ In our previous study, it was showed that NLRP6 could recognize *P. gingivalis* in gingival fibroblasts, and also could promote pyroptosis of fibroblasts.²⁶ Compared with periodontal connective tissue, the crevicular pocket epithelium is located at the frontline to dental plaque and act as a physical barrier of defending against bacteria and other external stimuli. Pyroptosis of periodontal epithelial cells induced by NLRP6 might not only promote the gingival inflammation, but also break the integrity of the epithelial barrier, making it easier for bacteria to invade deep tissues and exacerbate the destruction of periodontal tissues. Furthermore, the pyroptosis of the gingival junctional epithelium cells destroyed the sealing of the dentogingival junction, which might be the cause of periodontal attachment loss. Thus, these findings in this present study provided a stronger evidence that NLRP6 play a critical role in the pathogenesis of periodontitis.

Previous studies showed *P. gingivalis* infection influenced different types of cell death, including apoptosis, necroptosis and autophagy.^{27–29} Apoptosis is another kind of PCD, which is characterized by cell shrinkage, membrane expansion and formation of apoptotic bodies. Different from pyroptosis, apoptosis is usually regarded as a cell death process with low inflammation and immunogenicity.³⁰ Our results demonstrated that pyroptosis and apoptosis occurred in HaCaTs simultaneously under *P. gingivalis* infection. Different cell death patterns may reveal

the different clinical stages of periodontitis. The monitoring of cell death patterns may help us to understand the process of periodontitis. Furthermore, recently evidence showed different types of PCD existed extensive crosstalk and could even switch to each other under specific conditions.^{31–33} A20 (the tumor necrosis factor alpha-induced protein 3, TNFAIP3) has been shown to enhance autophagy and reduce pyroptosis in PDLCs.³⁴ This result suggests that we can alleviating inflammation by regulating the expression of some specific proteins associated with PCD. More importantly, our findings revealed when knocking down NLRP6, the balance of apoptosis and pyroptosis induced by *P. gingivalis* would break out, indicating that NLRP6 might be a key molecules for regulating the different kinds of PCD. Since pyroptosis is thought to be related to the inflammation burst while apoptosis is characterized by low inflammation, the switch from pyroptosis to apoptosis might change the inflammatory microenvironment from “explosive” to “limited”, which suggested as a potential therapeutic strategy for inflammatory disease control.

Virulence factors of periodontal pathogens could impair the epithelial barrier functions and activate inflammasomes or pyroptosis in periodontal tissues. Besides LPS, outer membrane vesicles (OMVs) and gingipain produced by *P. gingivalis* could induce inflammasome activation.^{35,36} Moreover, *E. coli* LPS could trigger caspase-11 dependent cell death.³⁷ Caspase family members are closely related to PCD. Caspase-3/-8/-9 have been reported to induce

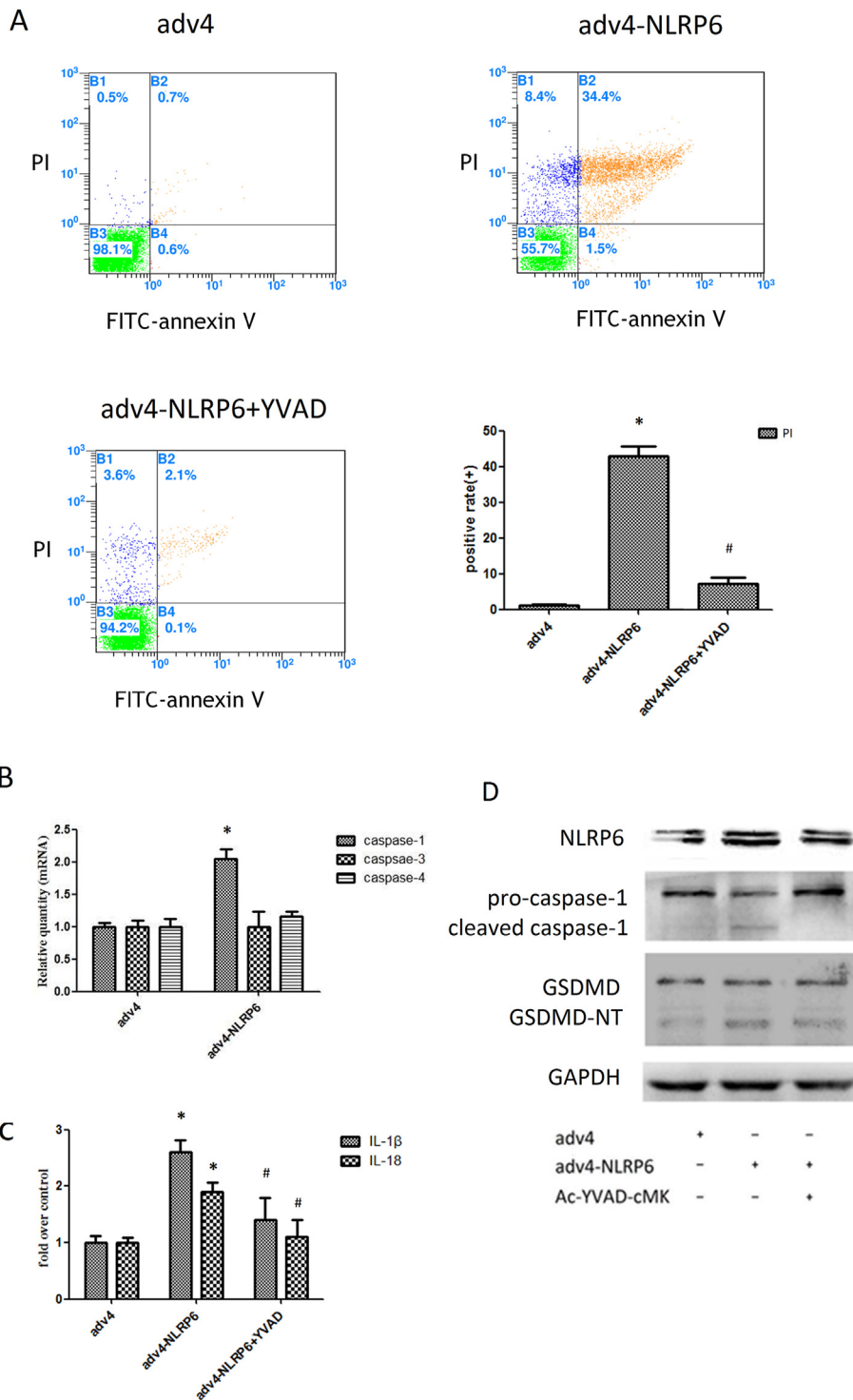


Figure 3 Detection of pyroptosis induced by NLRP6 overexpression in HaCaT cells. HaCaT cells were infected by Adv4-NLRP6 with or without addition of YVAD. Adv4 alone infection was treated as a control group. (A), Flow cytometry analysis of annexin-V and PI positive rates. (B), qPCR analysis of caspase-1/-3/-4. (C), ELISA results of secreted IL-1 β and IL-18. (D), Western blot analysis of NLRP6, caspase-1 and GSDMD. *: $P < 0.05$ compared with adv4 group. #: $P < 0.05$ compared with adv4-NLRP6 group.

apoptosis while caspase-1/-4/-11 have been reported to induce pyroptosis.^{38–40} Different from previous studies, *P. gingivalis*-LPS stimulation didn't induce pyroptosis according to our 48h observation (data not shown). Meanwhile, *P. gingivalis*-LPS alone is not able to activate NLRP6, and

caspase-3/-4 is not up-regulated by NLRP6. Hence, we assume that NLRP6 is irrelevant to LPS induced pyroptosis. However, which virulence factor and caspase family member affecting pyroptosis induced by NLRP6 need to be further studied.

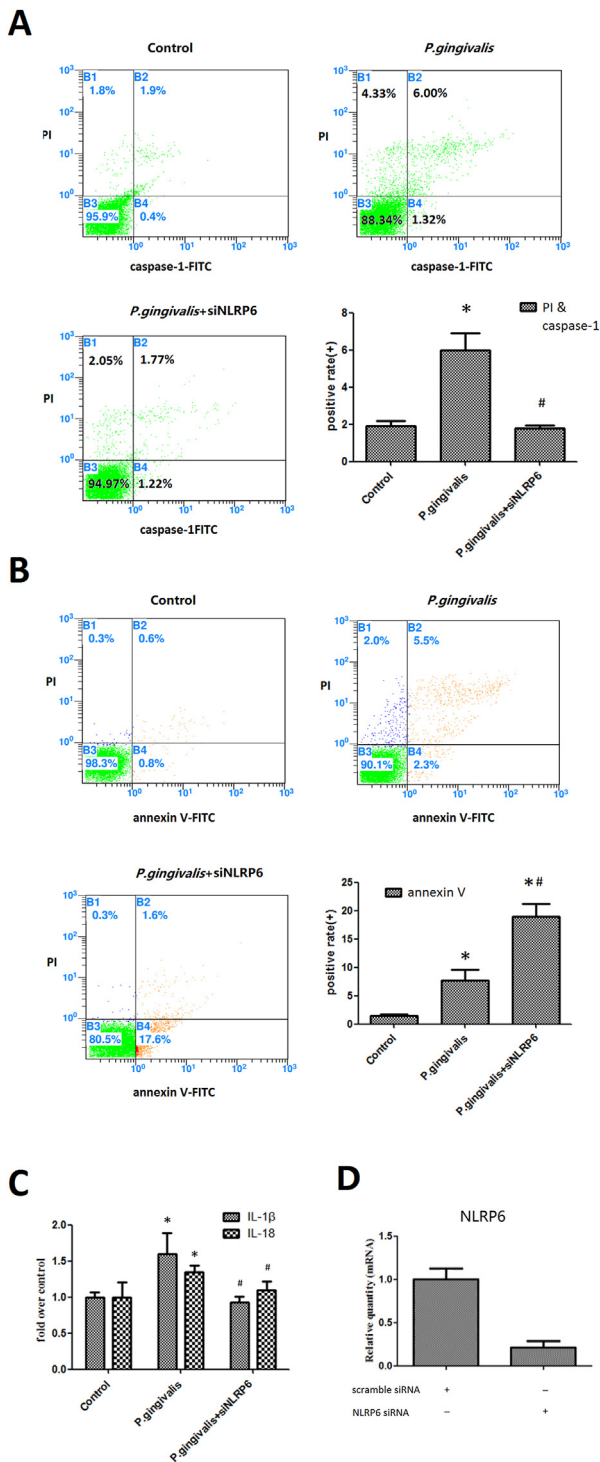


Figure 4 Role of NLRP6 in HaCaT during *P.g* infection. HaCaT cells were infected by *P.g* with NLRP6 or scramble siRNA transfection. Scramble siRNA alone transfection were treated as a control group. (A), Pyroptosis rates (caspase-1 and PI staining) detection by flow cytometry. (B), Apoptosis rates (annexin V and PI staining) detection by flow cytometry. (C), IL-1 β and IL-18 detection by ELISA. *: $P < 0.05$ compared with control group. #: $P < 0.05$ compared with *P. gingivalis* group.

Declaration of competing interest

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

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