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***Porphyromonas gingivalis* attenuates ATP-mediated inflammasome activation and HMGB1 release through expression of a nucleoside-diphosphate kinase**

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Abstract

Many intracellular pathogens evade the innate immune response in order to survive and proliferate within infected cells. We show that *Porphyromonas gingivalis*, an intracellular opportunistic pathogen, uses a nucleoside-diphosphate kinase (NDK) homolog to inhibit innate immune responses due to stimulation by extracellular ATP, which acts as a danger signal that binds to P2X₇ receptors and induces activation of an inflammasome and caspase-1. Thus, infection of gingival epithelial cells (GECs) with wild-type *P. gingivalis* results in inhibition of ATP-induced caspase-1 activation. However, *ndk*-deficient *P. gingivalis* is less effective than wild-type *P. gingivalis* in reducing ATP-mediated caspase-1 activation and secretion of the proinflammatory cytokine, IL-1 β , from infected GECs. Furthermore, *P. gingivalis* NDK modulates release of high-mobility group protein B1 (HMGB1), a pro-inflammatory danger signal, which remains associated with chromatin in healthy cells. Unexpectedly, infection with either wild-type or *ndk*-deficient *P. gingivalis* causes release of HMGB1 from the nucleus to the cytosol. But HMGB1 is released to the extracellular space when uninfected GECs are further stimulated with ATP, and there is more HMGB1 released from the cells when ATP-treated cells are infected with *ndk*-deficient mutant than wild-type *P. gingivalis*. Our results reveal that NDK plays a significant role in inhibiting P2X₇-dependent inflammasome activation and HMGB1 release from infected GECs.

Keywords

innate immunity; purinergic receptor; *Porphyromonas gingivalis*; interleukins; inflammation

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1. Introduction

Porphyromonas gingivalis is a Gram-negative opportunistic pathogen that colonizes the human oral epithelial tissues and plays a major role in the etiology of severe and chronic forms of periodontal disease [1]. The organism has been associated recently with a variety of other chronic and inflammatory conditions including orodigestive cancer, rheumatoid arthritis, liver disease, and diabetes [2, 3]. Gingival epithelial cells (GECs) are among the first cells in the oral cavity that are encountered and invaded by *P. gingivalis*, and they play an important barrier role while also sensing and responding to microbial colonizers [1, 2, 4].

Immune recognition of cellular damage and invading pathogens is carried out through pattern recognition receptors such as Toll-like receptors (TLRs) and purinergic P₂X receptors situated on the plasma membrane, and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) in the cytoplasm [5-7]. Ligation of the purinergic receptor, P₂X₇, typically leads to assembly of a complex of proteins, called the inflammasome, which results in activation of caspase-1 and subsequent processing and secretion of the pro-inflammatory cytokines, IL-1 β and IL-18. The most widely studied inflammasome contains the NLR member, NLRP3, the adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC), and the protease, caspase-1 [7, 8]. The NLRP3 inflammasome can be activated by multiple stimuli, including the “danger signal,” extracellular ATP, which is released by damaged, stressed, or infected cells [7, 9]. GECs express functional P₂X₇, whose ligation by ATP leads to production of reactive oxygen species (ROS) and assembly and activation of the NLRP3 inflammasome [7, 10-13].

Another danger signal molecule, high mobility group box 1 protein (HMGB1), can be secreted after inflammasome activation [14, 15]. In healthy cells, HMGB1 is associated with chromatin and enhances the binding of regulatory proteins to a variety of genes [14]. Outside the cell, HMGB1 has potent and diverse immunomodulatory functions after binding to its receptor, RAGE, TLR2 or TLR4 [16]. Extracellular HMGB1 has been linked with different inflammatory conditions, from endotoxemia, ischemia and liver injury to squamous cell carcinoma [17-19]. Increased HMGB1 release and RAGE expression is also described to possibly play a role in diabetes-associated periodontitis [20]. Recently, HMGB1 release has been shown to be regulated by inflammasomes [21-23]. However, there is still little known on how different microorganisms or their components regulate the expression and secretion of HMGB1.

As a successful facultative intracellular organism, *P. gingivalis* has evolved an array of virulence factors and associated mechanisms that allow the organism's survival and spread in the gingival epithelium, and evasion of recognition by the host immune system [1]. Prominent virulence factors include gingipains and a secreted homologue of nucleoside-diphosphate kinases (NDKs) [24-27].

NDKs catalyze the transfer of orthophosphates from nucleoside triphosphates, such as ATP, to nucleoside diphosphates, thus enabling it to control nucleotide pools inside the cells [24]. Additionally, secreted NDKs can be used by intracellular persistent bacteria, such as *Mycobacterium tuberculosis* and *P. gingivalis*, to mask their presence from the host immune

system by scavenging extracellular ATP [9, 28-30]. Unlike many other infectious bacteria, *P. gingivalis* can stimulate the production of biologically inactive pro-IL-1 β in infected GECs, but does not induce significant release of the secreted active form of IL-1 β [12]. Active IL-1 β secretion in *P. gingivalis*-infected GECs only occurs after stimulation with extracellular ATP [12]. Our previous studies have shown that *P. gingivalis* NDK secretion is important for maximal inhibition of ATP-triggered P2X₇-mediated apoptosis in infected GECs as well as ATP-induced generation of cellular ROS [13, 31, 32]. These studies imply a potentially critical role for *P. gingivalis* NDK in the modulation of the inflammasome-mediated host response and IL-1 β secretion. The current knowledge on the mechanisms of inflammasome activation has been predominantly derived from cells of the myeloid lineage [33]. Epithelial cells in general, including GECs, have attracted less attention. Additionally the mechanisms of regulation of other inflammasome-dependent danger-signals, such as HMGB1, are widely unexplored. Therefore, we aimed to examine the potential role of the effector molecule NDK on ATP-mediated inflammasome activation and pro-inflammatory cytokine release. We hypothesize that the release of NDK inhibits caspase-1 activation and secretion of pro-inflammatory cytokines and HMGB1 in *P. gingivalis*-infected GECs.

2. Materials and Methods

2.1. Primary and Immortalized GECs

Healthy gingival tissue was obtained by oral surgery to produce primary GECs cultures as previously described [34]. No subject recruitment per se was done. Adult patients were selected at random and anonymously from those presenting at the University of Florida Dental Clinics for tooth crown lengthening or impacted third molar extraction. Gingival tissue that would otherwise be discarded was collected after informed written consent by the patient. This study is approved by the Institutional Review Board under the University of Florida. Cells were cultured as monolayers in serum-free keratinocyte growth medium (Lonza) at 37°C in a 5% CO₂ incubator. The human immortalized GEC (HIGK) cell line was obtained as previously described [35]. Cells were cultured in defined keratinocytes serum-free media (K-SFM, Life Technologies) at 37 °C in a 5% CO₂ incubator.

2.2. *Porphyromonas gingivalis* Culture

Porphyromonas gingivalis ATCC 33277 and its isogenic *ndk*-deficient mutant were cultured anaerobically for 24 h at 37 °C in trypticase soy broth supplemented with yeast extract (1 mg/ml), hemin (5 mg/ml), and menadione (1 mg/ml). Erythromycin (10 mg/ml) was added to the media as a selective agent for the growth of the *ndk*-deficient mutant strain, which was previously described (29). Bacteria were grown for 24 h and collected by centrifugation at 6000 $\times$ g for 10 min at 4 °C. The bacteria were then washed twice and resuspended with Dulbecco's phosphate-buffered saline (PBS) (Life Technologies). Quantification of bacteria was determined using a Klett-Summerson photoelectric colorimeter.

2.3. Infection of Primary and Immortalized GECs with *P. gingivalis* and Treatment with ATP and Inhibitors

Primary GECs and HIGK cells were seeded onto 6-well plates and upon reaching 75-80% confluence the cells were infected with either wild-type or *ndk*-deficient *P. gingivalis* at a multiplicity of infection (MOI) of 100 which has previously been shown to be biologically relevant [27, 36, 37]. Cells used for HMGB1 Western blot assays were collected after 9 hours of infection. For cells treated with the caspase-1 inhibitor, YVAD (z-YVAD-fmk, R&D Systems) [10, 38, 39], YVAD was added to the media at a final concentration of 50 μ M and incubated at 37°C in a 5% CO₂ incubator. The cells were treated with 3 mM ATP (Sigma) 30 minutes after addition of YVAD, or 6 hour after infection and incubated for additional 3 h. Cell-culture fluids were collected and centrifuged at 100 $\times$ g to remove cell debris. Protease inhibitors (PhosStop, Roche) were added to all samples to prevent protein degradation.

2.4. Measurement of IL-1 β Secretion by ELISA

Samples from at least three separate experiments were collected for each infection and treatment condition. Samples were cleared of cellular debris by centrifugation at 800 $\times$ g for 5 min and cell-free supernatants were used for IL-1 β ELISA assay. IL-1 β secretion levels were measured using Human IL-1 β ELISA kit II (BD Biosciences). ELISAs were performed as per manufacturer's instructions. Briefly, samples, controls, and standards were diluted with assay diluent, loaded onto a 96-well plate, and incubated for 2 h at room temperature. Wells were washed and incubated with the detection antibody for 1 h at room temperature. After washing, substrate was added to all wells. The reactions were stopped with stop solution and optical densities were read at an absorbance of 450 nm using a Synergy MX plate reader (BioTek Instruments). P-values were calculated using two-tailed Student's *t*-test.

2.5. Western Blot

Proteins from cell-free culture fluids were concentrated using trichloroacetic acid (TCA) [40]. TCA at 100% was added to supernatants for a final concentration of 20% TCA. Samples were vortexed and incubated on ice. After incubation, samples were centrifuged and supernatants were discarded. The pellets were washed twice with cold acetone and allowed to air-dry. Pellets were resuspended with sample buffer (Thermo Scientific) and boiled. Protein concentration in samples was measured using the BioRad protein assay. Samples were loaded at equal final protein concentrations and run on SDS-PAGE gels, followed by transfer onto PVDF membranes. Membranes were blocked with 5% BSA for one hour at RT, and then incubated with primary antibodies against caspase-1 (Cell Signaling) or HMGB1 (Cell Signaling). After one hour, blots were washed and incubated with secondary anti-rabbit HRP (Millipore) for another hour. Finally, membranes were washed and exposed to Luminata Forte (Millipore) substrate. Images were acquired using ChemiDoc XRS+ system (Bio-Rad) and analyzed using NIH-ImageJ.

2.6. Immunofluorescence Examination of Intracellular IL-1 β and HMGB1

Primary GECs were cultured in 4-well culture plates (Nunc) containing round glass inserts. Cells were infected at a confluence of 75-80% with either wild-type *P. gingivalis* or the *ndk*-

deficient mutant strain at an MOI of 100. Six hours later, cells were treated with ATP at a final concentration of 3 mM for an additional 3 h. Cells were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min. Cells were permeabilized using 0.01% Triton X-100 solution in PBS and incubated with the primary antibodies. IL-1 β was detected using an FITC-conjugated mouse monoclonal antibody (R&D Systems). HMGB1 was detected using a rabbit polyclonal anti-human HMGB1 antibody (Cell Signaling). *P. gingivalis* infection was detected using anti-*P. gingivalis* rabbit polyclonal antibody, coupled with a secondary goat-anti-rabbit polyclonal antibody (Invitrogen), and all inserts were mounted onto glass slides using VectaShield mounting medium (Fisher) containing DAPI stain. The intensity of IL-1 β and HMGB1 staining was measured using NIH-ImageJ, and measurements of at least 25 cells, originating from at least three separate replicate experiments, were averaged.

2.7. Statistical Analysis

Data are reported as mean $\pm$ standard deviation (SD). Statistical significance was calculated by two-tailed Student's *t*-test and was considered significant at $P < 0.05$.

3. Results

3.1. NDK Inhibits Caspase-1 Activation

We previously showed that ATP stimulation of P2X₇ resulted in assembly with P2X₄ and pannexin-1 and generation of reactive oxygen species (ROS) [10]. Stimulation with ATP alone could induce caspase-1 activation in GECs, but *P. gingivalis* infection diminished the effect of ATP [12], likely through hydrolysis of extracellular ATP by secretion of the NDK homologue [31]. Therefore, we aimed here to evaluate the effects of *P. gingivalis* NDK on ATP-mediated inflammasome activation during infection of GECs. As shown in Figure 1A and B, HIGKs infected with wild-type *P. gingivalis* showed reduced ATP-induced caspase-1 activation, whereas cells infected with *ndk*-deficient *P. gingivalis* resembled uninfected cells, with higher levels of secreted (activated) caspase-1. This suggests that the NDK homologue of *P. gingivalis* regulates caspase-1 activation and may therefore influence secretion of pro-inflammatory cytokines.

3.2. NDK Inhibits IL-1 β secretion

Our previous studies have shown that *P. gingivalis*-infected or LPS-primed GECs require stimulation with a second danger signal, such as ATP, in order to release active IL-1 β [12]. In order to evaluate the role of NDK in inhibiting signaling by ATP, we infected primary GECs with *P. gingivalis* followed by stimulation with ATP, and examined the supernatant for IL-1 β by ELISA. The results demonstrated significantly lower ATP-induced IL-1 β secretion in wild-type *P. gingivalis*-infected GECs, compared to *ndk*-deficient *P. gingivalis*-infected GECs or uninfected cells (Fig. 2).

Immunofluorescence staining for IL-1 β showed that *ndk*-deficient *P. gingivalis* induces higher levels of IL-1 β accumulation within primary GECs (Fig. 3A) and there is a significant decrease in fluorescence intensity upon stimulation with ATP. In contrast, GECs infected with wild-type *P. gingivalis* showed reduced IL-1 β release and relatively higher

intracellular staining after ATP treatment, compared with cells infected with *ndk*-deficient *P. gingivalis* (Fig. 3B), suggesting that NDK produced by wild-type *P. gingivalis* inhibits ATP-mediated IL-1 β secretion.

3.3. NDK Modulates the Release of HMGB1

We next evaluated whether *P. gingivalis* NDK affects release of another danger signaling molecule, HMGB1. Under normal conditions, HMGB1 is found within the nucleus of eukaryotic cells and is associated with DNA, where it functions as a regulator of gene expression [17]. However, HMGB1 can act as a danger signaling molecule when released by stressed cells or cells undergoing necrosis [17]. In a previous study using *Salmonella*-infected macrophages, it was shown that HMGB1 release is caspase-1 activation-dependent [41]. We therefore hypothesized that NDK may inhibit HMGB1 release due to its ability to inhibit ATP-mediated caspase-1 activation. To study this possibility, we examined the release of HMGB1 by Western blot analysis. Our results showed that ATP treatment induces HMGB1 release from uninfected GECs, and that wild-type *P. gingivalis* infection is more effective than infection with *ndk*-deficient *P. gingivalis* in inhibiting ATP-induced HMGB1 release (Fig. 4A and B).

Due to the localization of HMGB1 in the nucleus under quiescent conditions, we hypothesized that HMGB1-release may require relocation from the nucleus through the cytosol and to the extracellular space. We therefore performed immunofluorescence staining against HMGB1 and confirmed the expected nuclear localization of HMGB1 in uninfected untreated primary GECs (Figure 5A). However, when uninfected cells were treated with ATP, we observed a relocation of HMGB1 from the nucleus to the extracellular space, which was attenuated in cells treated with the caspase-1 inhibitor. Moreover, there was relocation of HMGB1 from the nucleus to the cytosol in GECs infected with either the wild-type or *ndk*-deficient *P. gingivalis* strain. Upon ATP stimulation, the intensity of HMGB1 in the cytosol was significantly decreased in the cells infected with *ndk*-deficient *P. gingivalis*, consistent with the release of HMGB1 from the cell demonstrated in the Western blot assay (Figure 5A and B). The cells infected with the wild-type *P. gingivalis* showed a substantially higher intensity of HMGB1 in the cytosol after ATP stimulation, compared to cells infected with the *ndk*-deficient *P. gingivalis*, supporting the proposed role of *P. gingivalis* NDK in inhibiting ATP-mediated HMGB1 release.

4. Discussion

The healthy human oral mucosa is colonized by a diversity of organisms, including high numbers of different bacterial species in addition to fungi and protozoa [42]. These microbes can reside within pockets between the teeth and epithelium to form biofilms and carry on as commensals or potential pathogenic colonizers [43]. Some of these microorganisms have evolved elaborate virulence factors to ensure their survival within the oral mucosa without eliciting an immediate destructive host immune response [1]. Among these organisms, *P. gingivalis* uses its major fimbriae to adhere to the host-cell membrane and quickly localize to the endoplasmic reticulum rich perinuclear region [13, 27, 44]. *P. gingivalis* infection can also inhibit the host apoptotic response in GECs by activating the PI3K/Akt pathway and modulating expression of Bcl-2 family proteins [1, 45]. Finally, *P. gingivalis* secretes an

enzyme homologue of the evolutionarily conserved NDKs [13,31], which is critical for suppression of cellular ROS production and ATP-induced host cell death [13, 31]. In the present study, we show that the lack of NDK significantly reduces the inhibition of ATP-dependent inflammasome activation and subsequent release of pro-inflammatory cytokines by GECs. Our previous studies have elucidated the role of this effector of *P. gingivalis* as an important modulator of cellular ROS generation, as well as demonstrated that the *ndk*-deficient strain has significantly less intracellular survival ability compared to its isogenic wild strain upon ATP-stimulation in primary GECs [12, 13, 31]. Further, there was a significant intracellular recovery of the *ndk*-deficient strain after treatment with the potent ROS-inhibitor N-acetyl-cysteine (NAC) and complementation of the *ndk* gene restored the observed phenotype [13]. Since ROS generation contributes to inflammasome activation [46], our results showing a suppression of caspase-1 activation by wild-type *P. gingivalis* infection was expected. Our previous studies have extensively demonstrated that *P. gingivalis* is a facultative intracellular bacterium and in the GECs, the majority of bacteria rapidly enters and resides in the GECs [1, 36, 47]. Further, our previous studies displayed that GECs that are infected with *P. gingivalis* had intact membranes while their supernatant showed a time-dependent release of the small NDK molecule [13]. Altogether, this evidence points that the majority of the NDK in supernatants are derived from the intracellular bacteria, rather than incidental extracellular *P. gingivalis*.

Previous studies have shown that IL-1 β secretion requires two signals. Recognition of a pathogen-associated molecular pattern by a pattern recognition receptor such as TLR provides the first signal and stimulates pro-IL-1 β production [48]. A second signal, from a danger signal such as ATP, activates the inflammasome and caspase-1, resulting in processing of pro-IL-1 β and secretion of the mature cytokine, IL-1 β [48]. We hypothesized that ATP-dependent IL-1 β secretion would be reduced by infection with wild-type *P. gingivalis* due to its ability to produce NDK, which should scavenge extracellular ATP. The findings in this study confirm the anti-inflammatory role of NDK in infected GECs.

We also investigated whether *P. gingivalis* infection could affect the release by GECs of another danger signal, HMGB1 [49, 50]. Unexpectedly, our studies also revealed that *P. gingivalis* induces translocation of HMGB1 from the nucleus to the cytosol. However, similarly to IL-1 β secretion, ATP was required as a secondary stimulus to release HMGB1 from the cell (Fig. 6). Furthermore, *ndk*-deficient *P. gingivalis* infection was less effective than infection with wild-type *P. gingivalis* in inhibiting HMGB1 release from infected GECs, which also required caspase-1 activation.

NDKs have recently attracted attention due to their involvement in a variety of chronic inflammatory diseases, and increased expression of human NDK was recently described in affected tissues of oral squamous cell carcinoma patients [51]. In addition, *P. gingivalis* is associated with oral squamous cell carcinomas and other chronic diseases [3, 52]. Our findings also support studies showing that HMGB1 is another possible candidate for an early inflammatory disease marker [53, 54]. Collectively, these results suggest that *P. gingivalis* NDK dampens the host-immune response by decreasing release of pro-inflammatory cytokine, IL-1 β , and the pro-inflammatory danger signal, HMGB1, from infected GECs.

Identification of NDK as a key mediator used by *P. gingivalis* to modulate inflammation suggests that therapeutic strategies against periodontitis could also target this effector.

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References

1. Yilmaz O. The chronicles of Porphyromonas gingivalis: the microbium, the human oral epithelium and their interplay. Microbiology. 2008; 154:2897–2903. [PubMed: 18832296]
2. Darveau RP. Periodontitis: a polymicrobial disruption of host homeostasis. Nat Rev Microbiol. 2010; 8:481–490. [PubMed: 20514045]
3. Atanasova KR, Yilmaz O. Looking in the Porphyromonas gingivalis cabinet of curiosities: the microbium, the host and cancer association. Mol Oral Microbiol. 2014; 29:55–66. [PubMed: 24506890]
4. Krisanaprakornkit S, Kimball JR, Weinberg A, Darveau RP, Bainbridge BW, Dale BA. Inducible expression of human beta-defensin 2 by Fusobacterium nucleatum in oral epithelial cells: multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier. Infect Immun. 2000; 68:2907–2915. [PubMed: 10768988]
5. Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. Annu Rev Immunol. 2009; 27:229–265. [PubMed: 19302040]
6. Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, Washburn NR, et al. The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. Immunol Rev. 2007; 220:60–81. [PubMed: 17979840]
7. Said-Sadier N, Ojcius DM. Alarmins, inflammasomes and immunity. Biomed J. 2012; 35:437–449. [PubMed: 23442356]
8. Martinon F, Gaide O, Petrilli V, Mayor A, Tschopp J. NALP inflammasomes: a central role in innate immunity. Semin Immunopathol. 2007; 29:213–229. [PubMed: 17703304]
9. Coutinho-Silva R, Ojcius DM. Role of extracellular nucleotides in the immune response against intracellular bacteria and protozoan parasites. Microbes Infect. 2012; 14:1271–1277. [PubMed: 22634346]
10. Hung SC, Choi CH, Said-Sadier N, Johnson L, Atanasova KR, Sellami H, et al. P2X4 assembles with P2X7 and pannexin-1 in gingival epithelial cells and modulates ATP-induced reactive oxygen species production and inflammasome activation. PLoS One. 2013; 8:e70210. [PubMed: 23936165]
11. Schwiebert EM, Zsembery A. Extracellular ATP as a signaling molecule for epithelial cells. Biochim Biophys Acta. 2003; 1615:7–32. [PubMed: 12948585]
12. Yilmaz O, Sater AA, Yao L, Koutouzis T, Pettengill M, Ojcius DM. ATP-dependent activation of an inflammasome in primary gingival epithelial cells infected by Porphyromonas gingivalis. Cell Microbiol. 2010; 12:188–198. [PubMed: 19811501]
13. Choi CH, Spooner R, DeGuzman J, Koutouzis T, Ojcius DM, Yilmaz O. Porphyromonas gingivalis-nucleoside-diphosphate-kinase inhibits ATP-induced reactive-oxygen-species via P2X7 receptor/NADPH-oxidase signalling and contributes to persistence. Cell Microbiol. 2013; 15:961–976. [PubMed: 23241000]
14. Keyel PA. How is inflammation initiated? Individual influences of IL-1, IL-18 and HMGB1. Cytokine. 2014; 69:136–145. [PubMed: 24746243]
15. Vande Walle L, Kanneganti TD, Lamkanfi M. HMGB1 release by inflammasomes. Virulence. 2011; 2:162–165. [PubMed: 21422809]
16. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, et al. HMG-1 as a late mediator of endotoxin lethality in mice. Science. 1999; 285:248–251. [PubMed: 10398600]

17. Klune JR, Dhupar R, Cardinal J, Billiar TR, Tsung A. HMGB1: endogenous danger signaling. *Mol Med*. 2008; 14:476–484. [PubMed: 18431461]
18. Kamo N, Ke B, Ghaffari AA, Shen XD, Busuttil RW, Cheng G, et al. ASC/caspase-1/IL-1 β signaling triggers inflammatory responses by promoting HMGB1 induction in liver ischemia/reperfusion injury. *Hepatology*. 2013; 58:351–362. [PubMed: 23408710]
19. Pang X, Zhang Y, Wei H, Zhang J, Luo Q, Huang C, et al. Expression and effects of high-mobility group box 1 in cervical cancer. *Int J Mol Sci*. 2014; 15:8699–8712. [PubMed: 24837834]
20. Morimoto-Yamashita Y, Ito T, Kawahara K, Kikuchi K, Tatsuyama-Nagayama S, Kawakami-Morizono Y, et al. Periodontal disease and type 2 diabetes mellitus: is the HMGB1-RAGE axis the missing link? *Med Hypotheses*. 2012; 79:452–455. [PubMed: 22800803]
21. Lu B, Wang H, Andersson U, Tracey KJ. Regulation of HMGB1 release by inflammasomes. *Protein Cell*. 2013; 4:163–167. [PubMed: 23483477]
22. Lamkanfi M, Sarkar A, Vande Walle L, Vitari AC, Amer AO, Wewers MD, et al. Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia. *J Immunol*. 2010; 185:4385–4392. [PubMed: 20802146]
23. Willingham SB, Allen IC, Bergstralh DT, Brickey WJ, Huang MT, Taxman DJ, et al. NLRP3 (NALP3, Cryopyrin) facilitates in vivo caspase-1 activation, necrosis, and HMGB1 release via inflammasome-dependent and -independent pathways. *J Immunol*. 2009; 183:2008–2015. [PubMed: 19587006]
24. Spooner R, Yilmaz O. Nucleoside-diphosphate-kinase: a pleiotropic effector in microbial colonization under interdisciplinary characterization. *Microbes Infect*. 2012; 14:228–237. [PubMed: 22079150]
25. Grenier D, Imbeault S, Plamondon P, Grenier G, Nakayama K, Mayrand D. Role of gingipains in growth of *Porphyromonas gingivalis* in the presence of human serum albumin. *Infect Immun*. 2001; 69:5166–5172. [PubMed: 11447200]
26. Into T, Inomata M, Kanno Y, Matsuyama T, Machigashira M, Izumi Y, et al. Arginine-specific gingipains from *Porphyromonas gingivalis* deprive protective functions of secretory leucocyte protease inhibitor in periodontal tissue. *Clin Exp Immunol*. 2006; 145:545–554. [PubMed: 16907925]
27. Yilmaz O, Young PA, Lamont RJ, Kenny GE. Gingival epithelial cell signalling and cytoskeletal responses to *Porphyromonas gingivalis* invasion. *Microbiology*. 2003; 149:2417–2426. [PubMed: 12949167]
28. Sansom FM, Robson SC, Hartland EL. Possible effects of microbial ecto-nucleoside triphosphate diphosphohydrolases on host-pathogen interactions. *Microbiol Mol Biol Rev*. 2008; 72:765–781. [PubMed: 19052327]
29. Galgiani JN, Ampel NM, Blair JE, Catanzaro A, Johnson RH, Stevens DA, et al. Coccidioidomycosis. *Clin Infect Dis*. 2005; 41:1217–1223. [PubMed: 16206093]
30. Zaborina O, Li X, Cheng G, Kapatral V, Chakrabarty AM. Secretion of ATP-utilizing enzymes, nucleoside diphosphate kinase and ATPase, by *Mycobacterium bovis* BCG: sequestration of ATP from macrophage P2Z receptors? *Mol Microbiol*. 1999; 31:1333–1343. [PubMed: 10200955]
31. Yilmaz O, Yao L, Maeda K, Rose TM, Lewis EL, Duman M, et al. ATP scavenging by the intracellular pathogen *Porphyromonas gingivalis* inhibits P2X7-mediated host-cell apoptosis. *Cell Microbiol*. 2008; 10:863–875. [PubMed: 18005240]
32. Spooner R, Yilmaz O. The role of reactive-oxygen-species in microbial persistence and inflammation. *Int J Mol Sci*. 2011; 12:334–352. [PubMed: 21339989]
33. Yazdi AS, Drexler SK, Tschopp J. The role of the inflammasome in nonmyeloid cells. *J Clin Immunol*. 2010; 30:623–627. [PubMed: 20582456]
34. Yilmaz O, Jungas T, Verbeke P, Ojcius DM. Activation of the phosphatidylinositol 3-kinase/Akt pathway contributes to survival of primary epithelial cells infected with the periodontal pathogen *Porphyromonas gingivalis*. *Infect Immun*. 2004; 72:3743–3751. [PubMed: 15213114]
35. Oda D, Bigler L, Lee P, Blanton R. HPV immortalization of human oral epithelial cells: a model for carcinogenesis. *Exp Cell Res*. 1996; 226:164–169. [PubMed: 8660952]

36. Choi CH, DeGuzman JV, Lamont RJ, Yilmaz O. Genetic transformation of an obligate anaerobe, *P. gingivalis* for FMN-green fluorescent protein expression in studying host-microbe interaction. *PLoS One*. 2011; 6:e18499. [PubMed: 21525983]
37. Dawes C. Estimates, from salivary analyses, of the turnover time of the oral mucosal epithelium in humans and the number of bacteria in an edentulous mouth. *Arch Oral Biol*. 2003; 48:329–336. [PubMed: 12711376]
38. Bian ZM, Elner SG, Elner VM. Dual involvement of caspase-4 in inflammatory and ER stress-induced apoptotic responses in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 2009; 50:6006–6014. [PubMed: 19643964]
39. Chen CC, Tsai SH, Lu CC, Hu ST, Wu TS, Huang TT, et al. Activation of an NLRP3 inflammasome restricts *Mycobacterium kansasii* infection. *PLoS One*. 2012; 7:e36292. [PubMed: 22558425]
40. Koontz, L. Chapter One - TCA Precipitation. In: Jon, L., editor. *Methods Enzymol*. Academic Press; 2014. p. 3-10.
41. Lamkanfi M, Sarkar A, Vande Walle L, Vitari AC, Amer AO, Wewers MD, et al. Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia. *J Immunol*. 2010; 185:4385–4392. [PubMed: 20802146]
42. Zaura E, Nicu EA, Krom BP, Keijser BJ. Acquiring and maintaining a normal oral microbiome: current perspective. *Front Cell Infect Microbiol*. 2014; 4:85. [PubMed: 25019064]
43. Avila M, Ojcius DM, Yilmaz O. The oral microbiota: living with a permanent guest. *DNA Cell Biol*. 2009; 28:405–411. [PubMed: 19485767]
44. Yilmaz O, Watanabe K, Lamont RJ. Involvement of integrins in fimbriae-mediated binding and invasion by *Porphyromonas gingivalis*. *Cell Microbiol*. 2002; 4:305–314. [PubMed: 12027958]
45. Yao L, Jermanus C, Barbetta B, Choi C, Verbeke P, Ojcius DM, et al. *Porphyromonas gingivalis* infection sequesters pro-apoptotic Bad through Akt in primary gingival epithelial cells. *Mol Oral Microbiol*. 2010; 25:89–101. [PubMed: 20331797]
46. Tschopp J, Schroder K. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? *Nat Rev Immunol*. 2010; 10:210–215. [PubMed: 20168318]
47. Yilmaz O, Verbeke P, Lamont RJ, Ojcius DM. Intercellular spreading of *Porphyromonas gingivalis* infection in primary gingival epithelial cells. *Infect Immun*. 2006; 74:703–710. [PubMed: 16369027]
48. Lopez-Castejon G, Brough D. Understanding the mechanism of IL-1beta secretion. *Cytokine Growth Factor Rev*. 2011; 22:189–195. [PubMed: 22019906]
49. Eskin MA, Benakanakere MR, Rose BG, Zhang P, Zhao J, Stathopoulou P, et al. Interleukin-1beta modulates proinflammatory cytokine production in human epithelial cells. *Infect Immun*. 2008; 76:2080–2089. [PubMed: 18332211]
50. Erlandsson, Harris H.; Andersson, U. Mini-review: The nuclear protein HMGB1 as a proinflammatory mediator. *Eur J Immunol*. 2004; 34:1503–1512. [PubMed: 15162419]
51. Turhani D, Krapfenbauer K, Thurnher D, Langen H, Fountoulakis M. Identification of differentially expressed, tumor-associated proteins in oral squamous cell carcinoma by proteomic analysis. *Electrophoresis*. 2006; 27:1417–1423. [PubMed: 16568407]
52. Atanasova KR, Yilmaz O. Prelude to oral microbes and chronic diseases: past, present and future. *Microbes Infect*. 2015 In Press.
53. Nogueira AV, de Souza JA, de Molon RS, Pereira Eda S, de Aquino SG, Giannobile WV, et al. HMGB1 localization during experimental periodontitis. *Mediators Inflamm*. 2014; 2014:816320. [PubMed: 24692854]
54. Luo L, Xie P, Gong P, Tang XH, Ding Y, Deng LX. Expression of HMGB1 and HMGN2 in gingival tissues, GCF and PICF of periodontitis patients and peri-implantitis. *Arch Oral Biol*. 2011; 56:1106–1111. [PubMed: 21570059]

Figure 1. NDK modulates ATP-induced caspase-1 activation in GECs

Cells were infected or uninfected with wild-type *P. gingivalis* or *ndk*-deficient *P. gingivalis* for 6 h, and then treated with 3 mM ATP for 3 h. (A) Caspase-1 (20 kDa) activation in HIGK cell supernatants was detected by SDS-PAGE Western blot assay. (B) Average of three Western blot experiments, quantified by densitometry. Error bars represent the standard deviations ($\pm$ SD) of at least two independent experiments ran in at least duplicates (* $P < 0.05$ Student *t*-test).

Figure 2. NDK hydrolysis of ATP inhibits secretion of IL-1 β in ATP-induced GECs

Cells were infected or uninfected with wild-type *P. gingivalis* or *ndk*-deficient *P. gingivalis* for 6 h, and then treated with 3 mM ATP for 3 h. ELISA of IL-1 β secretion was measured in supernatants of *P. gingivalis*-infected primary GECs treated with ATP. Error bars represent the standard deviations ($\pm$ SD) of at least two independent experiments ran in at least duplicates (** $P < 0.001$ and *** $P < 0.0001$ Student *t*-test).

Figure 3. NDK affects intracellular IL-1 β accumulation and ATP-dependent release in primary GECs

GECs were infected or uninfected with wild-type *P. gingivalis* or *ndk*-deficient *P. gingivalis* for 6 h, and then treated with 3 mM ATP for 3 h. (A) Immunofluorescence staining of DAPI-labeled nuclei (blue), Alexa-Fluor-594-labeled *P. gingivalis* (red), and fluorescein-isothiocyanate (FITC)-labeled IL-1 β (green). Images were taken at 40X magnification, and scale bars represent 10 μ m. (B) Quantification analysis for mean fluorescence intensity was performed using NIH-ImageJ. Error bars represent the standard deviations ($\pm$ SD) of at least 25 independent cells (* P <0.05 and *** P <0.0001 Student *t*-test).

Figure 4. NDK affects release of HMGB1 in GECs

Cells were infected or uninfected with wild-type *P. gingivalis* or *ndk*-deficient *P. gingivalis* for 6 h, and then treated with 3 mM ATP for 3 h. (A) HMGB1 secretion in GEC cell supernatants was imaged by SDS-PAGE Western blot assay. (B) Average of two Western blot experiments, quantified by densitometry. Error bars represent the standard deviations ($\pm$ SD) (* $P < 0.05$ Student t-test).

Figure 5. NDK or *P. gingivalis* infection affects intracellular HMGB1 localization in primary GECs or ATP-mediated HMGB1 secretion

Cells were infected or uninfected with wild-type *P. gingivalis* or *ndk*-deficient *P. gingivalis* for 6 h, and then treated with 3 mM ATP for 3 h. YVAD inhibitor was administered at a final concentration of 50 μ M prior to ATP stimulation. (A) Immunofluorescence staining representing DAPI-labeled nuclei (blue), Alexa-Fluor-594-labeled *P. gingivalis* (red), and Alexa-Fluor-488-labeled HMGB1 (green). Images were taken at 40X magnification, and scale bars represent 10 μ m. (B) Quantification analysis for mean fluorescence intensity was

performed using NIH-ImageJ. Error bars represent the standard deviations ($\pm$ SD) of at least 25 independent cells (**P<0.001 Student *t*-test).

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Figure 6. Proposed model for the role of NDK in HMGB1 localization during *P. gingivalis* infection

(A) *P. gingivalis* inhibits ATP-mediated caspase-1 activation and maturation of IL-1β by NDK hydrolysis of ATP. (B) *P. gingivalis* modulates caspase-1 dependent HMGB1 release from the host cell [12, 13].