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Cell Microbiol. Author manuscript; available in PMC 2011 February 1.

Published in final edited form as:
Cell Microbiol. 2010 February ; 12(2): 188–198. doi:10.1111/j.1462-5822.2009.01390.x.

ATP-dependent activation of an inflammasome in primary gingival epithelial cells infected by *Porphyromonas gingivalis*

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Summary

Production of IL-1β typically requires two-separate signals. The first signal, from a pathogen-associated molecular pattern, promotes intracellular production of immature cytokine. The second signal, derived from a danger signal such as extracellular ATP, results in assembly of an inflammasome, activation of caspase-1, and secretion of mature cytokine. The inflammasome component, Nalp3, plays a nonredundant role in caspase-1 activation in response to ATP binding to $P2X_7$ in macrophages. Gingival epithelial cells (GECs) are an important component of the innate-immune response to periodontal bacteria. We had shown that GECs express a functional $P2X_7$ receptor, but the ability of GECs to secrete IL-1β during infection remained unknown. We find that GECs express a functional Nalp3-inflammasome. Treatment of GECs with LPS or infection with the periodontal pathogen, *Porphyromonas gingivalis*, induced expression of the *il-1β* gene and intracellular accumulation of IL-1β protein. However, IL-1β was not secreted unless LPS-treated or infected cells were subsequently stimulated with ATP. Conversely, caspase-1 is activated in GECs following ATP-treatment but not *P. gingivalis*-infection. Furthermore, depletion of Nalp3 by siRNA abrogated the ability of ATP to induce IL-1β secretion in infected-cells. The Nalp3-inflammasome is therefore a likely to be an important mediator of the inflammatory response in gingival epithelium.

Keywords

NLR; innate immunity; purinergic receptor; inflammasome; bacteria

Introduction

Within the gingival compartment, epithelial cells are among the first host-cells encountered by periodontal pathogens such as *Porphyromonas gingivalis*. In addition to constituting a physical barrier to invasion by microbial pathogens, epithelial cells can sense and respond to the presence of bacteria through stimulation of pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs) (Meylan *et al*., 2006; Inohara *et al*., 2005; Janeway Jr. and Medzhitov, 2002). Consequently, gingival epithelial cells (GECs) are an important component of the innate host response to periodontal bacteria and

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make a significant contribution to the gingival health of the host (Eskan *et al*., 2008; Hasegawa *et al*., 2008; Yilmaz, 2008; Darveau *et al*., 1998)

Most studies on the innate immune response against bacterial infections have focused on immune cells of hematopoietic origin, but epithelial cells infected by *P. gingivalis* and other oral bacteria can also produce proinflammatory cytokines, chemokines and defensins, and upregulate cell adhesion molecules (Hasegawa *et al*., 2008; Eick *et al*., 2006; Huang *et al*., 2004; Kusumoto *et al*., 2004; Asai *et al*., 2001; Scannapieco *et al*., 2001; Krisanaprakornkit *et al*., 2000; Sandros *et al*., 2000a; Darveau *et al*., 1998; Darveau *et al*., 1997). *P. gingivalis* is a successful host-adapted self-limiting intracellular bacterium that is capable of intracellular replication and can maintain viability for extended periods of time. Furthermore, *P. gingivalis* infection promotes an anti-apoptotic phenotype in primary GECs by rendering the host cells resistant to cell death induced by potent pro-apoptotic agents ((Mao *et al*., 2007; Yilmaz *et al*., 2004; Nakhjiri *et al*., 2001). Although much is known about virulence factors produced by *P. gingivalis* (Hajishengallis *et al*., 2008; Hintermann *et al*., 2002; Michalek *et al*., 2002; Nisapakultorn *et al*., 2001; Katz *et al*., 2000; Curtis *et al*., 1999; Holt *et al*., 1999; Lamont and Jenkinson, 1998; Cutler *et al*., 1995), recognition of the bacteria by PRRs on GECs, or the pathogen's ability to subvert an immune response, remain poorly understood.

Ligation of surface-exposed TLRs is sufficient for transcription, synthesis, and secretion of cytokines such as IL-8, IFNγ and IL-12 (Takeda *et al*., 2003; Janeway Jr. and Medzhitov, 2002). But given the key role played by IL-1β and IL-18 in fever and inflammatory disease (Dinarello, 2006; Ferrari *et al*., 2006), their production and secretion are tightly controlled, requiring typically two separate signals (Ferrari *et al*., 2006; Lich *et al*., 2006; Mariathasan *et al*., 2006; Meylan *et al*., 2006; Kahlenberg *et al*., 2005). The first signal, from a pathogen-associated molecular pattern (PAMP) such as flagellin or lipopolysaccharide (LPS), promotes production and intracellular accumulation of the immature cytokines. The second signal, usually derived from a "danger signal" (DS) such as ATP, results in processing and secretion of the mature cytokines. Danger signals released from infected, dying or stressed cells interact with "danger signal receptors" (DSRs) on neighboring cells to induce/alter the host response (Lich *et al*., 2006; Tournier and Quesnel-Hellmann, 2006; Lotze and Tracey, 2005; Sitkovsky and Ohta, 2005; Seong and Matzinger, 2004; Matzinger, 2002). Most studies on DSRs have focused on immune effector cells of hematopoietic origin (Ferrari *et al*., 2006; Lich *et al*., 2006; Meylan *et al*., 2006), but we find that at least one DSR, the purinergic receptor for extracellular ATP, called $P2X_7$ (Lich *et al*., 2006; Ferrari *et al*., 1997), is also expressed and is functional on GECs (Yilmaz *et al*., 2008). Moreover, *P. gingivalis*, a successful intracellular opportunistic bacterium, can inhibit GEC apoptosis induced by ATP ligation of $P2X_7$ (Yilmaz *et al*., 2008).

Recent studies show that DSs and PAMPs can synergize in promoting IL-1β secretion and inflammation. Thus, the NLR family member, Nalp3 (also known as cryopyrin or NLRP3) (Ting *et al*., 2008), is activated in response to stimulation of cells with extracellular ATP, some microbial products, or gout-associated uric acid crystals (Kanneganti *et al*., 2006; Lich *et al*., 2006; Martinon *et al*., 2006; Sutterwala *et al*., 2006; Mariathasan *et al*., 2004). Nalp3 then activates the protease caspase-1, which cleaves pro-IL-1β or pro-IL-18, resulting in their secretion. Hence, a pathogen must both express a PAMP capable of driving cytokine synthesis, and be viewed as potentially dangerous to the host organism, in order for mature IL-1β and IL-18 to be secreted efficiently.

We have observed that GECs express Nalp3, and that cells infected with *P. gingivalis* transcribe the gene encoding IL-1β. However, infected GECs do not secrete IL-1β protein. We therefore explored the possibility that $P2X_7$ ligation by ATP, through its ability to

activate a Nalp3 inflammasome in GECs, may stimulate processing and secretion of IL-1β during *P. gingivalis* infection.

Results

GECs express components of an inflammasome

We first verified that *P. gingivalis* infection of GECs leads to transcription of the *il-1β* gene by real-time PCR, using primers specific for human *il-1β*. In fact, the expression of the *il-1β* gene increased by a factor of 1.95 ± 0.04 ($P < 0.001$) after a 6 hr infection of primary GECs with *P. gingivalis* 33277 at a multiplicity of infection (MOI) of 100 (Fig. 1A), compared to basal levels. These results are consistent with a previous report using oligonucleotide arrays of host-cell genes, which showed a two-fold upregulation of the *il-1β* gene after a 30 min of *P. gingivalis* infection, and significant ($P < 0.001$) upregulation of *il-1β* transcription after a 2 hrs of infection (Handfield *et al*., 2005;Sandros *et al*., 2000b). These results imply that *P. gingivalis* produces at least the PAMPs necessary for inducing *il-1β* gene transcription in GECs.

In addition, by RT-PCR, we find that primary GECs express the Nalp3 gene at high levels (Fig. 1B). PCR amplification was carried out with primers specific for human Nalp3 (amplicon expected size, 200 bp). The RT-PCR control, without reverse transcriptase, did not produce a detectable band. Primary GECs also express the NLR family members Nalp1 and Ipaf (Fig. 1B). In humans, Nalp1 is a PRR for cytosolic peptidoglycan, while Ipaf is mainly a PRR for cytosolic flagellin. Thus primary GECs could potentially stimulate any of these three inflammasomes.

We had previously shown that a functional purinergic receptor, $P2X_7$, is expressed on primary GECs and *P. gingivalis* can inhibit the GEC apoptosis induced by ATP ligation of $P2X_7$ (Yilmaz *et al*., 2008). Furthermore, only ligation of $P2X_7$ by ATP is known to stimulate activation of the Nalp3 inflammasome and caspase-1, resulting in processing and secretion of the IL-1β protein, as demonstrated by studies with macrophages deficient in $P2X_7$ (Ferrari *et al*., 2006; Mariathasan *et al*., 2006). We therefore evaluated whether GECs may be capable of secreting IL-1β following stimulation of either LPS-primed or *P. gingivalis*-infected GECs with ATP.

Unless *P. gingivalis* can provide its own DS in addition to PAMPs, there should be no IL-1β secretion following infection alone. Consistent with this possibility, we find that primary GECs do not secrete any IL-1β (above basal levels) following either treatment with *E. coli* LPS for 6 hrs or *P. gingivalis* infection for 6 hrs (Fig. 2). There was a small, reproducible increase in IL-1β secretion when uninfected GECs were treated with 5 mM ATP for 3 hrs. However, a high level of IL-1β secretion was observed only in GECs that were infected with *P. gingivalis* for 6 hrs, followed by incubation with ATP for 3 hrs (Fig. 2).

Among several inflammasomes that have been characterized, only the Nalp3 inflammasome can be activated by extracellular ATP through $P2X_7$ ligation (Pétrilli *et al*., 2007). These results therefore suggest that GECs express a functional Nalp3 inflammasome. Furthermore, since *P. gingivalis* infection by itself does not induce IL-1β secretion, the results imply that *P. gingivalis* does not activate to a significant level either the Nalp1 or Ipaf inflammasome.

GEC infection by P. gingivalis leads to production of intracellular cytokine

IL-1β is first synthesized as pro-IL-1β, which is cleaved by caspase-1 into mature IL-1β. In order to rule out the possibility that ATP may be stimulating synthesis of pro-IL-1β, instead of simply promoting secretion of mature IL-1β protein, we also measured intracellular accumulation of IL-1β by immunofluorescence microscopy, using antibodies against IL-1β

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protein. This antibody can not distinguish between pro-IL-1β or processed IL-1β, but nonetheless allowed us to quantify the total amount of cytokine (pro-IL-1β and IL-1β) that had accumulated within the cell.

GECs were mock-infected or infected with *P. gingivalis* for 6 hrs, and ATP or control buffer was added for an additional 3 hrs. As shown in Fig. 3, there is no measurable IL-1β in uninfected cells treated with control buffer (Fig. 3A-iiii) or uninfected cells treated with ATP for 3 hrs (Fig.3A-iii). However, there is a large increase in IL-1β staining in GECs infected with *P. gingivalis* (Fig. 3A-i). The IL-1β staining is visibly lower in infected GECs that were subsequently incubated with ATP, indicating the release of accumulated IL-1β from infected GECs into the extracellular medium (Fig. 3A-ii).

We quantified the extent of ATP-induced release of IL-1β from infected cells by measuring relative fluorescence staining with NIH ImageJ analysis software, as we had previously done for quantifying *P. gingivalis* infection (Yilmaz *et al*., 2006). Thus, ATP alone, which is not thought to induce cytokine production on its own (Petrilli *et al*., 2007; Ferrari *et al*., 2006), has no effect on intracellular IL-1β staining in GECs, compared to untreated controls (Fig. 3B). However, a 6 hr infection with *P. gingivalis* resulted in a large increase in intracellular IL-1β staining. The intracellular IL-1β levels decreased substantially in infected cells that were then treated with ATP for 3 hrs (Fig. 3B).

Given that the concentration of IL-1β in the supernatant of *P. gingivalis*-infected GECs increased significantly after ATP treatment (Fig. 2), these results strongly suggest that *P. gingivalis* infection stimulates production of pro-IL-1β and its intracellular accumulation, but ATP induces release of IL-1β from the infected cells. This interpretation is also supported by the observation that addition of ATP to GECs infected with *P. gingivalis* does not further augment the two-fold increase in *il-1β* gene transcription due to infection alone (not shown).

Nalp3 depletion inhibits ATP-dependent IL-1β secretion from P. gingivalis infected GECs

Our results suggest that *P. gingivalis* infection of GECs leads to intracellular accumulation of pro- IL-1β, but the cytokine is not secreted unless the infected cells are subsequently treated with extracellular ATP, presumably via $P2X_7$ ligation. $P2X_7$-mediated inflammasome activation in macrophages requires participation of a Nalp3 inflammasome. Therefore, we investigated the potential involvement of Nalp3 in IL-1β secretion by depleting Nalp3 in GECs through RNA interference. As shown in Fig. 4A, Nalp3 expression in GECs was decreased by more than half in GECs treated with Nalp3-specific siRNA, while transfection with nontarget siRNA had no effect. Depletion of Nalp3 had no effect on IL-1β secretion from primary GECs treated with LPS (not shown) or infected with *P. gingivalis*. However, there was a significant effect of Nalp3 depletion on IL-1β secretion from cells that were infected with *P. gingivalis* and subsequently stimulated with ATP (Fig. 4B). The results demonstrate that activation of a Nalp3 inflammasome is required for secretion of most of the IL-1β in *P. gingivalis*-infected cells treated with ATP.

ATP treatment stimulates activation of caspase-1 in GECs

We further examined a role for an inflammasome by measuring caspase-1 activation in LPS-primed or *P. gingivalis*-infected GECs. Caspase-1 activation was measured by incubating LPS-treated or infected cells with the caspase-1 substrate, FAM-YVAD-FMK, whose relative fluorescence increases inside the cells that have activated caspase-1 (Thornberry *et al*., 1997). Neither 6 hr treatment with LPS nor 6 hr infection with *P. gingivalis* had a measurable effect on caspase-1 activation in primary GECs (Fig. 5). However, subsequent treatment with ATP for 3 hrs induced a large increase in caspase-1 activation. Incubation

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with ATP by itself, in the absence of LPS treatment or *P. gingivalis* infection, also induced caspase-1 activation (Fig. 5). Since extracellular ATP does not activate caspase-1 in macrophages that are deficient in Nalp3 (Pétrilli *et al*., 2007; Mariathasan *et al*., 2006) — and extracellular ATP has never been shown to activate inflammasomes containing NLRs other than Nalp3 — these results are consistent with ATP-mediated assembly of a Nalp3 inflammasome in GECs, which leads to caspase-1 activation. This interpretation is supported by our observation that ATP does not stimulate IL-1β secretion from infected GECs in Nalp3-deficient GECs (Fig. 4B). The results also suggest that caspase-1 is not activated in the cells in which Nalp3 had been depleted by siRNA.

Since a previous study of *P. gingivalis* infection in a monocytic cell line showed upregulation of Nalp3 during infection (Bostanci *et al*., 2009), we examined whether infection of primary GECs may have sensitized the cells to ATP-induced caspase-1 activation. Unexpectedly, expression of the Nalp3 gene decreases partially after *P. gingivalis* infection (Fig. 6). Infection by *P. gingivalis* had no effect on expression of the inflammasome adaptor protein, ASC, and ATP had no additional effect on Nalp3 or ASC expression in the infected GECs (Fig. 6). The results suggest that Nalp3 downregulation by *P. gingivalis* in GECs, by itself, does not have measurable consequences for Nalp3 inflammasome activity, since ATP could activate caspase-1 as efficiently in uninfected cells as in infected cells (Fig. 5). Further depletion by siRNA is required for a decrease in inflammasome activity (Fig. 4).

Discussion

Ligation of a PRR by a PAMP is sufficient for transcription and synthesis of pro-IL-1β in macrophages. However, a second signal is needed in most cases for processing and secretion of the mature cytokine, IL-1β (Ferrari *et al*., 2006; Lich *et al*., 2006; Mariathasan *et al*., 2006; Meylan *et al*., 2006; Kahlenberg *et al*., 2005). Processing of pro-IL-1β requires activation of caspase-1, which cleaves pro-IL-1β into IL-1β.

Recently, the molecular machinery behind caspase-1 activation has been revealed. Two caspase-1 molecules are recruited to a macromolecular complex containing an NLR family member and the adaptor protein, ASC, to form the "inflammasome". This leads to the activation of caspase-1 and subsequent IL-1β and IL-18 maturation. To date, four different inflammasomes have been characterized, containing the NLRs: Nalp1, Nalp2, Nalp3, and Ipaf (Lamkanfi *et al*., 2007; Pétrilli *et al*., 2007).

The human Nalp1 inflammasome is activated by cytosolic muramyl-dipeptide, a degradation product of peptidoglycan (Martinon *et al*., 2002). The Nalp2 inflammasome has been assembled in vitro, but the activators are still unknown (Bruey *et al*., 2004).

The Nalp3 inflammasome is ASC dependent and, as noted above, requires two signals. First a PAMP binds to its PRR (mainly TLRs); and second, a danger signal, for example ATP ligation to its purinergic receptor P2X7, stimulates formation of the Nalp3 inflammasome and activation of caspase-1 (Lich *et al*., 2006; Martinon *et al*., 2006).

The Ipaf inflammasome is activated during *S. typhimurium* and *L. pneumophila* infections. Studies have shown that flagellin from these bacteria are recognized by Ipaf, which can lead to activation of caspase-1 independently of Nalp3 (Creagh and óNeill, 2006; Meylan *et al*., 2006). Furthermore, Ipaf-dependent activation of caspase-1 can take place in the absence of surface TLRs such as TLR5, which recognizes extracellular flagellin (Franchi *et al*., 2006; Miao *et al*., 2006). Thus, ligation of only TLR5 initiates synthesis of pro-IL-1β but not its secretion, whereas Ipaf ligation leads to both cytokine synthesis and its processing by

caspase-1 (Franchi *et al*., 2006; Miao *et al*., 2006). Clearly, the presence of flagellin in the cytosol is sufficient for the immune system to recognize this PAMP as being dangerous.

The role played by the Nalp1, Nalp2, Nalp3 or Ipaf inflammasomes in cytokine secretion by epithelial cells infected with oral pathogens has never been investigated. The function of NLRs has also been characterized mainly in macrophages. Given that epithelial cells represent the preferred target host-cell for many pathogens and that *P. gingivalis* infects GECs, we have turned our attention to the possibility that primary GECs may express a functional inflammasome.

We have recently shown that primary GECs express a functional $P2X_7$ receptor (Yilmaz *et al*., 2008), which binds extracellular ATP. The source of the extracellular ATP remains to be shown, but is thought to be released from stressed or infected cells in oral tissue. *P. gingivalis* is found to modulate the $P2X_7$ activity and protect the host cells against cell death maximally around 60 hrs of infection, which in turn may also aid the organism's intracellular survival in the gingival epithelial tissues (Yilmaz *et al*., 2008). In this report, we show that infection of GECs with *P. gingivalis* stimulates transcription of the *il-1β* gene and production of intracellular pro-IL-1β, but the cytokine is not secreted unless the infected cells are treated subsequently with ATP. Moreover, Nalp3 depletion by RNA interference abrogated partially the ability of ATP to induce IL-1β secretion in *P. gingivalis* infected cells. Taken together, these results suggest that primary GECs indeed express a functional Nalp3 inflammasome. Although inflammasomes containing other NLR members have been characterized, none of them is sensitive to extracellular ATP (Pétrilli *et al*., 2007). Since infection by itself did not stimulate IL-1β secretion, the results therefore imply that *P. gingivalis* can not activate the Ipaf, Nalp1 or Nalp2 inflammasomes. Consistent with our results, the first histological characterization of the distribution of the Nalp3 protein in different tissues shows that Nalp3, unlike Nalp1, is expressed in epithelial cells of the oral cavity (Kummer *et al*., 2007).

Mutations in the Nalp3/cryopyrin inflammasome cause hereditary inflammatory diseases in humans. The most common of these disorders are autoimmune and are considered gain-of-function mutations. The diseases are marked by skin rashes and prolonged episodes of fever, including Muckle-Wells syndrome, familial cold autoinflammatory syndrome, and neonatal-onset multisystem inflammatory disease, and are collectively known as the Cryopyrin-Associated Periodic Syndromes (CAPS) (Kanneganti *et al*., 2007; Martinon and Tschopp, 2004). Studies have shown that mutations involved in CAPS result in enhanced caspase-1 activation and spontaneous IL-1β and IL-18 secretion (Kanneganti *et al*., 2007; Agostini *et al*., 2004). Confirmation of the mechanism of disease is revealed by the effectiveness of the treatment of CAPS using an IL-1 receptor antagonist (Kanneganti *et al*., 2007).

While the role of Nalp3 mutations in periodontal disease have not been investigated until now, a correlation has been observed between IL-1β single-nucleotide polymorphisms and periodontal disease (Akman *et al*., 2008). Significantly, higher levels of IL-1β have also been detected in saliva from patients with periodontitis, compared to healthy controls (Tobón-Arroyave *et al*., 2008), suggesting a role for this cytokine in development of disease. Future studies are thus likely to also uncover a role for Nalp3 and IL-1β polymorphisms in periodontal disease. As mutations of Nalp3 and $P2X_7$ are associated with autoinflammatory disease or cancer, they should allow us to identify genetic markers for host susceptibility to periodontitis.

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Experimental Procedures

Bacteria and cell culture

P. gingivalis ATCC 33277 was cultured anaerobically for 24 hrs at 37°C in trypticase soy broth supplemented with yeast extract (1 mg ml^{-1}), haemin (5 mg ml^{-1}) and menadione (1 mg ml^{-1}). Bacteria were grown for 24 hrs, harvested by centrifugation at 6000 g and 4°C for 10 min, washed twice, and resuspended in Dulbecco's Phosphate-buffered saline (PBS, from Sigma), pH 7.3, before incubation with host cells (Yilmaz *et al*., 2008). Bacteria were quantified using a Klett-Summerson photometer.

Primary GECs were obtained after oral surgery from healthy gingival tissue as previously described (Yilmaz *et al*., 2004). Cells were cultured as monolayers in serum-free keratinocyte growth medium (KGM) (Lonza) at 37°C in 5% CO_2. GECs were used for experimentation at 75–80% confluence and cultured for 48 hrs before infection with bacterial cells or exposure to other test reagents in KGM.

Analysis of mRNA expression for Nalp3 and the other NLRs—In order to determine whether GECs express the Nalp3 (Nlrp3), Nalp1 (Nlrp1) and Ipaf (Nlrc4) genes, PCR amplification was carried out with primers specific for the human genes. The sequences of the primers used were as follows. Nalp3: forward primer, 5′ CTTCTCTGATGAGGCCCAAG 3′; reverse primer, 5′ GCAGCAAACTGGAAAGGAAG 3′ (amplicon expected size, 200 bp). Nalp1: forward primer, 5′-ACCTGATCCCAAGTGACTGC-3′, reverse primer, 5′-TCTTCTCCAGGGCTTCGATA-3′. And Ipaf: forward primer 5′-CTCTCATGGTGGAAGCCAGTCC-3′, reverse primer 5′-GACAGAGACTTGACTATGTAATCC-3′.

The PCR cycling protocol for all primers was 94°C at 5 sec, 55°C at 5 sec, and 68°C at 15 sec. The protocol was repeated for 45 cycles and included an initial 5-min enzyme activation step at 94°C and a final 10-min extension step at 72°C. PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

Measurement of IL-1β, Nalp3, and ASC gene transcription—Transcription of the *il-1β*, *nalp3*, and *asc* genes was measured by quantitative, real-time PCR (qPCR), using primers specific for human *il-1β*, *nalp3*, and *asc* and an Mx3000P (Stratagene) instrument. Briefly, GECs were infected with *P. gingivalis* 33277 at an MOI of 100 for 6 hrs. RNA was prepared and 2 μg per sample were reverse-transcribed from uninfected, infected, and infected and 5 mM ATP treated GECs, as described (Darville *et al*., 2003). Quantitative PCR was performed with 1/50 of the cDNA preparation in the Mx3000P (Stratagene) in 25 μl final volumes with the Brilliant QPCR Master Mix (Stratagene). cDNA was amplified using 200 nM of each specific sense and antisense primers. Quantitative PCR was conducted at 95°C for 10 min, followed by 40 cycles at 95°C for 30 sec, 55°C for 1 min and 72°C for 30 sec. The expression levels of cytokine cDNA and Nalp3, ASC were compared with GAPDH and normalized to uninfected GEC responses by the comparative cycle threshold method, as described by the manufacturer (Stratagene). The primers for the genes coding IL-1β, Nalp3, ASC, and GAPDH were as follows. For human IL-1β: 5′ CAGCCAATCTTCATTGCTCA 3′ (forward), 5′ TCGGAGATTCGTAGCTGGAT 3′ (reverse). For human Nalp3: 5′ CTTCCTTTCCAGTTTGCTGC 3′ (forward), 5′ TCTCGCAGTCCACTTCCTTT 3′ (reverse). For human ASC: 5′ AGTTTCACACCAGCCTGGAA 3′ (forward), 5′ TTTTCAAGCTGGCTTTTCGT 3′ (reverse). For human GAPDH: 5′ AACGGATTTGGTCGTATTGGGC 3′ (forward), 5′ CTTGACGGTGCCATGGAATTTG 3′ (reverse).

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Depletion of Nalp3 by RNA interference

Primary cultures of GECs at 50% confluence were transfected in GEC growth media using 125 nM multiple short interfering RNA (siRNA) duplexes in 3 μl siRNA DharmaFECT1 agent (Dharmacon). Briefly, 3 μl transfection agent was added drop-wise into 100 μl of GEC growth media. After gentle mixing, the incubation was performed for 10 min at room temperature. Then 125 nM siRNA Nalp3 sequences were added to diluted transfection agent, mixed gently, and incubated for 10 min at room temperature. Finally, 195 μl of this mixture was added to each well, the plate was rocked gently, and further incubated for 48 hrs at 37 °C 5% CO_2. The sequences of the siRNA for Nalp3 consisted of the following mixture: 5′-GGAUCAAACUACUCUGUGAUU-3′ UUCCUAGUUUGAUGAGACACU 5′-UGCAAGAUCUCUCAGCAAAUU-3′ UUACGUUCUAGAGAGUCGUUU 5′-GAAGUGGGGUUCAGAUAAUUU-3′ UUCTTCACCCCAAGUCUAUUA 5′-GCAAGACCAAGACGUGUGAUU-3′ UUCGUUCUGGUUCUGCACACU Non-target pool siRNA (Dharmacon) and transfection agent alone were used as negative controls. The qRT-PCR analysis was performed up to 48 hrs post-transfection to confirm the Nalp3 gene knockdown. The expression level for the each condition was normalized to housekeeping gene GAPDH using the comparative cycle threshold (Ct) method. The fold changes were calculated as $2^{-\Delta\Delta Ct}$, by comparing target siRNA with non-target siRNA (normalized to 1) from the ΔΔCt value. Results were then expressed as percentages.

Measurement of cytokine secretion by ELISA

GECs were either treated with 2 μg/ml of *Escherichia coli* LPS (InvivoGen) for 6 hrs or infected with *P. gingivalis* (MOI = 100) for 6 hrs. Some cells were subsequently treated with 5 mM ATP in cell culture medium for an additional 3 hrs. Cell culture supernatants from LPS-treated, *P. gingivalis*-infected or control GECs were collected and assayed for cytokine activity by ELISA using a commercial cytokine ELISA kit for IL-1β (BD Biosciences Pharmingen).

Analysis of intracellular cytokine production by fluorescence microscopy

Intracellular accumulation of IL-1β protein (pro-IL-1β and IL-1β) was visualized by immunofluorescence microscopy, using antibodies against IL-1β protein (R&D Systems). GECs growing on 4-well chambered glass slides (Nalge-Nunc International) were infected with *P. gingivalis* at an MOI of 100 for 6 hrs, and 5 mM ATP or control buffer was added for an additional 3 hrs. Infected cells were fixed in 10% neutral buffered formalin for 20 min. The cells were permeabilized for 10 min with 0.1% Triton X-100, and the slides were incubated with fluorescein-conjugated anti-IL-1β monoclonal antibody at a 1:100 dilution for 1 hr at room temperature. The samples were incubated with anti-*P. gingivalis* 33277 antibody and reacted simultaneously with Alexa Fluor 633 secondary antibody (Invitrogen) (Yilmaz *et al*., 2008). Samples with no primary antibody incubation were included as control. The samples were treated with 4,6-diamidino-2-phenylindole (DAPI) 1 mg ml^{-1} (Sigma) to visualize the nuclei. After washing, the slides were mounted in Vectashield mounting medium and examined with an epifluorescence microscope. The images were captured with a Zeiss Axio imager A1 fluorescence microscope equipped with a cooled CCD camera (Qimaging) controlled by QCAPTURE software v.1394. The levels of intracellular IL-1β in infected and uninfected cells were quantified by measuring the intensity of fluorescence emitted from the acquired images with NIH ImageJ analysis software, as previously done for quantifying *P. gingivalis* infection (Yilmaz *et al*., 2006). Briefly, the threshold of relative fluorescence intensity was determined with samples that were uninfected and untreated based on pixel intensity in each field of interest. The results were then expressed as a percentage of the fluorescence intensity, compared to the control samples. The same microscopy settings were employed throughout all samples.

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Measurement of caspase-1 activity

GECs were treated with 2 μg/ml of *E. coli* LPS or control buffer, or infected with *P. gingivalis* (MOI = 100) for 6 hrs. LPS-treated, infected, or control cells were then incubated for an additional 3 hrs with 5 mM ATP or control buffer. Caspase-1 activation was measured by incubating cells with the caspase-1 substrate, FAM-YVAD-FMK at a 1:30 dilution (Immunochemistry Technologies), whose relative fluorescence increases inside whole living cells that have activated caspase-1 (Thornberry *et al*., 1997). Specificity of substrate binding to caspase-1 was confirmed by pretreating the cells with excess of nonfluorescent z-YVAD,FMK, before addition of FAM-YVAD-FMK. The fluorescence increase in cells with activated caspase-1 was measured with a 96-well fluorescence plate reader (excitation at 488 nm, emission at 520 nm with black microtiter plates). Caspase-1 activation was quantified as the amount of green fluorescence emitted from the FLICA probes bound to the enzyme. The relative fluorescence intensities were obtained by subtracting the value of the sample with the smallest fluorescence intensity from each sample. The results were then expressed as percentages of the sample with highest intensity normalized to 100.

Statistical analysis

Student's *t*-test was used to calculate the statistical significance of the experimental results between two conditions (significance at $P < 0.05$). The uninfected cell populations were rejected from the analysis.

Acknowledgments

This study was supported by NIDCR grants R01DE016593 and R01DE019444. We also thank Dr. Chulhee Choi, University of Florida, and Department of Periodontology for his kind assistance.

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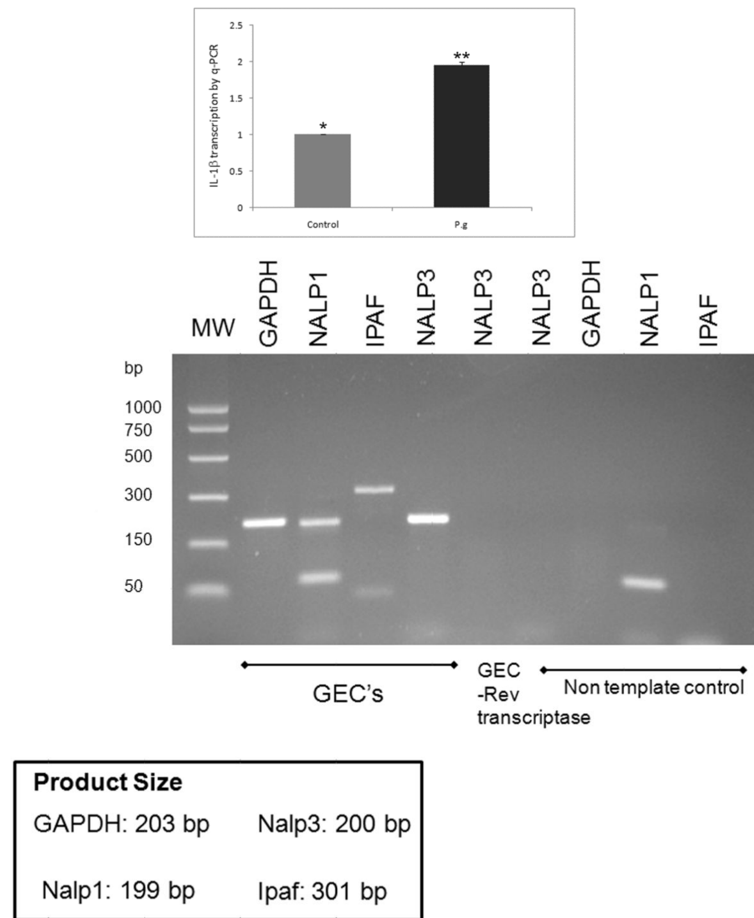

Figure 1.

Primary GECs express mRNA for NLR family members and *P. gingivalis* infection stimulates the *il-1β* gene transcription.

(A) *il-1β* gene expression was measured by quantitative, real time PCR (qPCR) from GECs infected with *P. gingivalis* at an MOI = 100 for 6 hrs, and uninfected (control). GAPDH was included as an internal control. Fold change was calculated by the comparative cycle threshold method. $P < 0.001$ for cells uninfected, control (*), compared with cells infected with *P. gingivalis* (**). Data are representative of two independent experiments performed in duplicates.

(B) PCR amplification was carried out with primers specific for Nalp3, Nalp1 and Ipaf, as described in Experimental Procedures. An amplicon of the expected size (200 bp) was found for Nalp3. The control, without primers (NTC), did not produce a detectable band.

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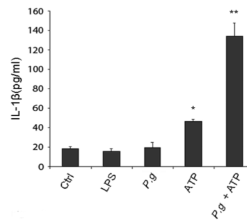

Figure 2.

Primary GECs express a functional inflammasome. IL-1β secretion was measured in supernatants from GECs incubated with control buffer (Ctrl), incubated with 2 μg/ml of *E. coli* LPS for 6 hrs, infected with *P. gingivalis* (*P.g.*) at an MOI = 100 for 6 hrs, incubated with 5 mM ATP for 3 hrs, or infected with *P. gingivalis* for 6 hrs and then incubated for an additional 3 hrs with 5 mM ATP. IL-1β concentrations were quantified with an IL-1β ELISA kit. The values show averages and S.D. from 3 samples of a representative experiment, and represent results obtained from at least three experiments. $P < 0.05$ for cells infected with *P. gingivalis* and cells treated with ATP (*), compared with cells infected with *P. gingivalis* and then incubated with ATP (**).

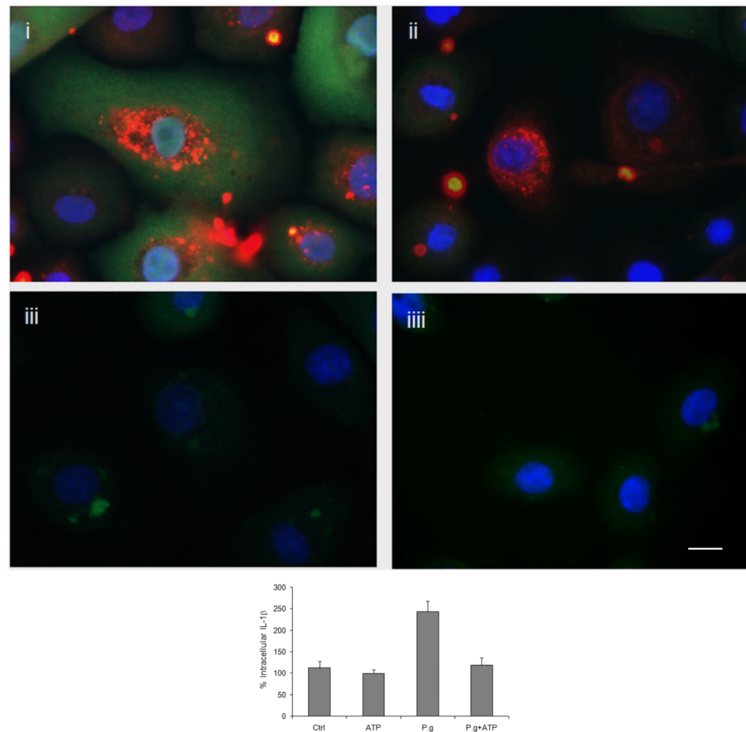

Figure 3.

Cytokine is produced intracellularly during *P. gingivalis* infection but is released from cells following ATP treatment.

(A) Intracellular cytokine (pro-IL-1β and IL-1β) was detected by immunofluorescence using antibodies against IL-1β (green). The samples were also stained with *P. gingivalis* antibody (red) and DAPI (blue) to visualize the nuclei. (i) There is a large increase in IL-1β staining in GECs infected with *P. gingivalis* at an MOI = 100 for 6 hrs. (ii) Most cytokine is released from cells that had been infected with *P. gingivalis* for 6 hrs and then incubated with 5 mM ATP for an additional 3 hrs. (iii) There is very little cytokine in GECs incubated with 5 mM ATP (iiii) or control buffer for 3 hrs. The images are representative of 150 cells studied per sample from at least two separate experiments performed in duplicate. Bar 10 μm.

(B) Images of cells prepared as in Fig. 3A were captured with a fluorescence microscope equipped with a cooled CCD. The levels of intracellular cytokine (pro-IL-1β and IL-1β) in cells incubated with control buffer (Ctrl), treated with 5 mM ATP for 3 hrs, infected with *P. gingivalis* at an MOI = 100 for 6 hrs (*P.g.*), or infected with *P. gingivalis* for 6 hrs and then treated with ATP for 3 hrs, were quantified by measuring relative fluorescence with NIH ImageJ analysis software, as described in Experimental Procedures. Data were expressed as a percentage of the fluorescence intensity of the control samples. Results are representative images of 150 cells studied per sample from at least two individual experiments performed in duplicate. The values show averages and S.D. from a representative experiment. $P = 0.05$ for cells infected with *P. gingivalis*, compared with cells infected with *P. gingivalis* and then incubated with ATP.

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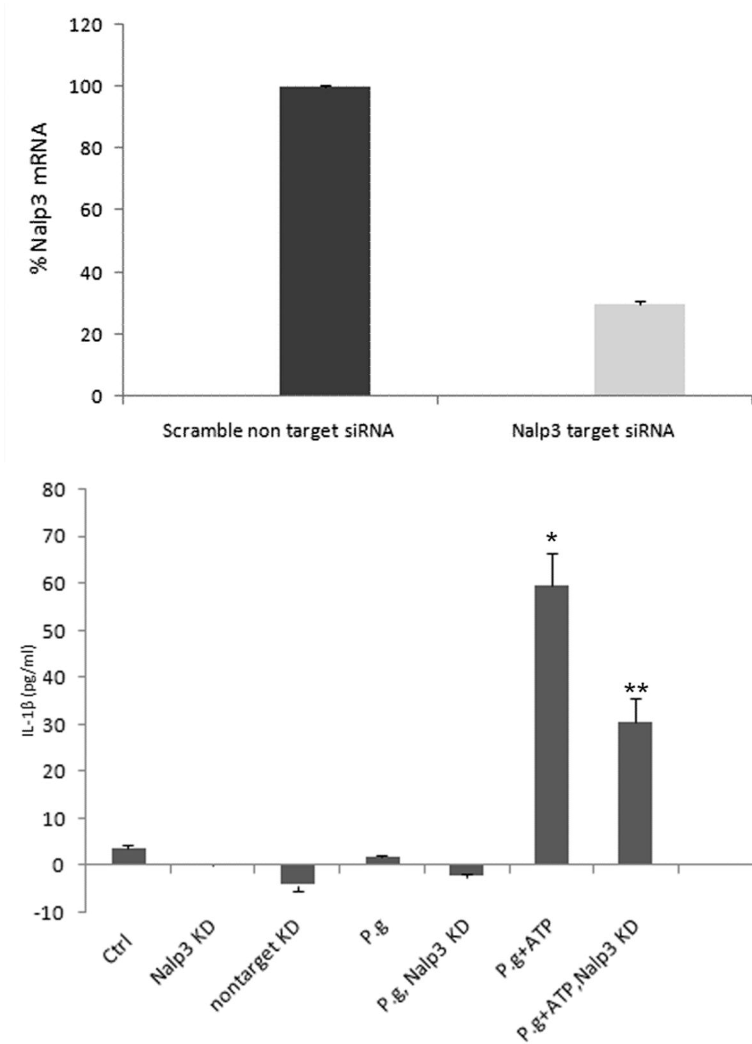

Figure 4.

Depletion of Nalp3 abrogates the ability of ATP to induce IL-1β secretion from *P. gingivalis*-infected GECs.

(A) Nalp3 gene expression was transiently silenced in primary by siRNA transfection, as described in Experimental Procedures. The expression levels for each condition were analyzed by qRT-PCR using the Ct method, and were represented as percentages of the non-target siRNA sample normalized to 100. The knockdown experiments were repeated at least three separate times demonstrating consistently ~ 70% decrease in the Nalp3 gene transcript in each time.

(B) Primary GECs were treated with Nalp3 or non-target siRNA reagents, and IL-1β secretion from Nalp3 knockdowns (KD) and controls was measured by ELISA. The cells infected with *P. gingivalis* (*P.g.*) with or without transfection with siRNA (Nalp3 KD) did not produce any noticeable IL-1β secretion. On the other hand, depletion of Nalp3 in cells infected with *P. gingivalis* and then incubated with ATP (Nalp3 KD + *P.g.* + ATP) resulted in significant inhibition in secretion of IL-1β, compared with GECs that were not treated with siRNA (*P.g.* + ATP). The values show averages and S.D. from duplicate samples of a representative experiment, and show results obtained from at least 2 separate experiments. *P* = 0.05 for cells infected with *P. gingivalis* and then incubated with ATP (*P.g.* + ATP *),

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compared with cells that were transfected with Nalp3 siRNA, infected with *P. gingivalis*, and then incubated with ATP (*P.g*. + ATP, Nalp3 KD **).

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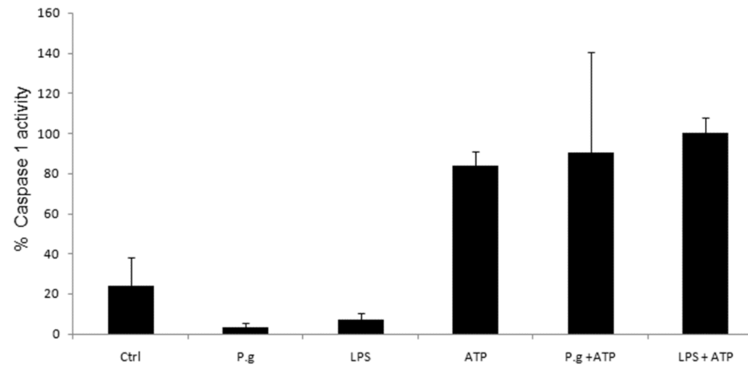

Figure 5.

Stimulation with ATP activates caspase-1 in GECs. Primary GECs were infected with *P. gingivalis* at an MOI = 100 for 6 hrs (*P.g.*), treated with 2 μg/ml of *E. coli* LPS for 6 hrs, incubated with 5 mM ATP for 3 hrs, or incubated with 5 mM ATP for 3 hrs after 6 hrs of infection or LPS-treatment. Caspase-1 activation was measured by incubating treated or infected cells with a fluorescent caspase-1 substrate, as described in Experimental Procedures. LPS treatment or *P. gingivalis* infection have no effect on caspase-1 activity, but subsequent treatment with ATP induces a large increase in caspase-1 activation. The values show percent averages and S.D. from 3 samples of a representative experiment, and represent results obtained from at least three experiments. $P = 0.02$ for cells infected with *P. gingivalis*, compared with cells infected with *P. gingivalis* and then incubated with ATP.

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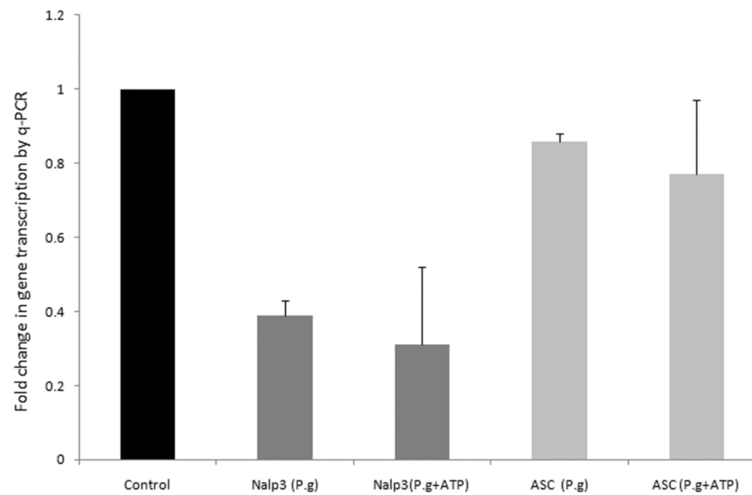

Figure 6.

The gene expression levels of Nalp3 but not ASC decreased partially after *P. gingivalis* infection. GECs were uninfected and untreated (control), infected with *P. gingivalis* at an MOI of 100 for 6 hrs, or infected and treated for an additional 3 hrs with 5 mM ATP. The expression levels of Nalp3 and ASC were compared with GAPDH and normalized to uninfected GEC responses, as described in Experimental Procedures. Data are representative of two independent experiments performed in duplicates.