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ROLE of NLR PROTEINS NLRP3 AND NLRX1 in ORAL BACTERIA INFECTED GINGIVAL EPITHELIAL CELLS

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Quantitative and Systems Biology

by

SHU-CHEN HUNG

Dissertation Committee:
Professor Wei-Chun Chin, Chair
Professor Kirk Jensen
Professor Masashi Kitazawa (UC Irvine)
Professor David M Ojcius (UOP Arthur A. Dugoni School of Dentistry)

2016

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The Dissertation of Shu-Chen Hung is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Dr. Wei-Chun Chin, Chair

University of California, Merced
2016

Dedication

This thesis is dedicated to my parents, sisters and loved ones. Their support and encouragement have made my path pursuing PhD possible.

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List of Abbreviations

ADP: Adenosine diphosphate

ASC: Apoptosis-associated speck-like protein containing a CARD

Atg5: Autophagy protein 5

Atg12: Autophagy protein 12

ATP: Adenosine triphosphate

BPE: Bovine pituitary extract

CARD: Caspase recruitment domain

CD4: Cluster of differentiation 4

CDC: Centers for disease control and prevention

DAMP: Damage-associated molecular patterns

DPI: Diphenyleneiodonium chloride

eATP: Extracellular adenosine triphosphate

FBS: Fetal bovine serum

GEC: Gingival epithelial cell

HBSS: Hanks' balanced salt solution

HIGK: Human immortalized gingival keratinocyte

HMGB1: High mobility group box 1

ICAM: Intracellular adhesion molecule

IgG: Immunoglobulin G

IL: Interleukin

Kgp: Lysine gingiapin

LRR: Leucine-rich repeat

MAPK: Mitogen-activated protein kinases

M.O.I.: Multiplicity of infection

mtDNA: Mitochondrial DNA

mROS: Mitochondrial ROS

NADPH: Nicotinamide adenine dinucleotide phosphate

NF-κB: Nuclear factor-κB

NK: Natural killer

NLR: NOD-like receptor

NLRC: NLR family CARD domain-containing protein

NLRP3: NLR Family, Pyrin Domain Containing 3

NLRX1: NLR family member X1

NOD: Nucleotide-binding oligomerization domain-containing protein

OCP: Osteoclast precursor

OPG: Osteoprotegrin

PAMP: Pathogen-associated molecular patterns

PBS: Phosphate-buffered saline

PRR: Pattern-recognition receptor

RANK: Receptor activator of NF-κB

RANKL: RANK-ligand

RgpA: Arginine gingiapin A

RgpB: Arginine gingiapin B

SCI: Spinal cord injury

SerB: Phosphoserine phosphatase

SFM: Serum free medium

siRNA: Small interfering RNA

shRNA: short hairpin RNA

T5SS: Type V secretion system

T9SS: Type IX Secretion System

TLR: Toll-like receptor

TMS1: Methylation-induced silencing-1

TNF: Tumor necrosis factor

TRAF6: TNF receptor-associated factor 6

TSB: Trypticase soy broth

TUFM: Tu translation elongation factor

TXN: Thioredoxin

TXNIP: Thioredoxin interaction protein

UTP: Uridine-5'-triphosphate

UQCRC2: Ubiquinol-cytochrome C reductase core protein II

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I would like to thank the chair of my thesis committee, Dr. Wei-Chun Chin, who has mentored and given me guidance since my first year. Also, his support enables me to complete experiments in my thesis. I would also like to thank other members in my thesis committee. Dr. Masashi Kitazawa and Dr. Kirk Jensen's always give me best advice for troubleshooting and they also encourage me to implement new techniques in my projects.

Finally, I would also like to thank the financial support from UC Merced, University of the Pacific Arthur A. Dugoni School of Dentistry, and NIH for providing financial support as well as the support from my lab mates and co-workers at UC Merced and Dugoni School of Dentistry. Their support and assistance have made my thesis possible.

Curriculum Vitae

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EDUCATION

University of California at Merced - *Ph.D.*

AUG 2011 - AUG 2016, Merced, CA

Ph.D. in Quantitative and Systems Biology (QSB)

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AUG 2008 - JUL 2009, Tao-Yuan, Taiwan

M.S. in Molecular and Cellular Biology

Chang Gung University - *B.S.*

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B.S. in Life Science

EXPERIENCE

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- Uncover liposome induced NLRP3 inflammatory responses.
- Determine the role of calcifying nanoparticles (CNPs) in endodontics, and propose an R21 grant.
- Manage relocation and set up lab instruments from UC Merced to Dugoni School of Dentistry.

University of California at Merced, Merced - *Ph.D. candidate in Dr. David Ojcius' Lab*

AUG 2011 - AUG 2016

- Revealed the association of $P2X_4$ with $P2X_7$ and pannexin-1 and their roles in ATP-induced NLRP3 inflammasome activation.
- Discovered positive regulation of NLRX1 in NLRP3 inflammasome activation in ATP-treated gingival epithelial cells (GECs).
- Uncovered NLRX1 as a negative regulator in TLR pathway in *Fusobacterium nucleatum*-infected GECs.
- Discovered the mechanism of CNPs or *F. nucleatum* infection triggered-NLRP3 inflammasome activation and TLR pathway.

- Examined the role of *F. nucleatum* infection induced NLRP3 activation in lipidomic (collaboration with Dr. Fabian Fillip).
- Discovered the role of NDK from *Porphyromonas gingivalis* on ATP-mediated inflammatory responses.

University of California at Merced, Merced - *Teaching Assistant in School of Natural Sciences*

AUG 2013 - MAY 2015

- Introduction to Molecular Biology Lab, Spring 2012, Spring 2015
- Molecular Immunology Lab, Spring 2013
- Contemporary Biology, Fall 2013

Chang Gung University, Taiwan - *Visiting Scholar in Dr. John D. Young's Lab*

MAY 2014 - AUG 2014

- Characterize CNPs induced-NLRP3 inflammasome activation
- Determine the effects of CNPs sizes on *F. nucleatum* infection efficiency in GECs .

Chang Gung University, Taiwan - *Research Associate in Dr. Tzu-Chien Van Wang's Lab*

AUG 2009 - JUL 2011

- Uncovered the role of hnRNP A3 in telomere biogenesis.
- Examined the mechanism by which anti-cancer drug N-(1-pyrenyl) maleimide induced apoptosis in hematopoietic cancer cells.

Chang Gung University, Taiwan - *Master student in Dr. Tzu-Chien Van Wang's Lab*

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- Optimized the expression and purification of recombinant hnRNP A3.
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Publications (*: equal contribution)

1. **S.C. Hung**, P.R. Huang, P.Q. Bui, D.M. Ojcius (2016). NLRX1 modulates the balance between NLRP3 inflammasome activation and NF-kB signaling during Fusobacterium nucleatum infection. Cellular Microbiology, **In Press.**

2. P.Q. Bui, L. Johnson, J. Roberts, **S.C. Hung**, J. Lee, K.R. Atanasova, P.R. Huang, O. Yilmaz, & D.M. Ojcius (2015). Fusobacterium nucleatum Infection of Gingival Epithelial Cells Leads to NLRP3 Inflammasome-Dependent Secretion of IL-1b and the Danger Signals ASC and HMGB1. Cellular Microbiology, In Print.

3. L. Johnson, K.R. Atanasova, P.Q. Bui, J. Lee, **S.C. Hung**, O. Yilmaz, D.M. Ojcius, Porphyromonas gingivalis attenuates ATP-mediated inflammasome activation and HMGB1 release through expression of a nucleoside-diphosphate kinase, Microbes Infect, 17 (2015) 369-377.

4. P.R. Huang, **S.C. Hung**, C.C. Pao, T.C. Wang, N-(1-pyrenyl) maleimide induces bak oligomerization and mitochondrial dysfunction in jurkat cells, Biomed Res Int, 2015 (2015) 798489.

5. **S.C. Hung***, C.H. Choi*, N. Said-Sadier*, L. Johnson, K.R. Atanasova, H. Sellami, O. Yilmaz, D.M. Ojcius, P2X4 assembles with P2X7 and pannexin-1 in gingival epithelial cells and modulates ATP-induced reactive oxygen species production and inflammasome activation, PLoS One, 8 (2013) e70210.

6. P.R. Huang*, **S.C. Hung***, T.C. Wang, Telomeric DNA-binding activities of heterogeneous nuclear ribonucleoprotein A3 in vitro and in vivo, Biochim Biophys Acta, 1803 (2010) 1164-1174.

SKILLS

Human cancer cell / immortalized epithelial cell culture
Anaerobic bacteria culture
Gene Knockdown (siRNA, shRNA)
Lentivirus Package and transduction
RNA isolation / Reverse transcription
PCR / Real-Time PCR / qPCR
Site-Directed Mutagenesis
Immunofluorescence Staining
Confocal Microscopy
Electrophoretic Mobility Shift Assay (EMSA)
Telomeric Repeat Amplification Protocol (TRAP)
Terminal Restriction Fragment (TRF) assay
Recombinant Protein Purification
Protein Dialysis
Immunoblotting / Co-immunoprecipitation
ELISA
2D Gel electrophoresis
Coomassie Blue / Silver staining
LC / MS / MS
Flow Cytometry
Biacore

Peer Reviewership

Journal Title: African Journal of Biotechnology (0.57)

Journal Title: PLOS ONE (3.234)

Journal Title: International Journal of Modern Physics B (0.455)

Presentation

1. June 20, 2014 *The Role of NLR and Inflammasome activation mechanism in Oral Bacteria Infected Gingival Epithelial Cells Predisposition*. Journal Club of the Center for Molecular and Clinical Immunology, Chang Gung University, Tao-Yuan, Taiwan (invited talk)
2. March 16, 2013 *$P2X_4$ Assembles with $P2X_7$ and Pannexin-1 in Gingival Epithelial Cells and Modulates ATP-induced Reactive Oxygen Species Production and Inflammasome Activation*. XVIth Bay Area Microbial Pathogenesis Symposium, San Francisco, CA (Poster)

3. May 14, 2016 *NLRX1 modulates the balance between NLRP3 inflammasome activation and NF-kB signaling during Fusobacterium nucleatum infection*. AAI Annual Meeting, Seattle, WA (Poster)

Abstract

ROLE of NLR PROTEINS NLRP3 AND NLRX1 in ORAL BACTERIA INFECTED GINGIVAL EPITHELIAL CELLS

By

Shu-Chen Hung

Doctor of Philosophy in Quantitative and Systems Biology

University of California, Merced 2016

Professor Wei-Chun Chin, Chair

Periodontitis, a chronic inflammatory disease results from both dysbiotic microbiota and dysregulated innate immune responses in the periodontal tissue, can affect up to 90% of the population in industrialized countries and developing countries globally. However, how dysbiosis triggers dysregulated inflammatory responses remains unclear. Many NOD-like receptors (NLRs) have been shown to contribute to the dysregulated inflammation in periodontitis. Thus, we investigated the role of NLRs in periodontitis in molecular level.

NLRs play an important role in regulation of host innate immunity, yet their role in periodontitis remains to be defined. NLRP3, one of the well-studied NLR family members, can form a multiprotein complex called inflammasome and has been shown to be one of the dysregulated inflammatory responses in periodontitis. We have previously reported that infection of gingival epithelial cells (GECs) with periopathogen *Porphyromonas gingivalis,* keystone pathogens for periodontitis, requires an exogenous danger signal such as extracellular ATP (eATP) to activate NLRP3 inflammasome and caspase-1, thereby inducing secretion of interleukin (IL)-1β. Stimulation with eATP also stimulates production of reactive oxygen species (ROS) in GECs. However, the mechanism by which ROS is generated in response to eATP, and the role that different purinergic receptors may play in inflammasome activation, is still unclear. In this study, we began with revealing that the purinergic receptor $P2X_4$ is assembled with the receptor $P2X_7$ and its associated pore, pannexin-1. eATP induces ROS production through a complex consisting of the $P2X_4$, $P2X_7$, and pannexin-1. $P2X_7$−mediated ROS production can activate the NLRP3 inflammasome and caspase-1 by oxidizing endogenous ligands for NLRP3. Furthermore, separate depletion or inhibition of $P2X_4$, $P2X_7$, or pannexin-1 complex blocks IL-1β secretion in *P. gingivalis*-infected GECs following eATP treatment. However, activation via $P2X_4$ alone induces ROS generation but not inflammasome activation. These results suggest that ROS is generated through stimulation of a $P2X_4$/$P2X_7$/pannexin-1 complex, and reveal an unexpected role for $P2X_4$, which acts as a positive regulator of inflammasome activation during microbial infection.

NLRX1, a new member of the NLR family that localizes to mitochondria, has been shown to modulate mitochondrial ROS (mROS) generation. mROS activates the NLRP3 inflammasome upon eATP stimulation or microbial infection, yet the role of

NLRX1 in NLRP3 inflammasome activation has not been examined. In this study, we further revealed the mechanism by which NLRX1 positively regulates eATP-elicited NLRP3 inflammasome activation through mROS in GECs. Fluorescence microscopy showed that depletion of NLRX1 by shRNA attenuates eATP-induced mROS generation and redistribution of the NLRP3 inflammasome adaptor protein, ASC. Furthermore, we have shown that another periopathogen *Fusobacterium nucleatum* infection activates NLRP3 inflammasome in GECs. Depletion of NLRX1 inhibits *F. nucleatum* infection-activated caspase-1, suggesting that it also inhibits the NLRP3 inflammasome. Therefore, we propose that NLRX1 may promote *F. nucleatum*-caused dysregulated pro-inflammatory responses in periodontitis. On the other hand, the mechanism by which *F. nucleatum* infection-induces IL-8 expression is still unclear. We showed that NLRX1 also acts as a negative regulator in NF-κB signaling to modulate IL-8 expression. Thus, NLRX1 stimulates detection of the pathogen *F. nucleatum* via the inflammasome, while dampening cytokine production. We expect that commensals should not activate the inflammasome, but NLRX1 should still decrease their ability to stimulate inflammation (to prevent hyper-regulated cytokine expression). Consequently, we conclude that NLRX1 may act as a potential switch in regards to the virulence of *F. nucleatum* in healthy or diseased oral cavity.

CHAPTER 1

Introduction

1.1 Periodontitis

Periodontitis, a chronic inflammatory disease results from both dysbiotic microbiota (altered microbiota) and dysregulated innate immune responses in the periodontal tissue, can affect up to 90% of the population in industrialized countries and developing countries globally (1). In the US, one out of every two adults aged over 29 have different degrees of periodontitis according to CDC, which shows the prevalence and importance of the diseases. In oral cavity, gingival epithelial cells (GECs) are among the first host cells invaded by pathogenic bacteria and lead to reversible inflammation of the gingiva called gingivitis (2-4). Untreated gingivitis will progress to periodontitis that have irreversible destruction of tooth-supporting tissues and alveolar bone, leading to bone loss (1,5). In addition to irreversible periodontium damages, periodontitis has also been shown to correlate with many systemic diseases such as heart disease, atherosclerosis, diabetes, and preterm birth (6-12). However, the aetiology of periodontitis remains unclear.

Our oral cavities encounter at least 100 billion microbes every day. Healthy periodontium can initiate adequate inflammatory responses to eradicate microbial infections and prevent periodontitis occurance. Healthy periodontium expresses inflammatory mediators such as IL-8, intracellular adhesion molecules (ICAMs) and E-selectin to defend against the constant challenge of dental plaque consisting of a polymicrobial biofilm community adjacent to the teeth generated on a daily basis (13). E-selectin expression in the endothelium facilitates neutrophil extravasation to the epithelium. IL-8, which acts as a neutrophil chemotactic factor, as well as ICAMs have been reported to gradually increase toward areas exposed to biofilm. The IL-8 and ICAMs gradients facilitate the locating of biofilm by neutrophil and the building of a wall between dental plaque and host epithelial cells (14).

However, in diseased periodontal tissue, the biofilm community alters from mainly gram-positive to gram-negative bacteria (dysbiosis). The tooth surface can be easily colonized by gram-positive commensals like, streptococci, via recognition of receptors of salivary pellicle. But the rising of the number of *Fusobacterium nucleatum* is important in the biofilm community transition due to its ability to coaggregate with various microbes, allowing colonization of gram-negative pathogens, such as “red complex.” Red complex primarily composed of *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia* that are always found together at periodontal sites and is highly correlated with periodontitis (15-17). Among the red complex bacteria, *P. gingivalis* has been reported to disrupt the established defense mechanism like IL-8 gradient to build milieu for other pathogens and promote disease progression by its structure and protease (14,18-20). Moreover, dysbiosis also results in enhanced expression of other inflammatory mediators such as IL-1β and TNF, which induces RANKL (receptor-activator of nuclear factor-κB ligand) and causes osteolysis (21). Although much is known about the symptoms of periodontitis induced by dental plaque, the mechanism of dysregulated inflammatory

responses in host upon infection remains poorly understood. Thus, we intended to reveal the host's dysregulated inflammatory responses using *F. nucleatum* or *P. gingivalis* infected GECs as a model for periodontitis.

1.2 *Porphyromonas gingivalis*

Porphyromonas gingivalis (named *Bacteroides gingivalis* before reclassification to genus, *Porphyromonas*) is an obligate anaerobic Gram-negative bacillus under genus *Porphyromonas* that belongs to the family *Bacteroidaceae*. The name of *Porphyromona* comes from Greek *porphyreos* (purple) and *monas* (unit) due to its characteristic of forming black-pigmented colonies on blood agar (22). *P. gingivalis* is non-motile, non-sporeforming, asaccharolytic, and requires hemin (iron) and vitamin K for growth (23).

P. gingivalis is one of the red complex periopathogens commonly found in advanced periodontitis. It has been hypothesized to be the keystone pathogen that dampens normal host-microbiota homeostasis and lead to dysbiosis in periodontitis due to its ability to produce various virulence factors (24). *P. gingivalis* secrets serine phosphatase enzyme, SerB, to internalize epithelial cells by modulating actin depolymerizing factor cofilin (25). SerB also can dephosphorylate p65 of NF-κB to down-regulate IL-8 (26). *P. gingivalis* also secretes virulence cysteine proteases known as gingiapins (RgpA, RgpB, and Kgp) possibly through Type IX Secretion System (T9SS) (27,28). *P. gingivalis* utilizes gingipains to degrade cytokine and complements to suppress host immune system and promote dysbiosis (29). Besides modulating host innate immunity, *P. gingivalis* may also corrupt host adaptive immunity by regulating NLRP3 inflammasome (30,31). NLRP3 inflammasome activates caspase-1 to process IL-1β maturation, which enhances $CD4^+$ T cells proliferation (18,32-35). However, the detailed mechanism by which *P. gingivalis* modulates NLRP3 inflammasome in periodontitis remains unclear.

1.3 *Fusobacterium nucleatum*

Like *P. gingivalis, Fusobacterium nucleatum* belongs to family *Bacteroidaceae*, but is under genus *Fusobacterium*. The name of *Fusobacterium* originates from Greek *fusus* (spindle) and *bacterion* (rod) owing to its morphology. *F. nucleatum* is a non-motile, non-sporeforming, asaccharolytic, Gram-negative anaerobe that can survive in the presence of 6% oxygen (36,37).

F. nucleatum is the most abundant micro-organism in the oral cavity that has been linked to periodontitis (37). The severity of periodontitis is positively associated with *F. nucleatum* numerically. Its ability to co-aggregate with diverse microbes makes it a bridge connecting early (commensals) and late (pathogens) colonizers, which leads to dental plaque formation (38). Due to its presence in healthy gum, *F. nucleatum* was considered as commensal until recent studies revealed its pathogenicity. It has been shown that *F. nucleatum* exacerbates periodontitis through modulating host inflammatory responses triggered by other pathogens. It synergistically enhances other pathogens-induced cytokines expression, such as Il-1β, IL-6, β-defensin-2 (39-41). Besides, *F. nucleatum* can induce human lymphocytes aggregation and cell death via type Va secretion systems (T5SSs) as well as stimulate natural killer (NK) cells to secrete TNF-α, which may be important for *F. nucleatum*-

triggered bone loss in mice model (39,42-44). Recent studies showed that *F. nucleatum* infection activates NLRP3 inflammasome in human monocytes and GECs (18,45). However, the detailed mechanism by which NLRX1 modulates *F. nucleatum*-triggered NLRP3 inflammasome activationin healthy and diseased periodontium remains unclear.

1.4 Host innate immune responses

Periodontitis is the outcome of dysregulated host inflammatory responses caused destruction of the periodontium that ultimately leads to bone loss. The mechanism of regulating bone resorption and deposition depend on the ratio of receptor–activator of nuclear factor-κB ligand (RANKL) and osteoprotegrin (OPG) (46,47). RANKL binds to RANK on osteoclast precursor (OCP) to promote its maturation to degrade bones, whereas OPG blocks the interaction between RANKL and RANK to preclude osteoclasts. The expression of RANKL is regulated by inflammatory mediators including IL-6, IL-1β and tumor necrosis factor (TNF) (48,49). Therefore, bacterial infection caused hyper-regulated inflammatory cytokines could influence osteoclasts though augmenting the RANKL/OPG ratio (50). However, the host's initial innate immune responses to trigger expression of inflammatory mediators upon microbes challenging remain concealed. GECs, the first barrier in oral cavity, have been shown to play a role more than just mechanical defense. Upon encountering microbes, GECs secret inflammatory mediators to recruit leukocytes for defense in healthy periodontium (30,51). However, in diseased periodontium, periodontopathogens infection of GECs lead to local chemokine paralysis that results in bone loss (20). Thus, understanding the inflammatory responses of GECs upon commensals and pathogens will illuminate the etiology of periodontitis. GECs express a wide spectrum of pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs) and Nod-like receptors (NLRs), to recognize conserved pathogen-derived components that are known as pathogen-associated molecular patterns (PAMPs) or host-released factors by damaged or dying cells called damage-associated molecular patterns (DAMPs) (52-56). Among NLRs, some of them can form inflammasomes, which modulate proinflammatory cytokine IL-1β maturation that are associated with periodontitis. Therefore, we intend to investigate the role of NLR, especially NLRP3, in commensal and pathogens-triggered inflammatory responses in GECs.

NLRs typically are composed of a central conserved NOD domain, a C-terminal leucine-rich repeat (LRR), and a N-terminal effector domain (57). By the N-terminal effector domain, NLRs can be subclassifed into different groups, e.g. NLRP, NLRC, and NLRX1 (58). Each of NLRs plays a non-redundant role in recognizing specific PAMPs or DAMPs, but the responses they trigger can be categorized into inflammasome dependent or independent. For example, NOD1, NOD2 and NLRX1 induce pro-inflammatory responses through NF-κB or MAPK signaling upon microbial infections. On the other hand, NLRP1, NLRP3 and NLRC4 can form inflammasomes to activate caspase-1 upon sensing PAMPs and DAMPs (58,59).

Inflammasomes are large multiprotein complexes that act as a caspase-1-activating platform for IL-1β and IL-18 maturation. They can be categorized by the composition of their integral PRR family member, which acts as a scaffold protein

that contributes to caspase-1 recruitment, clustering, and auto-activation (60-62). The best-characterized inflammasome is the NLRP3 inflammasome. It contains NLRP3 as a scaffold protein, an apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and caspase-1 (63).

Several mechanisms have been proposed to induce NLRP3 inflammasome activation, including reactive oxygen species (ROS) production, lysosomal destabilization, K^+ efflux, and apoptosis (64-67). In particular, eATP stimulation of cells has been shown to induce caspase-1 activation following ROS production, and treatment with the $P2X_7$ antagonist, oxATP, attenuates eATP-induced ROS generation (68-73). In addition to $P2X_7$ agonists, agonists of other purinergic receptors also promote ROS generation, implying that other purinergic receptors may also contribute to eATP-induced ROS production (74-76). It has been shown that knockdown of $P2X_4$ inhibits ATP-triggered NLRP3 inflammasome activation in kidney epithelium upon high glucose stimulation (77). However, until now, no other purinergic receptor has been implicated in eATP-induced activation of the NLRP3 inflammasome other than $P2X_7$ in human GECs.

P2X purinergic receptors are cell surface ligand-gated ion channels for eATP that are expressed in almost all cell types (78). So far, seven P2X receptor subunits ($P2X_{1-7}$) have been identified in mammals. Each subunit is composed of two transmembrane domains separated by an extracellular loop domain (ectodomain) and is either heterotrimer or homotrimer (79). $P2X_4$, shares highest amino acid identity with $P2X_7$ among P2X receptors, is usually co-expressed with $P2X_7$ and has higher affinity to ATP in comparison to $P2X_7$ (79,80). Recently, emerging evidence show the involvement of $P2X_4$ in $P2X_7$-triggered signaling pathways and its association with $P2X_7$. Knockdown or express mutant $P2X_4$ attenuated $P2X_7$ channel-mediated current and $P2X_7$ agonist BzATP-induced cell death (81,82). Furthermore, knockout of $P2X_4$ reduced spinal cord injury (SCI) triggered capase-1 activation and IL-1β maturation in neurons, suggesting $P2X_4$ is involved in inflammasome activation (83,84). Although $P2X_4$ is also an ATP-gated ion channel, it has not been previously described to participate in eATP-mediated caspase-1 activation in GECs. Therefore, we investigate whether $P2X_4$ is involved in $P2X_7$-triggered NLRP3 inflammasome activation in GECs.

Cells stimulated with PAMPs or DAMPs, including eATP, elicit ROS production, a molecule that oxidizes ligands for NLRP3 recognition and activation (62). It has been shown that treatment of H_2O_2 alone leads to IL-1β secretion through NLRP3 inflammasome activation in THP1 cells. Further analysis showed that thioredoxin interacting protein (TXNIP) dissociates from thioredoxin (TRX) and interacts with NLRP3 as ROS is generated upon NLRP3 agonist stimulation, suggesting that released TXNIP may serve as a NLRP3 ligand when TRX is oxidized by ROS (70). In addition, recently, other studies have shown that mitochondrial damages, including mitochondrial ROS (mROS) and mitochondrial membrane potential ($\Delta\psi m$), play an important role in NLRP3 activation (71). Shimada et al. (2012) show that eATP elicited release of cytosolic oxidized mitochondrial DNA (mtDNA) that can be sensed by NLRP3 (85). However, the detailed mechanisms by which mitochondrial damages are induced to activate NLRP3 inflammasome are not well characterized.

NLR family member X1 (NLRX1), the only NLR identified to localize in mitochondria so far (86,87). Due to its localization, its inflammatory role has been linked to mitochondria, which is involved in multiple inflammatory responses, such as autophagy and inflammasome. Proteomic analysis has identified the association between NLRX1 and mitochondrial protein TUFM (Tu translation elongation factor), which interacts with autophagy-related proteins Atg5-Atg12. The indirect association between NLRX1 and Atg5-Atg12 is necessary to induce autophagy upon viral infection in mice (88,89). In addition, NLRX1 has been shown to modulate mROS upon infection possibly through interaction with UQCRC2, a subunit in mitochondrial respiratory chain complex III (90). Overexpression of NLRX1 enhanced ROS generation in response to TNF-α, poly(I:C), *Shigella* and *Chlamydial* infection, whereas knockdown of NLRX1 abrogated pathogens-induced mROS generation (91-93). Recently it has been shown that mROS activate NLRP3 inflammasome (85,94-96). Therefore, we have investigated whether NLRX1 modulates NLRP3 inflammasome via mROS.

In addition to the role in mitochondria, NLRX1 has also been reported to be involved in TLR signaling (97). Depletion of NLRX1 augments expression of LPS-induced NF-κB-responsive genes, such as IL-1β and IL-6 in mice (91,98,99). Immunoprecipitation shows constitutive binding between NLRX1 and TLR downstream signaling molecule, TRAF, in unstimulated conditions. However, upon LPS stimulation, NLRX1 dissociates from TRAF6 to IKK complex to free and activate NF-κB (98). All together, it shows that NLRX1 acts as a negative regulator in TLR signaling. Therefore, we explored the role of NLRX1 in NF-κB signaling in *F. nucleatum*-infected GECs.

CHAPTER 2

Materials and Methods

2.1 Cell culture and oral bacterial infections

2.1.1 Media, enzymes, oligonucleotides, and antibodies.

Defined Keratinocytes-serum free medium (SFM), Bovine Pituitary Extract (BPE), fetal bovine serum (FBS), TRIzol reagent, CM-H_2−DCFDA DCF (D3999), MitoSOX^{TM} Red Mitochondrial Superoxide Indicator (M36008), JC-1 dye (T3168) and all oligonucleotides were from Thermo Fisher Scientific. ATP, ADP, UTP, oxATP, PPADS, probenecid, puromycin, polybrene, hemin, and menadione were from Sigma-Aldrich. Antibodies against NLRX1 (8583), NF-kB RelA (8242) were from Cell Signaling. Anti-TMS1 (ab155970 for Immunoblotting; ab64808 for microscopy) and cleaved caspase-1 p10 (ab108326) were purchased from Abcam. Anti-actin (MAB1501) and cleaved caspase-1 p20 (06503) were obtained from Millipore. Anti TOPO-I (sc-5342) was purchased from Santa Cruz. Gel electrophoresis reagents were from Bio-Rad. Anti-$P2X_4$ (APR-002) and $P2X_7$ (APR-008) were obtained from Alomone Labs.

2.1.2 Cells and bacterial Culture

The human immortalized gingival epithelial cells (GECs) were obtained as previously described (95). Cells were routinely cultured in defined keratinocyte-SFM with 5 ug/ml plasmocin and grown at 37 °C in a humidified incubator containing 5% CO_2.

Porphyromonas gingivalis ATCC 33277 was cultured anaerobically for 24 h at 37°C in trypticase soy broth (TSB) supplemented with yeast extract (1 mg/ml), hemin (5 μg/ml) and menadione (1 μg/ml) and used for infection as described (76).

Fusobacterium nucleatum (ATCC 25586) was cultured anaerobically for 24 h at 37°C in brain-heart infusion broth supplemented with yeast extract (5 mg/ml), hemin (5 μg/ml), and menadione (1 μg/ml) and used for infection as previously described (100).

2.2 Molecular biology methods

2.2.1 Lentivirus infection and selection of shRNA-expressing Cells

GECs stably expressing shRNA against NLRX1, $P2X_4$, $P2X_7$, and pannexin-1 were generated by transducing the cells with lentiviral particles purchased from Sigma-Aldrich. The vectors encoding each shRNA sequence were:

Table 1. List of shRNA

Gene	TRC Number	Clone ID
$P2X_4$	TRCN0000044960	NM_002560.2-949s1c1
	TRCN0000044962	NM_002560.2-563s1c1
$P2X_7$:	TRCN0000045095	NM_002562.4-1149s1c1
	TRCN0000045097	NM_002562.4-843s1c1
Pannexin-1:	TRCN0000156046	NM_015368.3-866s1c1
	TRCN0000155348	NM_015368.3-1065s1c1
NLRX1:	TRCN0000129459	NM_024618.2-1992s1c1
	TRCN0000130268	NM_024618.2-3523s1c1
	TRCN0000218246	NM_024618.2-1414s21c1
	TRCN0000130325	NM_024618.2-3335s1c1
	TRCN0000128446	NM_024618.2-3337s1c1

Transduction was carried out following the manufacturer's instructions in the presence of 8 μg/ml polybrene. Nontarget shRNA control cells were also generated using an irrelevant sequence (SHC002V, Sigma). Briefly, GECs were plated at 70% confluency 24 h prior to transduction, and the corresponding lentiviral transduction particles were added at M.O.I. of 3 overnight. Fresh media was added the next day, and stably infected cells were selected by addition of media containing 0.6 μg/ml puromycin for 1 week.

2.2.2 Transient RNA depletion with SiRNA in Primary GECs

Expression of $P2X_4$ and $P2X_7$ in primary GECs were repressed with different siRNA sequences as previously described (92). The siRNA sequences were: 5′-GCUUUCAACGGGUCUGUCATT-3′ and 5′-UGACAGACCCGUUGAAAGCTA-3′ for $P2X_4$ (Ambion, LifeCell Technologies, S9957, Cat. #: 4392420); and 5′-ACAAUGUUGAGAAACGGACUCUGAT-3′ for $P2X_7$ (27 mer siRNA duplexes OriGene Technologies, Cat. #: SR303325). Briefly, cells were treated with siRNA using Glycofect Transfection Reagent (Kerafast) mixed with 10 nM siRNA (stock concentration of siRNA was 20 nM) in a total volume of 100 μl. Four hours later, new cell-medium was added to the cells without removal of the transfection mixture, and cells were incubated for 36 hours.

2.2.3 Measurement of mitochondrial membrane potential and ROS

Cells were stained with the cationic carbocyanine dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) or CM-H_2−DCFDA DCF or MitoSOX mitochondrial superoxide dye as described previously (95,101). In brief, cells were loaded with 7.7 μM JC-1 or 2.5 μM DCF or 5 μM MitoSOX in HBSS for 10 min or in PBS for 30 min at 37°C, washed with HBSS, and treated with 5 mM ATP for indicated time at 37°C followed by 3 washes with HBSS. Finally, the cells were observed under wide-field fluorescence microscope (Leica, Deerfield, IL). Quantitative analysis was performed using Image J software from NIH.

2.2.4 RNA extraction, reverse transcription, PCR and Quantitative PCR

Total RNA was isolated from GECs after indicated treatments using TRIzol reagent according to the manufacture's protocol. Total RNA was reversed transcribed to cDNA using Taqman Reverse Transcription Reagents kit (Applied Biosystem) followed by PCR or qPCR as previously described (95). The following primers were used:

Table 2. List or Primers

Gene		Sequence (5' to 3')
$P2X_1$	Forward	CGCCTTCCTCTTCGAGTATGA
	Reverse	AGATAACGCCCACCTTCTTATTACG
$P2X_2$	Forward	GCCTACGGGATCCGCATT
	Reverse	TGGTGGGAATCAGGCTGAAC
$P2X_3$	Forward	GCTGGACCATCGGGATCA
	Reverse	GAAAACCCACCCTACAAAGTAGGA
$P2X_4$	Forward	CCTCTGCTTGCCCAGGTACTC
	Reverse	CCAGGAGATACGTTGTGCTCAA
$P2X_5$	Forward	CTGCCTGTCGCTGTTCGA
	Reverse	GCAGGCCCACCTTCTTGTT
$P2X_6$	Forward	AGGCCAGTGTGTGGTGTTCA
	Reverse	TCTCCACTGGGCACCAACTC
$P2X_7$	Forward	TCTTCGTGATGACAAACTTTCTCAA
	Reverse	GTCCTGCGGGTGGGATACT
Pannexin1	Forward	GGTGAGACAAGACCCAGAGC
	Reverse	GGCATCGGACCTTACACCTA
NLRX1	Forward	GAAACCGCAGATCTCACCAT
	Reverse	TTGTAGTGACCCGGAGGAAC
IL-8	Forward	AATCTGGCAACCCTAGTCTGCTA
	Reverse	AGAAACCAAGGCACAGTGGAA
GAPDH	Forward	CTCTGCTCCTCCTGTTCGAC
	Reverse	TTAAAAGCAGCCCTGGTGAC
F. nucleatum 16S rRNA	Forward	CCGCGGTAATACGTATGTCACG
	Reverse	TCCGCTTACCTCTCCAGTACTC

2.3 Protein analysis

2.3.1 Immunofluorescence staining

For NF-κB staining, cells infected with *F. nucleatum* were washed with HBSS, fixed with 4% paraformaldehyde and permeabilized followed by 0.1% Triton-X for 40 min. Following one wash with PBST (PBS containing 0.05% Tween-20) and two washes with PBS, cells were blocked with 2% BSA in PBS for 30 min before hybridization with anti-NF-κB p65 antibody overnight. Cells were then washed with PBST once and PBS twice and incubated with goat anti-rabbit IgG antibody conjugated with red fluorescent Alexa Fluor 546 dye (Invitrogen) for 40 min. DAPI

was then used to stain nuclei for another 10 min followed by washed with PBST once and PBS twice and the fluorescence was visualized under wide-field fluorescence microscope (Leica) or laser scanning confocal microscope (Eclipse Ti C1, Nikon).

Nuclear/total NF-κB p65 ratio was quantified by CellProfiler and divided by the mean ratio of NF-κB p65 from corresponding uninfected samples. Number bigger than 1.2 is considered as NF-κB translocation. At least 300 cells were analyzed under each condition.

For ASC staining, the procedure is similar to NF-κB staining except the cells were permeabilized for 20 min and anti-ASC and goat anti-rabbit IgG (H+L) secondary antibody conjugated with Alexa Fluor 488 was used. Fluorescence intensity was analyzed using ImageJ. Mean fluorescence intensity of ASC was normalized to total fluorescence intensity of nuclei.

2.3.2 Co-Immunoprecipitation of purinergic receptors

Co-immunoprecipitation was performed with Dynabeads (Invitrogen) according to the manufacturer's instructions. Cells were lysed with the extraction buffer, and cell extracts were incubated for 3 h at 4°C with beads pre-coupled overnight with $P2X_4$ antibody. Precipitates were washed with extraction buffer and LWB with the use of a magnet and were subjected to 2X sample buffer and heated to 99°C for 10 min. The eluted proteins were analyzed by Western blot as previously described (102).

2.3.3 Nuclear/cytosol fractionation

To detect NF-κB protein levels in nuclear and cytosol, these extracts were prepared using Nuclear/Cytosol Fractionation kit from BioVision. Briefly, 2 x 10^6 uninfected or infected cells were collected and lysed with Cytosol Extraction Buffer-A (CEB-A) containing DTT and protease inhibitors. Following vigorously vortex and cold incubation on ice, CEB-B were added and mixed before centrifugation at 16,000 g at 4°C. The supernatant (cytoplasmic extract) was collected for further analysis, and the pellet was resuspended using Nuclear Extraction Buffer (NEB). The mixture was vortexed for 15s every 10 min for a total 40 min and finally centrifuged at 16,000 *g* for 10 min at 4°C. The supernatant (nuclear extract) was collected for future use.

2.3.4 Measurement of caspase-1 activation by ELISA

GECs were treated with 100 μM or 3 mM ATP for 3 h and supernatants were collected and subjected to human caspase-1 immunoassay (R&D) according to manufacturer's instructions. In brief, the caspase-1 ELISA uses monoclonal and polyclonal antibodies specific for the caspase-1 p20 subunit as capture and detection antibodies, respectively. One hundred μl of supernatant were first mixed with 50 μl of RD1W buffer and loaded onto caspase-1 monoclonal antibody coated-wells for 1.5 hrs. One hundred μl of caspase-1 antiserum was then used as detection antibodies. Anti-rabbit IgG-HRP conjugate was used for quantification. Activated caspase-1 was measured using a plate reader at 450 nm with wavelength correction at 540 nm.

2.3.5 Measurement of IL-1β secretion by ELISA

Secretion of IL-1β was measured using a commercial cytokine ELISA kit (BD Biosciences Pharmingen) as described (34).

2.3.6. Statistical analysis

All immunoblot and fluorescence microscopy data were quantitatively analyzed using ImageJ software or CellProfiler software. Statistics were carried out using two-way ANOVA with Bonferroni's *post hoc* test. For experiments examining one variable, t test was used for two groups and one-way ANOVA was carried our if there are more than two groups . A p value less than 0.05 was considered significant.

CHAPTER 3

3.1 The role of purinergic receptors in ATP- or *P. gingivalis*-treated GECs

3.1.1 ATP induces ROS generation in GECs

It has been shown that stimulation with eATP results in high levels of ROS generated in alveolar macrophages and primary GECs (68,103). In order to characterize eATP-induced ROS production in GECs, we used a stable GECs cell line, the human immortalized gingival keratinocyte cell line (HIGK) (104), stained with carboxy-H_2DCFDA (DCF), which remains nonfluorescent until its deacetylation and oxidation. Intracellular ATP concentration was shown to range from 0.5 mM to 5 mM, and 5 mM ATP has been reported to activate inflammasome in mice macrophages (105,106). Therefore, we treated cells with 3 or 5 mM ATP in our studies. Fluorescence microscopy images showed a significant increase of DCF fluorescence in 3 mM ATP stimulated HIGK cells (Figure 1A). Quantitative analysis of fluorescence microscopy data showed that the fluorescence in eATP-treated cells was about 9 times higher than in cells without treatment. Furthermore, the cells responded quickly to eATP stimulation within 5 minutes and reached a steady state from 30 minutes to at least 3 hours (Figure 1, B and C). Treatment with other extracellular nucleotides such as ADP, AMP, or UTP was unable to induce significant ROS generation in the cells (Figure 1C). These results suggest that stimulation with eATP, unlike other nucleotides, can induce ROS production in GECs.

3.1.2 Involvement of purinergic receptors in eATP-induced ROS generation

It has been proposed that eATP induces ROS production through ligation of the ATP-gated $P2X_7$ ion channel, in association with the pore-forming hemi-channel, pannexin-1, in macrophages and neurons (62,107,108). To investigate whether eATP-induced ROS production in GECs take place through $P2X_7$, we examined this unexpected result by stably depleting $P2X_4$, $P2X_7$ and pannexin-1 by lentiviral delivery of specific shRNA. Depletion efficiency in each cell line was validated individually by qPCR. As shown in Figure 2A, the mRNA levels of $P2X_4$, $P2X_7$, and pannexin-1 were reduced by at least ~70% in comparison to cells transduced with control shRNA virus particles. Depletion of $P2X_7$ or pannexin-1 resulted in attenuation of ROS production after eATP stimulation, compared to GECs transduced with control shRNA. Although depletion of $P2X_4$ by RNA interference was less efficient than for $P2X_7$, $P2X_4$ depletion resulted in a dramatic decrease in eATP-mediated ROS production (Figure 2, B and C). Collectively, these findings indicate that both $P2X_4$ and $P2X_7$ contribute to ROS generation after eATP treatment of GECs.

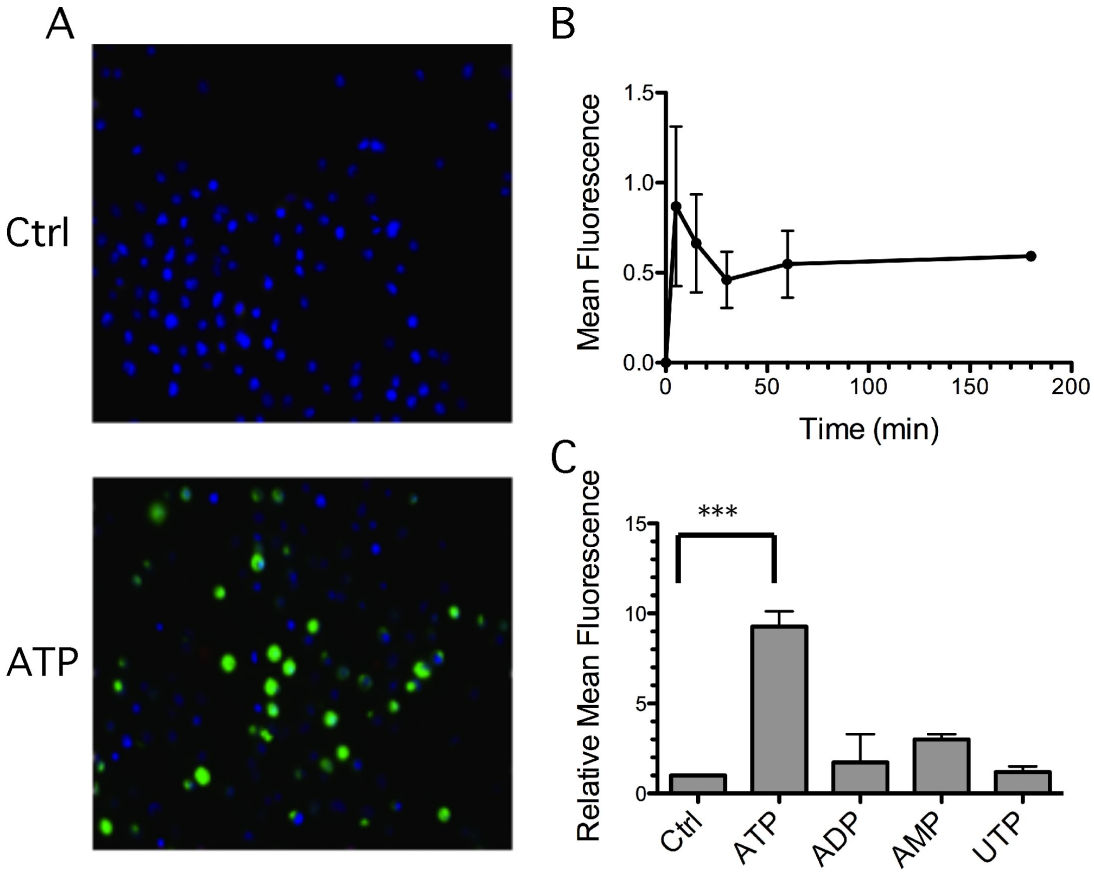


Figure 1. ROS production in response to ATP in GECs. (A) Immortalized GECs (HIGK) were treated for 1 hour with 3 mM ATP or left untreated as a control and stained with DCF (green) to detect ROS by fluorescence microscopy. Hoechst (blue) was used to stain nuclei. (B) ROS generation was measured with DCF at the indicated time points after 3 mM ATP stimulation. The mean fluorescence was quantified by image J and normalized for the number of cells. (C) Quantification of ROS production induced by different nucleotides. GECs were treated with 3 mM of ATP, ADP, AMP, or UTP, or left untreated for 1 hour, and analyzed by image J as in (B). The bars show the average values and SD of three independent experiments performed in duplicates, and *** $P < 0.05$ using one-way ANOVA.

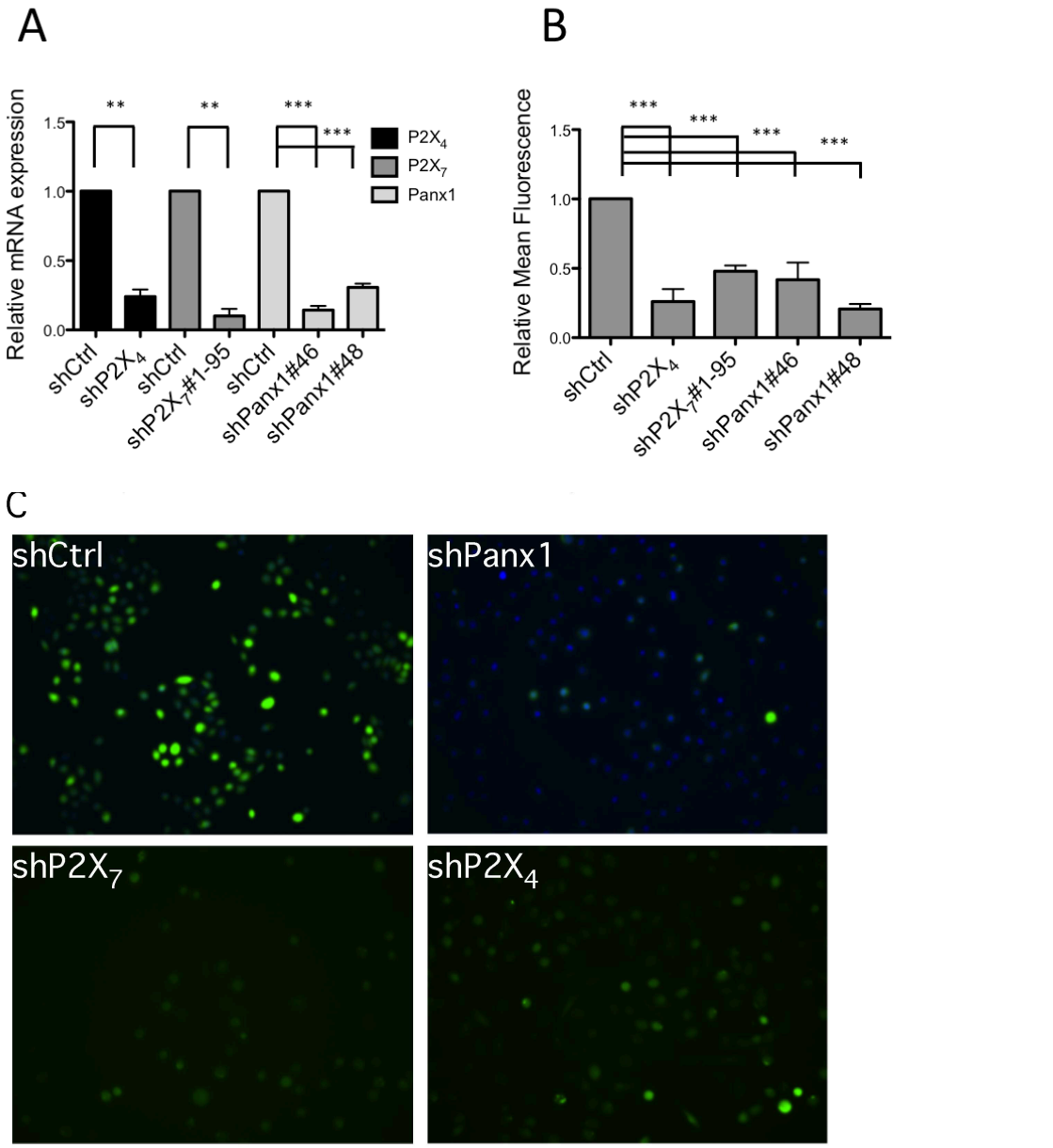


Figure 2. Diminished ATP-induced ROS production due to depletion of $P2X_4$ or $P2X_7$ by RNA interference. (A) Immortalized GECs were transduced with lentiviruses carrying the indicated shRNA-expressing plasmid for 1 day and selected with puromycin. After selection, cells were collected and total RNA was analyzed by qPCR to confirm knockdown efficiency. Paired t test was used for determine knockdown efficiency for $P2X_4$ and $P2X_7$, and one-way ANOVA was used for pannexin-1. **P<0.05, ***P<0.0001 (B) DCF staining of ROS production after 3 mM ATP stimulation for 1 hour in different cell lines. The fluorescence shown in (C) was quantified as in Figure 1 and normalized to shCtrl, which was transduced with control, non-mammalian shRNA.

3.1.3 ATP ligation by $P2X_4/P2X_7$/pannexin-1 complex leads to inflammasome activation in GECs

We previously showed that eATP treatment of GECs lead to NLRP3 inflammasome activation (34). As ROS production has been associated with inflammasome and caspase-1 activation (68,71,109,110), we evaluated whether eATP-mediated caspase-1 activation in GECs take place through $P2X_4/P2X_7$ ligation. Using ELISA to measure secretion of caspase-1, we observed that treatment of GECs with 100 μM ATP was insufficient for caspase-1 activation (Figure 3A), even though ROS generation was induced (data not shown). In contrast, 3 mM ATP treatment resulted in high levels of caspase-1 activation in GECs stably-expressing the control shRNA (Figure 3A); but the activation of caspase-1 by 3 mM ATP treatment was abrogated when either $P2X_4$ or $P2X_7$ were depleted in GECs (Figure 3A). Thus, treatment with 3 mM ATP induced ROS production via the $P2X_4/P2X_7$ complex and activated the NLRP3 inflammasome. However, 100 μM ATP stimulation induced ROS generation, but stimulation with this concentration of ATP was not sufficient to activate the inflammasome.

The non-redundant roles of $P2X_4$ and $P2X_7$ in ATP-induced ROS generation led us to hypothesize that $P2X_4$ and $P2X_7$ may be associated in the membrane and function as a physical complex in eATP-mediated responses in GECs. Therefore, we examined physical associations between $P2X_4$ and $P2X_7$ in GECs by performing co-immunoprecipitation experiments. After precipitating endogenous $P2X_4$ using an anti-$P2X_4$ antibody, we observed that $P2X_7$ and pannexin-1 were detected in the immunoprecipitate (Figure 3B). Taken together, for the first time, these data indicate that $P2X_4$, $P2X_7$, and pannexin-1 form a heterocomplex in GECs, and play non-redundant roles in eATP-induced ROS generation.

3.1.4 ATP ligation of $P2X_4/P2X_7$/pannexin-1 contributes to secretion of pro-inflammatory cytokines in primary GECs infected with *P. gingivalis*

Previously we had reported that infection of GECs with *P. gingivalis* leads to expression of pro-IL-1β and its accumulation within the infected cell. However, secretion of IL-1β requires a second signal, such as the danger signal ATP, in order to activate the NLRP3 inflammasome and caspase-1, allowing processing and secretion of the mature IL-1β (34).

Given the unexpected observation that $P2X_4$ can modulate eATP-dependent caspase-1 activation in the immortalized GECs, we examined whether a similar effect could be observed in primary GECs during infection with *P. gingivalis*. We have shown that primary GECs have functional NLRP3 inflammaosme in reponse to ATP treatment (34). To further confirm a role for $P2X_4$ and $P2X_7$ in IL-1β, we used siRNA to deplete $P2X_4$ and $P2X_7$ in primary GECs individually. Figure 4A showed that $P2X_4$ or $P2X_7$ mRNA levels were depleted with an efficiency of over 80% in primary GECs. Similarly to our previous results (34), *P. gingivalis* infection followed by 3 mM ATP treatment caused IL-1β secretion by the primary GECs that had been treated with control siRNA. However, depletion of $P2X_4$ or $P2X_7$ reduced significantly IL-1β secretion, which again showed a non-redundant role for $P2X_4$ and $P2X_7$ in eATP-

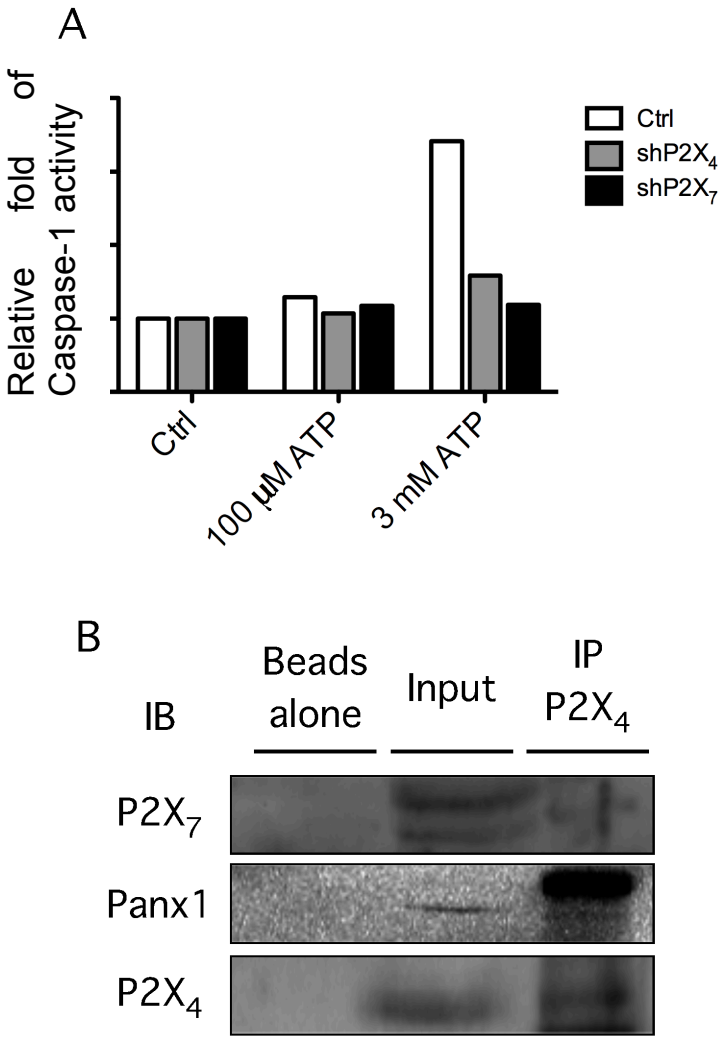


Figure 3. Impaired ATP-stimulated caspase-1 activation due to disruption of the complex containing $P2X_4$, $P2X_7$, and pannexin-1. (A) Immortalized GECs that had been depleted of $P2X_4$ or $P2X_7$ using lentiviral particles were treated with 100 μM or 3 mM ATP for 3 hours, and supernatants were collected for caspase-1 activity measurement. Caspase-1 activity was measured by ELISA as described in Material and Methods. (B) Total proteins isolated from GECs were subjected to immunoprecipitation (IP) by a polyclonal anti-$P2X_4$ antibody or Dynabeads as a control. Precipitates or total protein extract (as input) were resolved on SDS-PAGE and analyzed on immunoblots with anti-$P2X_7$ (top), anti-pannexin-1 (middle), or anti-$P2X_4$ (bottom) antibodies.

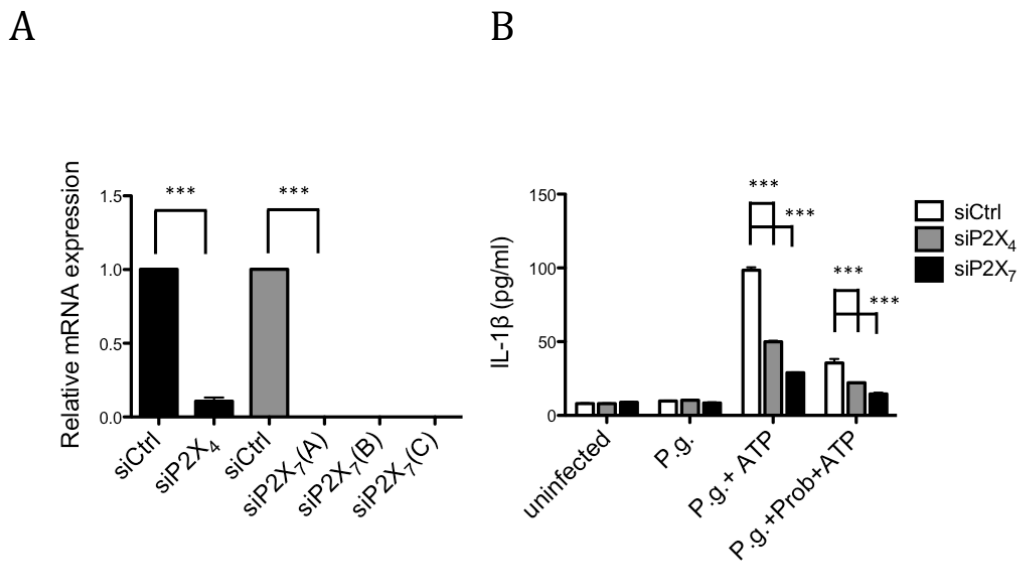


Figure 4. Abrogation of ATP-induced IL-1β secretion in *P. gingivalis*-infected GECs by inhibition of $P2X_4$, $P2X_7$, or pannexin-1. (A) Primary GECs were transfected with siRNA sequences against $P2X_4$ or $P2X_7$ for one day, and mRNA levels were detected by qPCR. Paired two-tailed t test was used for $P2X_4$, and one-way ANOVA was used for $P2X_7$. *** P-value<0.0001. (B) Primary GECs depleted of $P2X_4$ or $P2X_7$ were infected with *P. gingivalis* (*P.g.*) and pre-treated with 1 mM probenecid for 10 minutes and stimulated with 3 mM ATP for 1 hour. IL-1β secretion in the supernatants was analyzed by ELISA. The values showed averages and SD from duplicate samples, which were obtained from three separate experiments. Two-way ANOVA was used. ***P<0.001

dependent IL-1β secretion. Probenecid, pannexin-1 inhibitor, treatment prior to eATP stimulation repressed even further the IL-1β secretion in $P2X_4$ and $P2X_7$ knockdown cells, consistent with a role for pannexin-1 in IL-1β secretion by primary GECs. All these results imply that a $P2X_4$/$P2X_7$/pannexin-1 complex is required for IL-1β secretion in response to eATP treatment of *P. gingivalis*-infected cells (Figure 4B).

3.1.5 ATP-induced ROS is from both NADPH and mitochondria in GECs

Two possible sources of ROS that activate the inflammassome have been described. One may be due to activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, triggered by frustrated phagocytosis (111). The other main intracellular source of ROS is the mitochondria (71). Recent studies have uncovered the existence of cross-talk between the NADPH oxidase and mitochondria (112). In GECs, it was demonstrated that eATP induces both cytosolic and mROS production (76). To verify whether NADPH oxidase-induced ROS by eATP may modulates mROS production in GECs, we measured eATP-induced mROS generation after inhibiting NADPH oxidase with diphenyleneiodonium chloride (DPI), which was previously shown to block caspase-1 activation in human macrophages and is known as a NADPH oxidase inhibitor (113-115). As shown in Figure 5A and B, DPI profoundly attenuated eATP-triggered cytosolic ROS production, as detected by DCF, confirming that eATP treatment induces ROS through NADPH oxidase. To examine if NADPH oxidase could also modulate eATP-induced mROS production, we used MitoSOX to detect superoxide in mitochondria. Quantitative analysis of fluorescence micrographs confirmed that eATP treatment also triggers mROS production, in agreement with previous results (116). The increase of mROS can be partially inhibited by DPI, implying that NADPH oxidase plays a significant role in mROS generation (Figure 5, A and B). Further experiments using $p22^{phox}$ knockdown cells can be performed to confirm the results. Taken together, these results suggest that eATP-induced NADPH activation can synergistically promote mROS production in GECs.

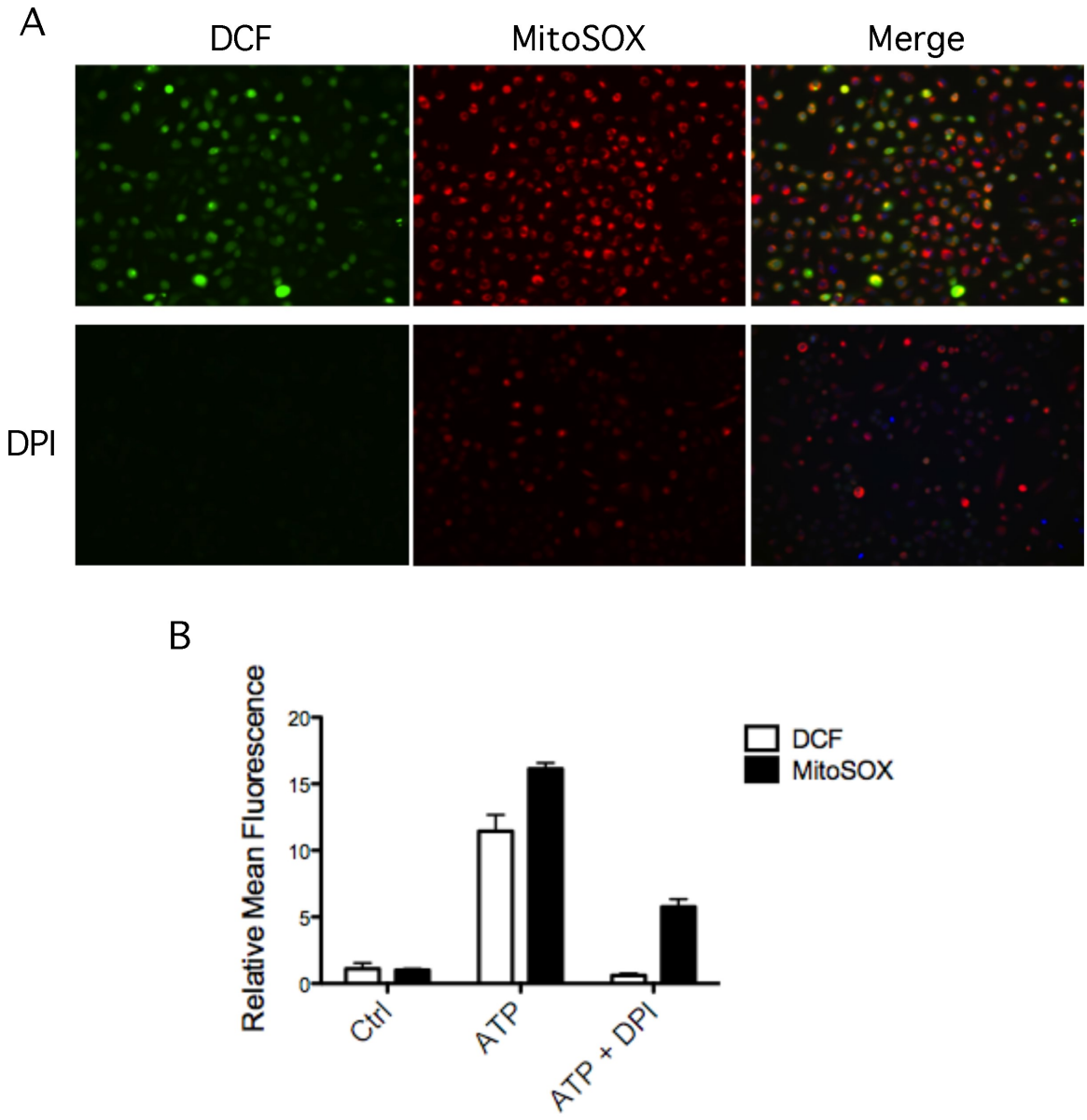


Figure 5. Synergistic effects of NADPH oxidase and mitochondria in ROS production.
(A) GECs were pretreated with 50 μM DPI for 30 minutes followed by 3 mM ATP stimulation, and stained with DCF (green) or MitoSOX (red) to detect ROS by fluorescence microscopy. (B) The fluorescence was quantified in three independent duplicate experiments as in Figure 1.

3.2 The role of NLRX1 in ATP- or *F. nucleatum*-treated GECs

3.2.1 Attenuated ATP-induced mROS in NLRX1-deficient GECs

We have previously reported that exogenous DAMPs, ATP, induced mROS generation to activate NLRP3 inflammasome (95). However, the mechanism by which mROS were generated remains to be understood. NLRX1, the only NLR in mitochondria, has been shown to mediate mROS production upon microbial infection (91-93). Therefore, we first constructed NLRX1 knockdown GECs. GECs were transduced with lentivirus carrying shRNA specific against NLRX1 or tGFP as a control. qRT-PCR and Western blot showed that NLRX1 was efficiently knocked down in GECs transduced with shRNA sequence 325 and 446 compared to tGFP (Figure 6, A and B). These data showed that we have successfully obtained stable NLRX1 knockdown GECs.

To investigate whether NLRX1 is involved in eATP-induced mROS generation, cells were stained with MitoSOX, which specifically detects mitochondrial superoxide. Fluorescence microscopy showed that eATP led to profound increase of mitochondrial superoxide production in wild-type GECs and GECs carrying shRNA control as previously described, whereas knockdown of NLRX1 inhibited the increased mitochondrial superoxide (95). Furthermore, quantification analysis of the fluorescence images verified that eATP-promoted ROS production decreased ~25% in NLRX1 knockdown cells compared to wild type (Figure 6, C and D). These results suggest that NLRX1 contributes to eATP-triggered mROS generation.

3.2.2 Depletion of NLRX1 inhibited ATP-caused mitochondrial damages

Recent studies have revealed the critical role of mitochondria in NLRP3 inflammasome activation. Shimada *et al.* showed that NLRP3 agonists like eATP led to unrecoverable mitochondrial damages by disrupting mitochondrial membrane potential, which accompanies with mROS generation and subsequent oxidized mitochondrial DNA release to cytosol to activate NLRP3 inflammasome (85). To further our study to the role NLRX1 in NLRP3 inflammasome activation, the effect of NLRX1 on mitochondrial membrane potential ($\Delta\psi_m$) were examined by mitochondrial membrane potential probe, JC-1. We found that JC-1 shifts profoundly from red J-aggregate to green monomer in both WT and shCtrl GECs upon eATP stimulation, suggesting reduction of $\Delta\psi_m$. However, depletion of NLRX1 reduced the eATP-elicited $\Delta\psi_m$ decrease (Figure 7A). Quantitative analysis of the fluorescence micrographs confirmed that depletion of NLRX1 abrogates eATP-induced $\Delta\psi_m$ reduction (Figure 7B).

eATP-induced mitochondrial ROS has been shown to induce cytosolic translocation of mitochondrial DNA (mtDNA), which is recognized by NLRP3 inflammasome (85,117). We therefore determined whether NLRX1 participated in eATP-triggered mtDNA release to cytosol. Cytosol were separated and collected from eATP-treated or untreated cells to examine mitochondrial DNA encoding *Cyt b* by

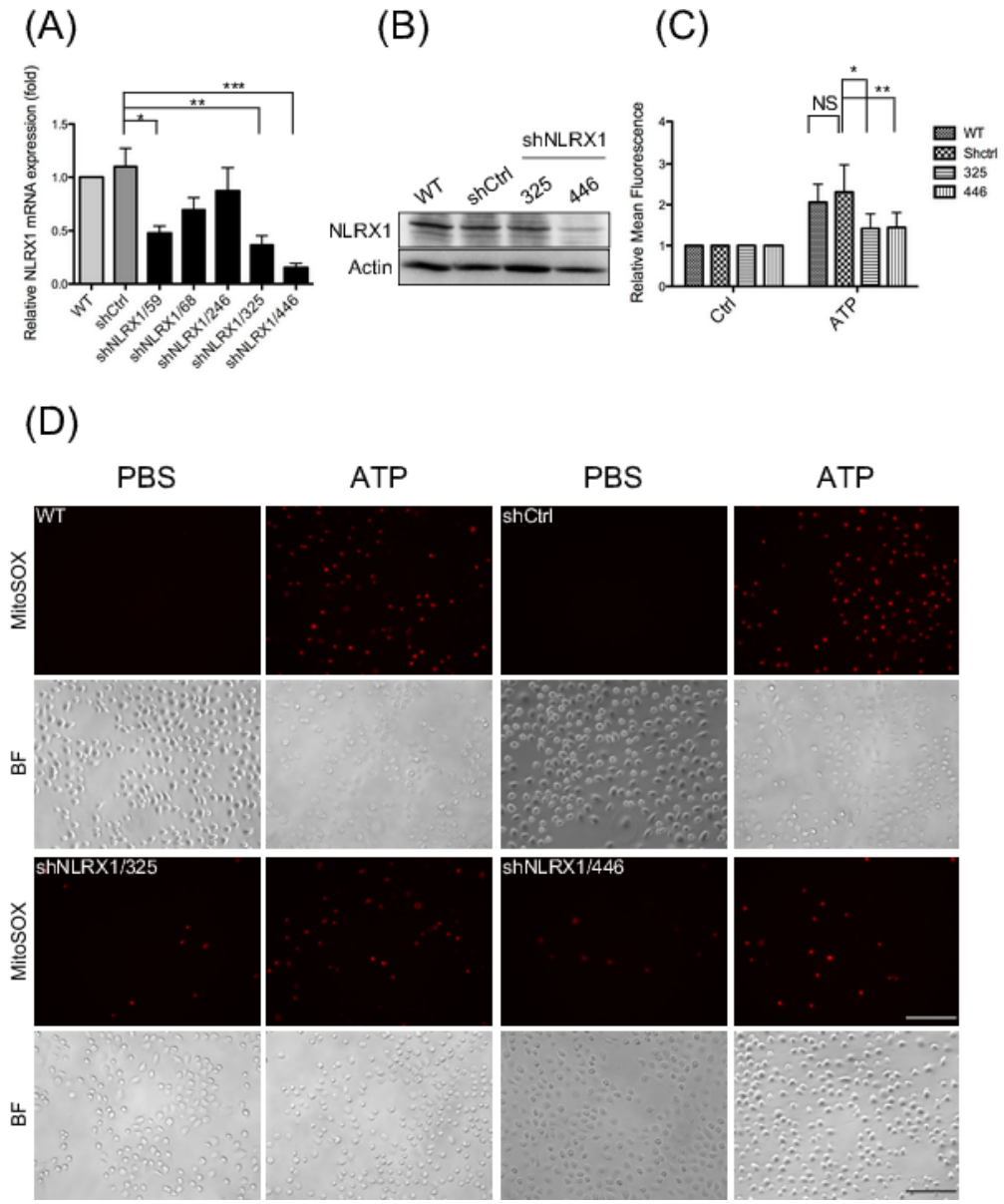


Figure 6. Depletion of NLRX1 reduced ATP-induced mROS in GECs
GECs were transduced with lentivirus carrying specific shRNA targeting NLRX1 (A) NLRX1 mRNA levels were measured by qRT-PCR in GECs expressing different shRNA. (B) Whole cell lysates were collected to measure protein expression levels of NLRX1. Actin was used as an internal control. (C and D) mROS generation was detected by fluorescence microscopy using MitoSOX after 5 mM ATP stimulation for 20 min. Quantification of mROS production were analyzed by ImageJ as in (C). BF represents bright field. Mean fluorescence intensity was obtained by dividing total fluorescence intensity against total number of cells in each image. Relative mean fluorescence is determined by normalizing with control group in each cell lines. Scale bars represents 100 μm. Statistical significance determined using one-way ANOVA (A) and two-way ANOVA for (C). *P-value < 0.05; **P-value < 0.01; ***P-value < 0.001. Data were from a representative of at least three independent experiments that gave similar results.

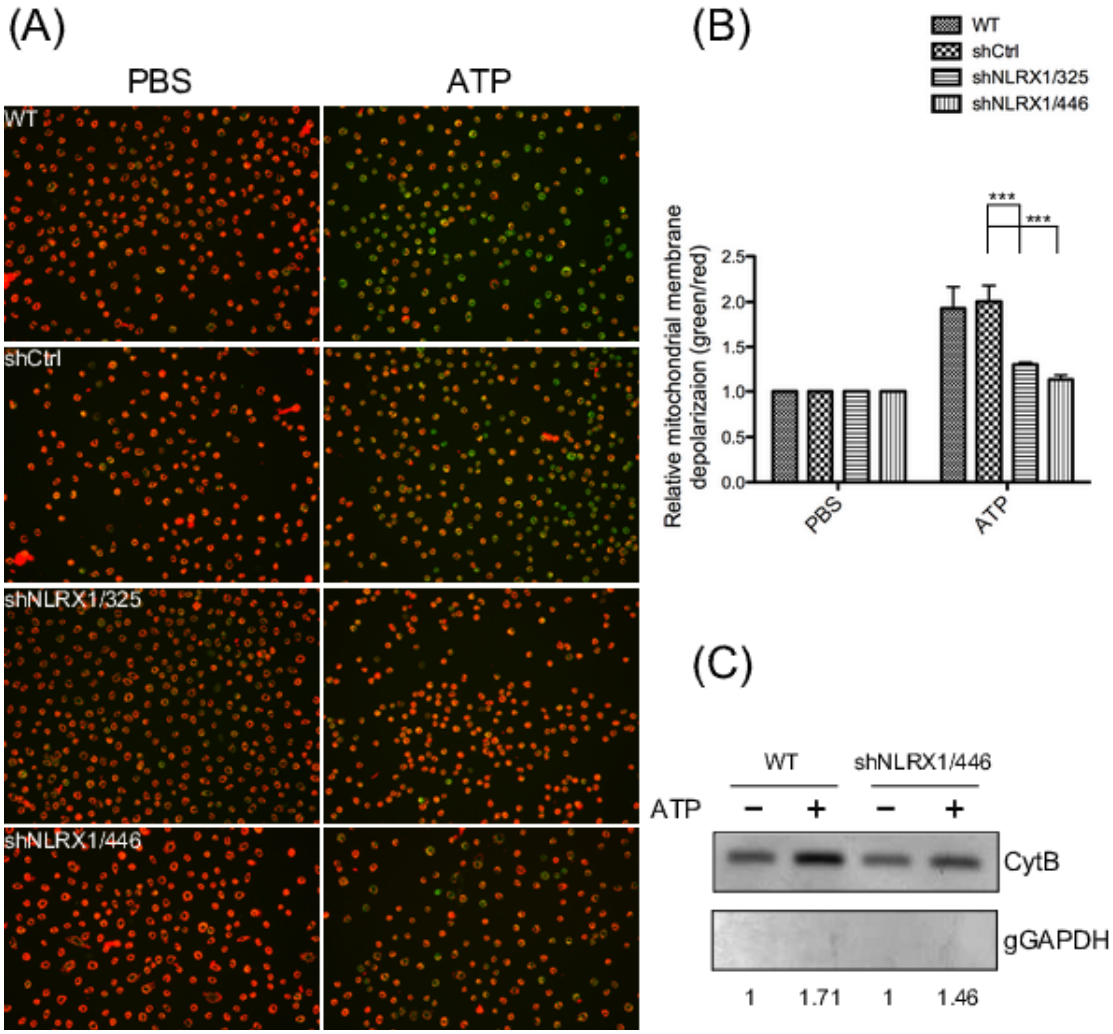


Figure 7. Suppression of ATP-elicited mitochondrial dysfunction in NLRX1-knockdown GECs. (A) GECs depleted of NLRX1 were stained with 5 μg/ml JC-1 followed by 5 mM ATP treatment for 20 min. The treated cells were subject to fluorescence microscopy for the analysis of the loss of mitochondrial membrane potential ($\Delta\Psi_m$). Red indicates JC-1 aggregates in healthy cells with high membrane potential. The red aggregates shits to green monomer in cytosol when mitochondrial potential is depolarized. Scale bars: 100 μm. (B) Quantification of mitochondrial membrane depolarization was obtained by dividing total fluorescence intensity of red J-aggregate over green monomer followed by normalization to the PBS control group. Statistical significance was determined using two-way ANOVA. ***P-value < 0.001. (C) Cytosolic fractions were isolated from GECs treated with or without 5 mM ATP for 1 h followed by Phenol-Chloroform-Isoamyl Alcohol precipitation. The cytosolic DNA were subject to PCR to detect mitochondrial gene CytB and genomic GAPDH. Data shown are representative of two or more independent experiments performed in duplicates (means ± SD).

PCR. Exposure to ATP significantly enhanced cytosolic mtDNA in GECs, whereas knockdown of NLRX1 compromised the enhancement (Figure 7C). Genomic DNA encoding *Gapdh* was not detected in all samples, excluding the possibility of contamination from organelles. All together, these results suggest that NLRX1 diminishes eATP-caused mitochondrial damages.

3.2.3 Compromised NLRP3 activation in NLRX1 knockdown GECs

Mitochondrial dysfunctions-caused cytosolic translocation of mitochondrial DNA is sensed by NLRP3, which triggers NLRP3 inflammasome assembly (85). Assembly of NLRP3 inflammasome requires recruitment of adaptor ASC (apoptosis-associated speck-like protein containing CARD) at perinuclear mitochondria-associated membranes (MAMs) of the endoplasmic reticulum (ER) (71,118,119). To further access whether NLRX1 was involved in ASC redistribution, the cells were treated with ATP for 10 min followed by immunofluorescence assays. Under untreated condition, ASC were barely detectable due to its general distribution throughout the cytoplasm. However, in eATP-stimulated GECs with or without shRNA against tGFP, ASC became predominantly perinuclear, and reduction of NLRX1 blocked eATP-induced ASC redistribution (Figure 8 A and B, and Supp. 1). In addition, the protein level of ASC was unaltered throughout the treatment (Figure 8C). These results suggest that NLRX1 abolishes eATP-triggered ASC redistribution.

NLRP3 inflammasome assembly relies on ASC to recruit pro-caspase-1 followed by caspase-1 autoprocessing, which cleaves pro-IL-1β to IL-1β (120). Therefore, to examine whether compromised ASC redistribution affected NLRP3 inflammasome activation, we analyzed the protein level of caspase-1. As expected, immunoblotting analysis showed that ATP stimulation significantly enhanced caspase-1 activation in wt and shCtrl GECs.

We further examined whether ATP-triggered activated caspase-1 increase is inhibited in NLRX1 knockdown cells. Keratinocytes have been shown to express basal level of caspase-1, but ATP treatment enhanced activated caspase-1 level in wild-type GECs as shown in Figure 8D (121,122). The level of caspase-1 was also enhanced in NLRX1 knockdown cells after ATP stimulation, but to a lesser extent than wild-type cells. Caspase-1 activation was impaired more sever in cells carrying shNLRX1 sequence 446 which may be owing to its better knockdown efficiency of NLRX1 (Figure 8D). Taken together, these data suggest that NLRX1 positively mitigates NLRP3 inflammasome activation.

3.2.4 Positive regulator role of NLRX1 in *F. nucleatum* infection-mediated caspase-1 activation

Previous studies including ours have shown that *F. nucleatum* infection leads to secretion of proinflammatory cytokine IL-1β as well as danger signal ASC and HMGB1 through NLRP3 inflammasome, whereas *P. gingivalis* suppresses NLRP3 inflammasome activation (35,100,123). However, the detailed mechanism by which *F. nucleatum* activate NLRP3 ifnlammasome remains unclear. In this article, so far, we showed that NLRX1 modulates eATP-induced NLRP3 inflammasome activation via

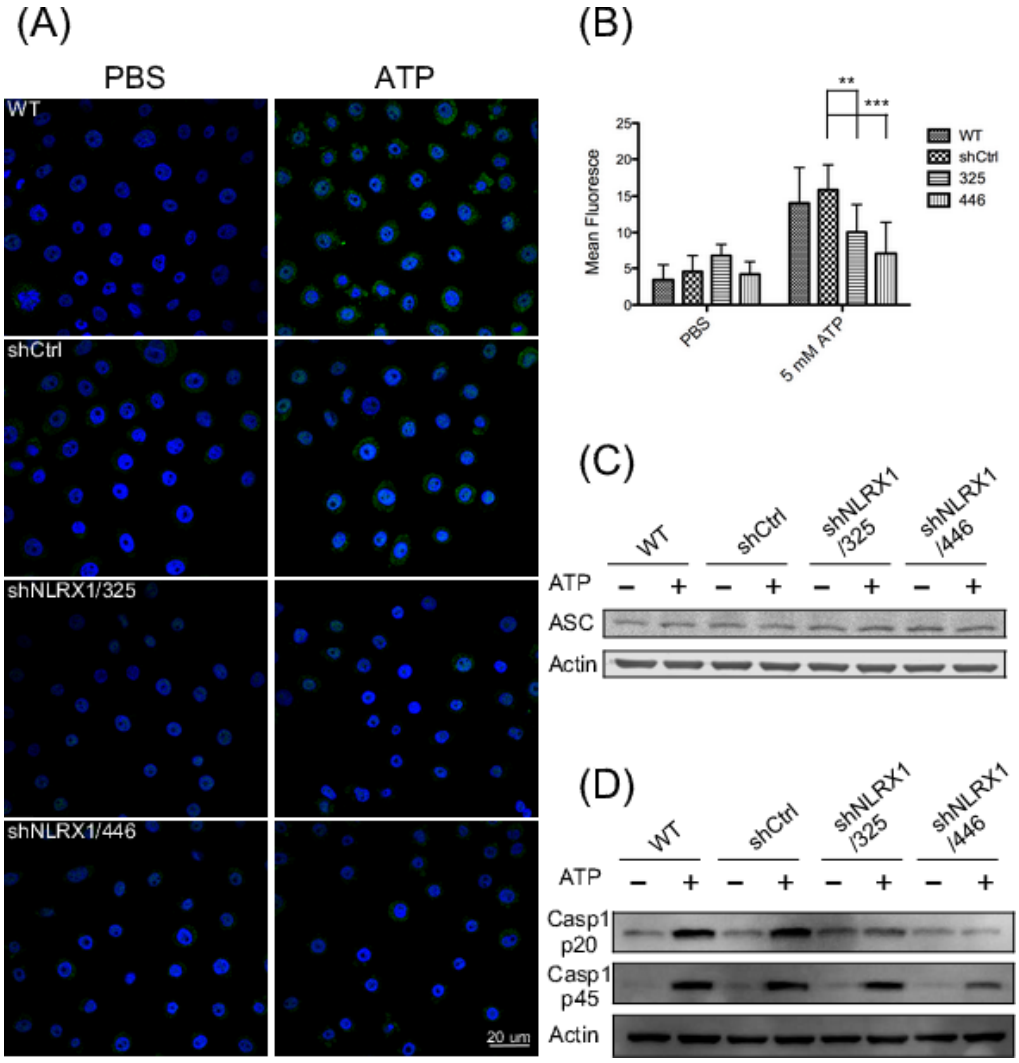


Figure 8. Reduction of NLRX1 abrogated ATP-triggered NLRP3 inflammasome activation.

(A) GECs were treated or untreated with 5 mM ATP for 10 min before fixation. The cells were then immunostained for ASC (green) antibody and subjected to confocal microscopy. Nuclei were stained by DAPI (blue). Scale bar, 20 μm. (B) Fluorescence microscopy images were quantified by dividing mean ASC fluorescence intensity over total DAPI fluorescence intensity of each cell. Statistical significance was determined using two-way ANOVA. **P-value < 0.01; **P-value < 0.001. (C) Western blot analysis of ASC protein levels of whole cell lysates from GECs treated as in (A). Actin was chosen as loading control. (D) Caspase-1 protein levels were examined by Western blot after GECs were left untreated or treated with 5 mM ATP for 1 h. Actins was used as internal control. Data shown are representative of two or more independent experiments performed in duplicates (means ± SD).

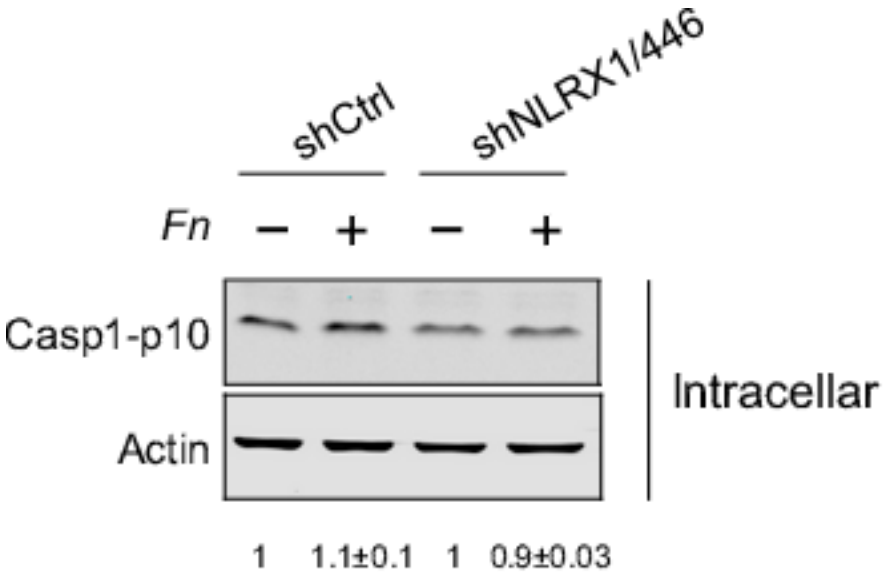


Figure 9. Engagement of NLRX1 in *F. nucleatum*-triggered caspase-1 activation. GECs were infected with or without F. nucleatum (Fn) at an M.O.I of 100 for 24 h. Supernatants were collected and subjected to TCA precipitation. Cells were lysed in RIPA buffer. Western blot was assayed to determine the protein levels of caspase-1 in supernatants (extracellular) and whole cell lysates (intracellular). Actin served as loading control. Data are representative of two independent experiments.

mROS. It has been shown that *F. nucleatum* infection triggers ROS production (124,125). Therefore, we postulated that NLRX1 may modulate *F. nucleatum*-mediated NLRP3 inflammasome activation via ROS. To substantiate the possible mechanism, caspase-1 activation was examined followed by *F. nucleatum* infection at an M.O.I. of 100. Correspondingly, results revealed that *F. nucleatum* infection-induced caspase-1 activation was inhibited in NLRX1 knockdown cells (Figure 9). Altogether, these results suggest that NLRX1 positively modulates *F. nucleatum* infection-elicited NLRP3 inflammasome activation.

3.2.5 Enhanced *F. nucleatum* infection-induced IL-8 gene expression in NLRX1 knockdown cells.

It has been shown that IL-8 is upregulated via NF-κB signaling to recruit neutrophils in responses to *F. nucleatum* infection (126,127). However, the mechanism by which *F. nucleatum* upregulates IL-8 remains elusive. NLRX1 has been reported to negatively regulate NF-κB signaling by targeting IKK complex (98). Therefore, we further examined whether NLRX1 participates in *F. nucleatum*-induced IL-8 upregulation. GECs were infected with or without *F. nucleatum* at an M.O.I. of 50 and 100 followed by qPCR examination of IL-8 expression. As shown in in Figure 10A, IL-8 gene expression increased in a *F. nucleatum* dose-dependent manner in shCtrl and wt cells. In addition, infection of *F. nucleatum* at the same M.O.I. in NLRX1 knockdown significantly led to more IL-8 transcripts in comparison to WT and shCtrl cells. 16S rRNA gene was examined by RT-PCR to confirm the infection (Figure 10B). Hence, these data suggest that NLRX1 negatively regulates *F. nucleatum* infection-induced IL-8 expression.

3.2.6 NLRX1 mitigates *F. nucleatum* infection-induced IL-8 via NF-κB.

Previously, NLRX1 has been revealed to negatively regulate NF-κB signaling (98). Therefore, to determine whether NLRX1 is involved in *F. nucleatum* infection-induced IL-8 expression, nuclear translocation of RelA (p65), subunit of NF-κB, was examined in NLRX1 knockdown GECs. As expected, confocal microscopy showed that infection of *F. nucleatum* at an M.O.I. of 50 resulted in nuclear translocation of RelA in 30% of cell in wt and shctrl cells, whereas depletion of NLRX1 enhanced RelA translocation to 60% (Figure 11 A and B). In addition, the cytosolic and nuclear extracts from infected cells were also analyzed for RelA by immunoblotting. NLRX1 knockdown cells showed increased RelA in nucleus, but reduced cytoplasmic RelA upon infection in comparision to wt and shctrl cells (Figure 11C). These results suggest that NLRX1 negatively regulates *F. nucleatum* infection induced IL-8 expression by NF-κB.

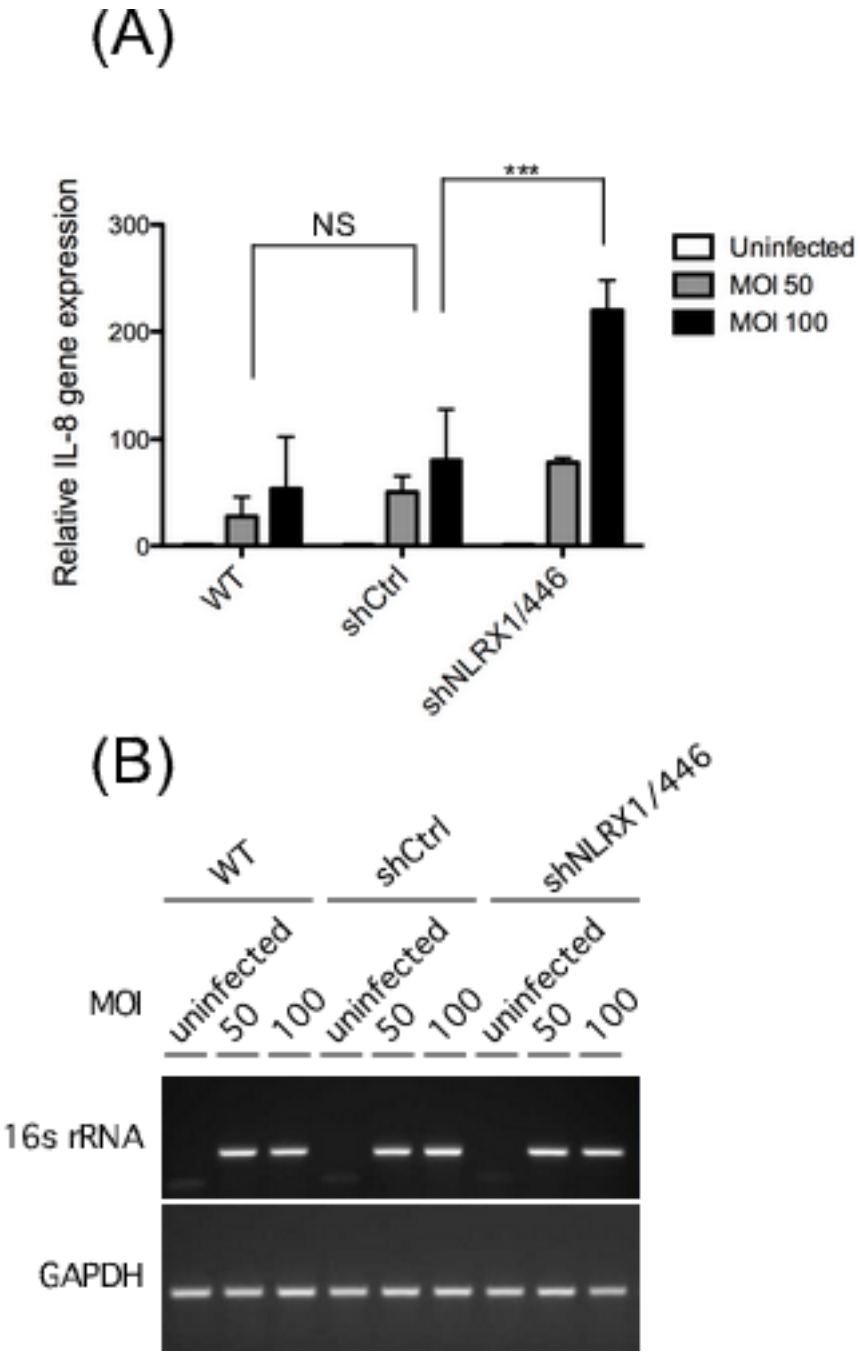


Figure 10. Down-regulation of NLRX1 enhanced IL-8 expression upon *F. nucleatum* infection.

(A) GECs were left uninfected or infected with *F. nucleatum* at an M.O.I of 50 for 24 h. RNA extracted from the cells were reverse transcribed to cDNA followed by qPCR to detect the IL-8 transcripts. Statistical significance was determined using two-way ANOVA. ***P-value <0.001. (B) PCR were used to detect 16s rRNA of *F. nucleatum* to confirm the infection in (A). GAPDH was used as an internal control. Data are representative of three independent experiments performed in triplicates (means ± SD).

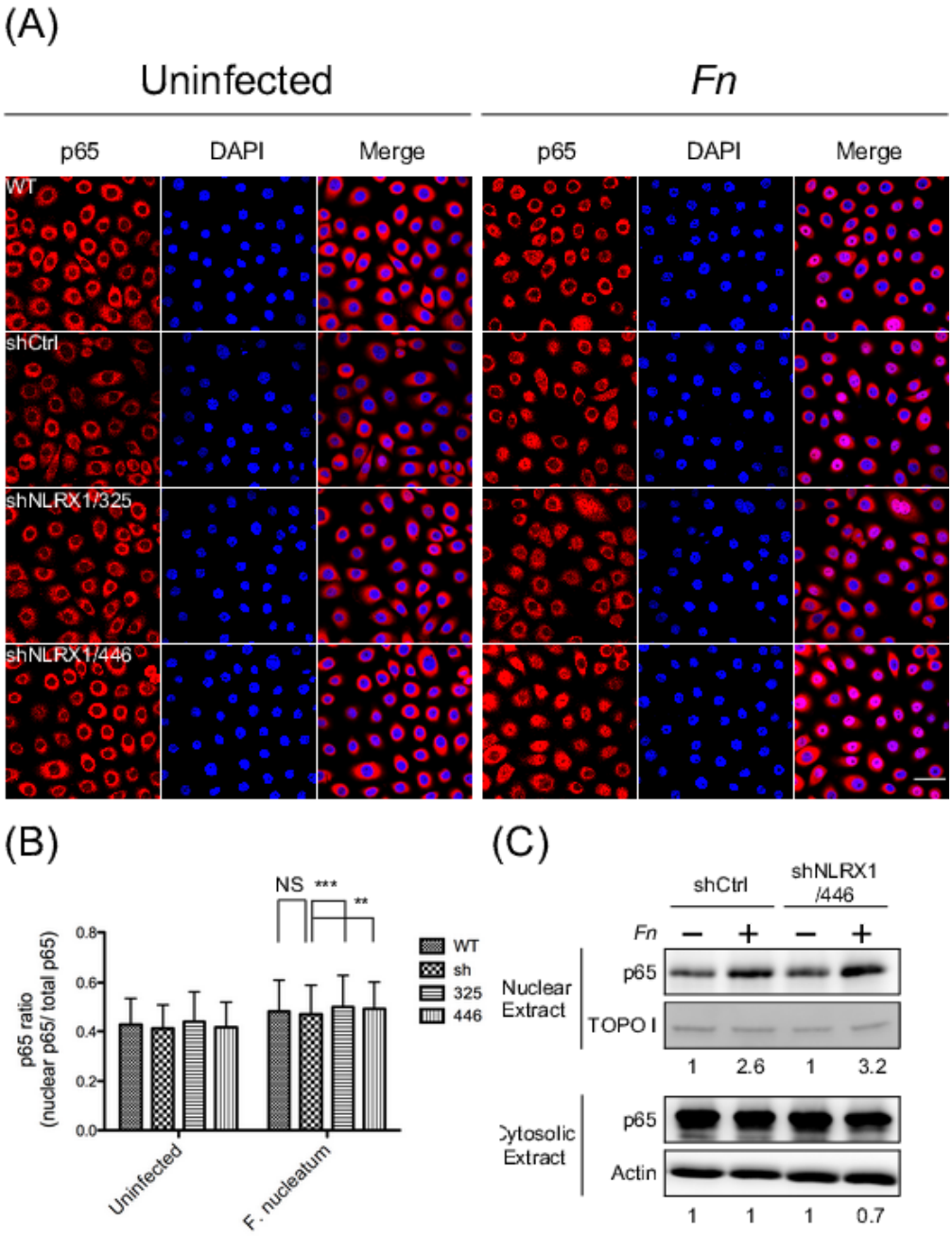


Figure 11. NLRX1 regulated *F. nucleatum*-elicited NF-κB nuclear translocation. (A) Confocal microscopy of GECs uninfected or infected with *F. nucleatum* at an M.O.I. of 50 for 1h followed by fixation. Cells were then immunostained with anti-NF-κB p65 (red) and stained nuclei with DAPI (blue). Scale bar, 20 μm. (B) Quantitative analysis of fluorescence microscopy images obtained under condition as in (A). Fluorescence intensity of p65 in nuclei and total cells was measured by CellProfiler. Statistical significance was determined using two-way ANOVA. **P < 0.01, ***P< 0.001. (C) Nuclear and cytosol fraction of cells were isolated followed by immunoblotting using anti-NF-κB p65 antibody. TOPO I and actin served as loading control for nuclear and cytosol fractions, separately. All data are representative of at least three independent experiments performed in duplicates.

3.3 Discussion

Periodontitis is a chronic inflammatory disease caused by dysbiotic microbiota induced-dysregulated host innate immune response in the periodontal tissue. However, how a host's immune system recognizes the bacteria and become dysregulated is unclear. Thus, here we use *F. nucleatum* or *P. gingivalis* infected GECs as the periodontitis model to study how the host cells recognize and respond to infection.

Previously, we demonstrated that GECs have a functional NLRP3 inflammasome. We have reported that *P. gingivalis* infection of GECs requires an exogenous danger signal such as eATP to activate an inflammasome and caspase-1, thereby inducing secretion of IL-1β. Stimulation with eATP alone also stimulates production of ROS in GECs. However, the mechanism by which ROS is generated in response to eATP, and the role that different purinergic receptors may play in inflammasome activation, remained unclear (34).

In this work, we examined the detailed molecular mechanism of NLRP3 activation upon eATP stimulation. Our results showed that $P2X_4$, $P2X_7$, and pannexin-1 contribute to ROS generation and are associated with inflammasome activation in GECs. Consistent with this possibility, previous studies have suggested that $P2X_4$ and $P2X_7$ may behave as heteromeric receptors in murine bone marrow-derived macrophages (BMDM) (81,128). Similarly, eATP-induced cell death of mouse macrophages was shown to involve the $P2X_4$ receptor, initiating Ca^{2+} influx upon stimulation with ATP and contributing to pore formation by activation of the $P2X_7$ receptor (82,129). These findings suggest the functionality and dependence of the $P2X_4$ and $P2X_7$ receptors on each other.

In human GECs, we found that inhibitors of $P2X_4$, $P2X_7$, and pannexin-1 reduced significantly ATP-dependent production of ROS. Depletion of either purinergic receptor or pannexin-1 by RNA interference showed that both purinergic receptors and pannexin-1 are required for efficient eATP-induced ROS production in primary or immortalized GECs. Our findings differ from another study, which showed that depletion of the $P2X_4$ receptor increased eATP-mediated ROS production in the macrophage cell line, RAW264.7 cells (82,130). The conflicting results may be attributed to different cells lines, but we also used primary GECs and found similar results as with GECs.

Our studies show that that pannexin-1 is indispensable for eATP-induced NLRP3 activation in GECs. However, recent genetic evidence showed normal NLRP3 inflammasome function in macrophages derived from *Panx1*-deficient mice (131,132). This discrepancy may be explained by assuming that pannexin-1 plays a different role in different species or cell types. For example, in neurons, pannexin-1 is involved in inflammasome-induced cell death, as shown through the use of pannexin-1 depletion and *Panx1*-deficient mice (108,133,134). In addition, recent studies have shown that PAMPs alone can activate NLRP3 inflammasome, and human cells are more sensitive to stimuli in comparison to mice cells (135). The difference possibly is through a newly discovered non-canonical inflammasoem pathway. It has been shown that certain bacteria such as enterohemorrhagic *Escherichia Coli* (EHEC), are capable

of activate NLRP3 inflammaosme through caspase-4 and caspase-5 in human (caspase-11 in mice), the non-canonical inflammasome (136).

We have previously reported that treatment of GECs with eATP leads to activation of the inflammasome and caspase-1 through $P2X_7$ (34). However, we now find that depletion of either $P2X_4$ or $P2X_7$ results in decreased caspase-1 activation in GECs. ROS is produced when either $P2X_4$ or $P2X_7$ are stimulated, but caspase-1 is activated only when GECs are treated with ATP concentrations that activate $P2X_7$. Similarly, IL-1β secretion from *P. gingivalis*-infected cells, which requires caspase-1 activation, could be induced by treatment of the infected cells with ATP that stimulate $P2X_7$, but inhibiting or depleting either $P2X_4$ or $P2X_7$ resulted in significantly lower levels of IL-1β secretion. Consistent with our finding, treatment of low concentration of ATP with ivermectin, antiparasitic agent that somehow enhances $P2X_4$-depedent signaling, triggered IL-1β secretion in $P2X_7$-KO macrophages (84,137).

Cl^- hasn't been linked with NLRP3 inflammasome. Recent studies of ivermectin show that it may activate NLRP3 inflammasome through $P2X_4$/$P2X_7$/Panx1 receptors. In addition, ivermectin-triggered Cl^- influx may contribute to cell swelling and lead to ATP release from cells (138).

Taken together, these results suggested that $P2X_4$ stimulation may not be sufficient for activation of caspase-1, but $P2X_4$ may form a complex with $P2X_7$, which could explain why $P2X_4$ depletion results in loss of $P2X_7$-mediated signaling. We confirmed this hypothesis by demonstrating by co-immunprecipitation experiments that $P2X_4$ is physically associated with $P2X_7$ and pannexin-1 in GECs. $P2X_4$ and $P2X_7$ have previously been shown to also form heteromeric receptors in BMDM (81). Thus, these results suggest that $P2X_7$ stimulation is required for caspase-1 activation, but $P2X_4$, through its presence in the $P2X_4$/$P2X_7$/pannexin-1

ROS have been shown to be an important step to activate NLRP3 inflammasome upon DAMPs stimulation (62). However, the intracellular origin of ROS remains debated. Previous studies demonstrated that inhibiting NADPH oxidases with pharmacological inhibitors such as DPI or depletion by siRNA significantly decreased caspase-1 activity and IL-1β maturation in macrophages stimulated with DAMPs, indicating that NADPH oxidase-elicited ROS play a role in inflammasome activation (139,140). Subsequently, another intracellular source of ROS, mitochondria, was also reported to activate NLRP3 in response to DAMPs by inducing oxidation and release of mitochondrial DNA (71,85). In GECs, a recent study demonstrated that ATP stimulation results in NADPH-induced ROS generation via $P2X_7$ ligation which also promotes mROS generation, indicating the existence of cross-talk between the NADPH oxidase and mitochondria (76). Consistent with these findings, we showed that inhibition of NADPH oxidiase also decreased oxATP-induced mROS generation.

The role of NLRX1 in innate immunity remains elusive. Previous reports identified a role of NLRX1 in ROS production upon TNF-α and *Shigella* stimulation and which suggests that NLRX is engaged in TLR-mediated NF-κB signaling (91,98). In this study, we uncover the surprising role of NLRX1 in orchestrating NLRP3 inflammasome and NF-κB signaling in GECs upon *F. nucleatum* infection. Depletion

of NLRX1 blocks both ATP- and *F. nucleatum* infection-activated NLRP3 inflammasome. Therefore, we hypothesize that NLRX1 promotes dysregulated inflammatory responses during *F. nucleatum* infection via the NLRP3 inflammasome in periodontitis. In contrast, *F. nucleatum* as a commensal induces NF-κB-regulated IL-8 expression, which is enhanced by knockdown of NLRX1. Thus, we propose that commensals should not activate the inflammasome, but NLRX1 should behave as a checkpoint to prevent hyperinflammatory responses.

F. nucleatum as a commensal is known to induce NF-κB regulated IL-8 expression in GECs to recruit neutrophils to eradicate infection (127,141). However, PRRs responsible for sensing and initiation of the cytokine production remain elusive. IL-8 production is induced by extracellular TLRs among most bacterial infections. Nevertheless, it has been reported that not all *F. nucleatum* strains infection-elicited IL-8 up-regulation is TLR-dependent (127,142,143). The ability of *F. nucleatum* in both attaching and invading cells suggest that intracellular PRRs also contribute to the IL-8 production (142,144). It has been shown that endosomal TLR9 up-regulates IL-8 in responses to *F. nucleatum* released bacterial DNA in GECs (145). In addition, crystal structure of NLRX1 has shown that its leucine-rich repeat (LRR) domain can directly sense PAMPs, such as nucleic acids (146). Here, we demonstrated that depletion of NLRX1 enhanced RelA nuclear translocation, thus augmenting IL-8 expression (Figure 10 and 11). Previous research showing NLRX1 negatively regulates NF-κB pathway through suppressing TRAF6 supports our result (97-99). These results suggest that NLRX1 may play a role in surveillance of *F. nucleatum*, albeit the detailed molecular mechanism by which NLRX1 senses *F. nucleatum* remains to be investigated. In addition, the fact that NLRX1 suppressed the inflammatory IL-8 responses suggests that it may act as a checkpoint to avert overreacting immune response upon commensal *F. nucleatum* infection.

Despite the fact that *F. nucleatum* is considered as an anaerobic oral commensal, the abundance of *F. nucleatum* is positively correlated to the severity of oral diseases and colon cancer (147-149). *F. nucleatum* plays a role as an opportunistical periodontopathogen in inducing dysregulated pro-inflammatory cytokines, metalloproteases and NLRP3 inflammasome, which are associated with periodontal destruction (17,100,150,151). Beside, the gene expression of NLRX1 and caspase-1 has been shown to increase significantly in periodontitis gingival tissues (152). Recent research has shown that NLRP3 inflammasome is activated through sensing mitochondrial damages-caused mtDNA release (85). Therefore, we here investigated whether mitochondrial protein NLRX1 is involved in NLRP3 inflammasome activation by inducing mitochondrial dysfunction. We first used extracellular ATP (eATP), one of the danger signals released by infected cells, to stimulate NLRP3 inflammasome activation (153,154). We showed that NLRX1 positively modulates eATP-induced mitochondrial dysfunction, including mROS generation, mitochondrial membrane potential disruption and mitochondrial DNA release, suggesting that NLRX1 regulates NLRP3 inflammasome (Figure 6 and 7). We further confirmed the engagement of NLRX1 in NLPR3 inflammasome activation by revealing reduced eATP-induced caspase-1 activation in NLRX1-depleted GECs. Depletion of NLRX1 also decreased eATP-induced ASC redistribution (Figure 8). Our findings suggest that down-regulation of NLRX1 inhibits eATP-elicited mitochondrial DNA release, thus blocking NLRP3 sensing and inflammasome

assembly.

Our previous findings showed that *F. nucleatum* infection activates NLRP3 inflammasome and facilitates the release of DAMPs like HMGB1 and ASC to amplify inflammation in GECs without significant cell death observed after infection for 24 h (100). However, the mechanism by which *F. nucleatum* infection activates NLRP3 inflammasome remains to be investigated. Here, we have demonstrated that NLRX1 behaves as a positive regulator in *F. nucleatum* infection-elicited NLRP3 inflammasome activation from the NLRX1 knockdown GECs. Down-regulation of NLRX1 reduced *F. nucleatum* infection triggered intracellular caspase-1 activation, albeit more activated extracellular caspase-1 was observed (Figure 9). The released DAMPs, such as eATP from infected or stressed cells could be one mechanism to activate NLRP3 inflammasome through $P2X_4/P2X_7$-gated Pannexin-1 channels, which triggers mROS generation modulated by NLRX1 (95). Another possible mechanism is that internalized *F. nucleatum* in lysosomes lead to Ca^{+2} influx or cathepsin B release to activate NLRP3 inflammasome via causing mitochondrial damages, which including mROS regulated by NLRX1 (124,155-158). Our finding for the regulation role of NLRX1 in eATP-induced-mROS production suggests that NLRX1 may employ a vital mechanism to activate NLRP3 inflammasome through mROS upon *F. nucleatum* infection. Further experiments showing direct correlation between *F. nucleatum* infection-triggered ROS production and NLRP3 inflammasome activation in NLRX1 knockdown GECs will be performed to confirm the role of NLRX1 in *F. nucleatum*-triggered NLRP3 ifnlammasome activation.

Emerging evidence has shown the virulence of *F. nucleatum* in not only periodontal diseases, but also other systematic diseases. However, it is puzzling why *F. nucleatum* plays different roles in healthy and diseased cells. Periodontitis is a chronic inflammatory disease caused by dysbiosis. This dysbiosis may allow other pathogens to promote the virulence of *F. nucleatum* and further up-regulate the triggered inflammatory responses. Animal studies support this notion by showing exacerbated bone loss upon co-infection of *F. nucleatum* with other periodontal pathogens, e.g. *P. gingivalis* or *Streptococci* (159,160). On the other hand, our finding suggests that NLRX1 plays an important role in regulating inflammation in response to *F. nucleatum* as commensal or pathogen. Therefore, further study using co-infection with other pathogens is needed to understand whether NLRX1 contributes to dysregulated inflammatory responses due to dysbiosis.

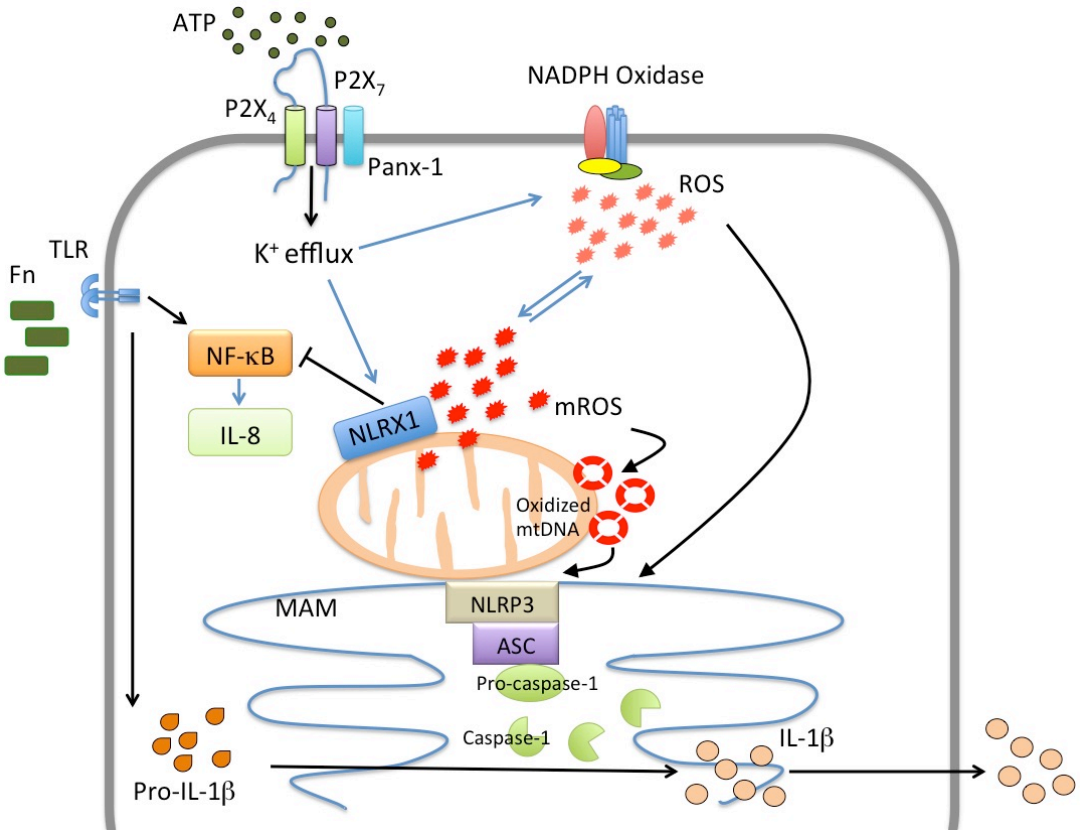
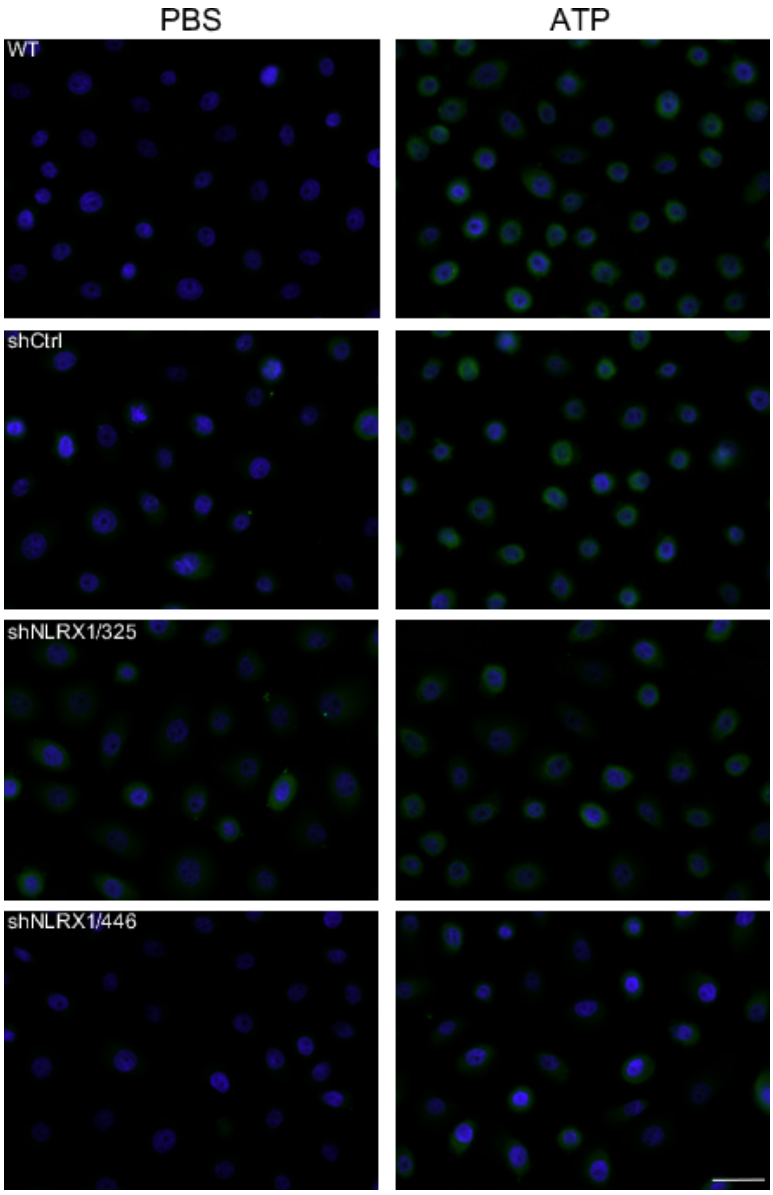


Figure 12. Model of NLRX1 as a modulator in different inflammatory responses in healthy or diseased periodontitium.



Supplementary 1. Knockdown of NLRX1 reduced ATP-triggered ASC redistribution.

GECs were treated or left untreated with 5 mM ATP for 10 min. The cells were then stained with anti-ASC antibody (green) and DAPI (blue) followed by fluorescence microscopy imaging. Scale bar: 20 μm.

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