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Danger Signal Adenosine via Adenosine 2a Receptor Stimulates Growth of *P. gingivalis* in Primary Gingival Epithelial Cells

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SUMMARY

Extracellular signaling during inflammation and chronic diseases involves molecules referred to as ‘Danger Signals’ (DS), including the small molecule adenosine. We demonstrate that primary gingival epithelial cells (GECs) express a family of G-protein coupled receptors known as adenosine receptors, including the high affinity receptors A1 and A2a and low affinity receptors A2b and A3. Treatment of *Porphyromonas gingivalis*-infected GECs with A2a receptor-specific agonist CGS-21680 resulted in elevated intracellular bacterial replication as determined by fluorescence microscopy and antibiotic protection assay. Additionally, A2a receptor antagonism and knockdown via RNA interference significantly reduced metabolically active intracellular *P. gingivalis*. Furthermore, analysis of anti-inflammatory mediator cyclic AMP (cAMP) following A2a receptor selective agonist CGS-21680 stimulation induced significantly higher levels of cAMP during *P. gingivalis* infection, indicating adenosine signaling may attenuate inflammatory processes associated with bacterial infection. This study reveals that the GECs express functional A2a receptor and *P. gingivalis* may utilize the A2a receptor coupled DS Adenosine signaling as a means to establish successful persistence in the oral mucosa, possibly via down-regulation of pro-inflammatory response.

Keywords

purinergic signaling; A2a receptor; persistence; epithelial mucosa; periodontal disease

INTRODUCTION

Porphyromonas gingivalis is a Gram-negative opportunistic pathogen that has been strongly involved in severe forms of periodontitis and recently associated with a number of other chronic pathologies. Gingival epithelial cells (GECs), which form the initial barrier to the colonizing bacteria in the gingival crevice and function as an important arm of the immune system, are among the first host cells populated by *P. gingivalis* (Yilmaz *et al.*, 2008). The organism has been demonstrated to successfully enter and replicate in GECs and exhibit highly specialized host-adaptive mechanisms to establish persistence in the oral epithelium (Yilmaz *et al.*, 2008; Yao *et al.*, 2010, Choi *et al.*, 2011, Choi *et al.*, 2013).

Infected, dying, or stressed cells release “danger signals (DSs)” that are normally found in the cytosol and nucleus of healthy cells, including ATP and adenosine. A key DS molecule is adenosine-triphosphate (ATP), a nucleoside molecule involved in cellular energetics.

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Despite its commonly understood role as an energy source, it is becoming increasingly evident that ATP is a potent regulator of inflammation (Ishii & Akira, 2008). ATP released from inflamed tissues acts through ionotropic purinergic receptors, notably P2X₇ receptor, to activate specific pro-inflammatory signaling cascades (Miller *et al.*, 2011). These downstream effects of ATP-P2X₇ coupling can also limit the ability of opportunistic pathogens to establish intracellular infections (Coutinho-Silva & Ojcius, 2012). We previously demonstrated that *P. gingivalis* effectively subverts ATP-P2X₇ mediated host response in GECs to support its colonization in the oral mucosa (Yilmaz *et al.*, 2008; Choi *et al.*, 2013).

A less appreciated component of the innate immune armamentarium is the DS adenosine, a metabolite of ATP that is generated via a series of enzymatic reactions in normal, stressed, and infected tissues (Yegutkin, 2008). The pro-inflammatory features of ATP, especially for limiting of intracellular infections, have been thoroughly studied, while the anti-inflammatory nature of adenosine during infection remains largely unexplored (Bours *et al.*, 2006). Adenosine receptors are G-protein coupled receptors (GPCRs) belonging to the P1 superfamily, including A1, A2a, A2b, and A3 subtypes, all of which have varying degrees of sensitivity to adenosine. The A2a receptor is highly sensitive to adenosine and suppresses inflammation by relying on cAMP-dependent activation of downstream effectors including Akt, CREB, and NF-κB (Jacobson & Gao, 2006). Recent studies demonstrate that stimulation of the A2a receptor with receptor-specific agonist CGS-21680 reduces lung inflammation by interfering with neutrophil migration (Impellizzeri *et al.*, 2011) and protects activated T-cell lymphocytes from activation-induced cell death (Himer *et al.*, 2010). During infection, the A2a receptor has been shown to suppress inflammation caused by gastric T-cell lymphocytes while simultaneously promoting persistence of *Helicobacter pylori* (Alam *et al.*, 2009). While additional studies have evaluated the importance of other adenosine receptors in infection, notably A2b during the infections by *Chlamydia* (Pettengill *et al.*, 2009) and *Klebsiella* (Barletta *et al.*, 2012) in neutrophils and HeLa cells, little is known about A2a receptor function in controlling intracellular bacterial infections. Furthermore, the role of adenosine signaling in the context of oral bacteria and gingival epithelium interaction remains completely uncharacterized.

Recent literature supports the importance of adenosine signaling in the oral cavity, particularly for periodontal disease. Bitto *et al.* (2013) reported an adenosine-dependent reduction in periodontal inflammation in rat models, while another study detected elevated A2a receptor mRNA expression in gingival tissues of patients diagnosed with chronic periodontal disease (Sun *et al.*, 2011). Furthermore, macrophages have been shown to upregulate A2a receptor mRNA when challenged with LPS (Streitová *et al.*, 2011). For the opportunistic pathogens, *Staphylococcus aureus* and *Bacillus anthracis*, manipulation of adenosine concentration appears to be a key survival mechanism and contributor to pathogenesis (Thammavongsa *et al.*, 2009). Given the anti-inflammatory nature of *P. gingivalis* infection in the GECs, including antagonism of pro-inflammatory cytokine IL-8 induced by other pathogens (Takeuchi *et al.*, 2013) and attenuation of host cell apoptosis, it became logical for us to explore adenosine and the A2a receptor coupling during *P. gingivalis* infection.

The present study demonstrates for the first time that primary GECs express the full complement of adenosine receptors, including the A2a receptor that is distributed across the cell membrane. Stimulation of *P. gingivalis*-infected GECs with A2a receptor-specific agonist CGS-21680 strongly induces proliferation of intracellular *P. gingivalis*, while treatment with broad-spectrum adenosine receptor agonist NECA has much lesser effect on the amount of infection. Antibiotic protection assay of *P. gingivalis*-infected GECs treated with A2a receptor-specific agonist CGS-21680 revealed significantly higher amounts of

recoverable *P. gingivalis* than the un-stimulated infected cells. Treatment of *P. gingivalis*-infected GECs with A2a receptor-specific antagonist SCH-58261 inhibited intracellular growth of bacteria. Additionally, siRNA depletion of A2a receptor resulted in substantially reduced levels of metabolically active flavin mononucleotide-based fluorescent protein-expressing *P. gingivalis* (PgFbFP), further strengthening the results of this study. Furthermore, stimulation of GECs with A2a receptor-specific agonist CGS-21680 resulted in elevated cAMP, indicating activation of anti-inflammatory A2a receptor signaling. This study shows a novel anti-inflammatory immune response utilized by *P. gingivalis* to further promote successful subsistence in the oral mucosa.

METHODS

Bacteria and cell culture

The *P. gingivalis* ATCC 33277 was cultured anaerobically for 24 h at 37°C in trypticase soy broth supplemented with yeast extract (1 µg/ml), hemin (5 µg/ml), and menadione (1 µg/ml). Bacteria were grown for 24 h, harvested by centrifugation at 6000 g and 4°C for 10 min, washed and re-suspended in Dulbecco's phosphate-buffered saline (PBS), pH 7.3, before incubation with host cells. Bacteria were quantified using a Klett–Summerson photometer.

Primary GECs were obtained after oral surgery in the clinics of the University of Florida from healthy gingival tissue as previously described (Yilmaz, *et al.*, 2002). Cells were cultured as monolayers in serum-free keratinocyte growth medium (Lonza, Walkersville, MD) at 37°C in 5% CO₂. GECs were used for experimentation at 80% confluence and cultured for 48 h before infection with bacterial cells or exposure to other test reagents in keratinocyte growth medium. The passage number of the primary GECs utilized for the experiments ranged from 3–7 with consistently similar results.

Reverse transcription PCR analysis for adenosine receptors

Total RNA was isolated from primary GECs using RNeasy kit (Qiagen) following the manufacturer's instructions. Total RNA was converted into cDNA by standard reverse transcription with M-MLV-Reverse Transcriptase (Promega). cDNAs were amplified using the MJ, Mini (BIO-RAD laboratories) in a 25 µl reaction mixture containing one-fifteenth of the cDNA generated from reverse transcription reaction, 10X PCR buffer, 2.5 mM MgCl₂, 0.25 mM (each) dNTPs, 0.5 µM forward and reverse primers, and 1 U GoTaq DNA polymerase (Promega). The sequences of the primers used for A1, A2a, A2b, and A3 were designed and obtained from Invitrogen (Table 1). The optimum annealing temperatures for each set of primers was determined prior to beginning PCR cycling (data not shown). The PCR protocol for the respective primers was initiated at 94°C for 1 min, pre-determined annealing temperature for 1 min, and 72°C for 1 min. The protocol was conducted for 30 cycles and included an initial 5 min enzyme activation step at 94°C and a final 5 min extension step at 72°C. "No reverse transcriptase" control was included in the assays. PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. At least three different GEC cell lines were used for the analysis.

Surface expression analysis of A2a adenosine receptor via immunofluorescence microscopy

Gingival epithelial cells were grown on 4-well chambered glass slides (Nalge-Nunc International), washed with ice-cold PBS, and fixed with 10% neutral buffered formalin for 1 h at room temperature. After washing twice with PBS, the cells were treated with permeabilization solution (0.1% Triton X-100) for 15 min. Samples were then washed twice with PBS and incubated with antibody raised in mice against full length recombinant human A2a receptor (Santa Cruz Biotechnology) and detected with Alexa-Fluor 594 anti-mouse

secondary antibody (Invitrogen). Samples with no primary antibody incubation were included as control. Glass coverslips to visualize the samples were mounted using media containing 4,6-diamidino-2-phenylindole (DAPI) 1 $\mu\text{g ml}^{-1}$ (Vector Labs) to visualize the nuclei. Finally, the samples were washed twice with PBS and analyzed using a Zeiss Axio Imager A1 fluorescence microscope equipped with band pass optical filter sets appropriate for imaging of the dyes and a cooled CCD camera (Qimaging). Single exposure images were captured sequentially and saved by Qcapture software v.1394.

Pharmacological treatment of adenosine receptors in *P. gingivalis*-infected GECs

Gingival epithelial cells were infected at a multiplicity of infection (moi) of 100 with *P. gingivalis* for 24 h at 37°C in a 5% CO₂ incubator. For analysis of effects of adenosine receptors on *P. gingivalis* infection, the infected GECs were treated at 1 h and 3 h post-infection (pi) with 10 μM A2a-receptor-specific agonist CGS-21680 (Tocris Biosciences), 100 μM broad spectrum adenosine receptor agonist 5'-N-Ethylcarboxamidoadenosine (NECA) (Sigma), or 10 μM A2a-specific antagonist SCH-58261 (Tocris Biosciences). Pharmacological reagents were chosen based on available literature [Jacobsen *et al.*, 2006]. All time points for the infections were carried backwards, so that all incubations could be stopped and assayed at the same time at the end of 24 h.

Impact of adenosine receptor on *P. gingivalis* infection via immunofluorescence microscopy analysis

Immunofluorescence labeling and microscopy for determining the level of infection were performed as previously described (Yilmaz *et al.*, 2006). Briefly, GECs cultivated on 4-well chambered cover-glass slides were infected with *P. gingivalis* at moi of 100 at 37°C for 24 h. The samples were incubated with anti-*P. gingivalis* 33277 antibody (a gift by Dr. Richard J Lamont) and reacted with Oregon Green 488 secondary antibody (Invitrogen), and glass coverslips were mounted using media with DAPI (Vector Labs). The samples were visualized using the fluorescence microscope system described above. Acquired images were analyzed for the intensity of fluorescence emitted from the infected samples with NIH ImageJ analysis software. An average of 175 fields per sample were studied from at least two separate experiments performed in duplicate.

Antibiotic protection assay

GECs were infected at moi of 100 with *P. gingivalis* for 24 h at 37°C in a 5% CO₂ incubator. A2a receptor-specific agonist CGS-21680 was added to culture at 1 h pi to a final concentration of 10 μM . Following 3 h infection, culture media was replaced with 400 μM metronidazole and 500 μM gentamicin diluted in PBS and cells were incubated for an additional 1 h at 37 °C. Cells were washed three times with PBS and lysed in 1 ml sterile H₂O at 37 °C for 30 m. Recovered lysate was then plated on blood agar plates and incubated at 37 °C for 48 h for analysis of colony formation. The average of total CFU/ml⁻¹ of un-stimulated infected GECs were set as 100 percent.

Flavin mononucleotide-based fluorescent protein-expressing *P. gingivalis* (PgFbFP)

Further analyses to verify immunofluorescence observations reported in this study were determined by using a *P. gingivalis* ATCC 33277 transformant strain ((PgFbFP)) previously developed in our laboratory (Choi *et al.*, 2011). GECs cultured in 4-well cover-glass slides were infected at moi of 100 with PgFbFP for 24 h at 37°C in a 5% CO₂ incubator. GECs were treated with A2a receptor-specific agonist CGS-21680 and A2a receptor-specific antagonist SCH-58261 at 3 h pi, and A2a-knockdown cells were also used. Cells were washed with ice-cold PBS, and fixed with 10% neutral buffered formalin for 1 h at room temperature. Pictures were taken using Zeiss Axio Imager A1 microscope and a cooled-

CCD camera (Qimaging). Fluorescence intensity analysis was carried out using NIH ImageJ software. An average of 175 fields per sample were studied from two separate experiments performed in duplicate.

Depletion of A2a by RNA interference

20 μ M siRNA solution was diluted in 1X siRNA buffer (Dharmacon) to final concentration of 5 μ M. Per culture dish well, 10 μ L of 5 μ M siRNA solution was added to 190 μ L serum-free solution and 2 μ L of DharmaFECT transfection reagent was added to 198 μ L serum-free media each solution was incubated at room temperature (RT) after mixing for 5 m. The siRNA and transfection reagent solutions were then mixed gently together and incubated at RT for 20 m. GECs were cultured in 6-well dishes at 37 °C for 24 h prior to siRNA transfection. 1.6 mL of antibiotic-free media was added along with 400 μ L of prepared siRNA/transfection reagent solution to yield a final concentration of 25 nM siRNA per culture well. Non-target pool siRNA (Dharmacon) and transfection agent alone were used as negative controls. A2a receptor knockdown GECs were verified using quantitative RT-PCR as described previously (Yilmaz *et al.*, 2010), resulting in ~67% depletion of A2a receptor in knockdowns (data not shown).

cAMP level detection

Assessment of intracellular cAMP levels was carried out by using cyclic AMP (Direct) Enzyme Immunometric Assay kit per manufacturer's instructions (Assay Designs). Un-infected, non-treated GECs were used as baseline controls. Analysis conditions included non-treated *P. gingivalis*-infected GECs, 10 μ M A2a receptor-specific agonist CGS-21680-treated un-infected GECs, *P. gingivalis*-infected GECs treated with 10 μ M CGS-21680 1 h post-infection, un-infected GECs treated with 10 μ M A2a receptor-specific antagonist SCH-58261 and subsequently stimulated with 10 μ M CGS-21680 1 h after initial treatment, and SCH-58261-treated GECs 1 h prior to infection with *P. gingivalis*. Samples were prepared per manufacturer specifications in microtiter plates and cAMP signal was detected via absorbance at 405 nm. Time courses of infections and pharmacological treatments were carried out backwards, so all conditions could be assayed at the same time.

RESULTS

Primary GECs express functional adenosine receptors

A2a receptors are expressed in a variety of tissues, and its presence in oral tissues has been reported to occur in gingival fibroblasts (Murakami *et al.*, 2001) and epithelium (Murakami *et al.*, 2002). To confirm the adenosine receptor expression profile of primary GECs, we utilized RT-PCR and immunofluorescence microscopy. RT-PCR analysis reveals that primary GECs express all four adenosine receptor subtypes, as amplicons were detected for A1, A2a, A2b, and A3 receptors (Figure 1A). Immunofluorescence microscopy confirmed the surface expression of the A2a receptor uniformly throughout the primary GEC cell membrane (Figure 1B). Thus, cells of the oral mucosa lining express all members of the adenosine receptor family including the A2a receptor.

Stimulation of the A2a receptor promotes enhanced infection by *P. gingivalis*

A variety of chemical compounds are known to serve as agonists for adenosine receptors. In order to ascertain the effects of the A2a receptor specifically, 10 μ M CGS-21680, an A2a receptor-specific agonist, was added to *P. gingivalis*-infected GEC culture medium at 1 h and 3 h post-infection. NIH software ImageJ was used to analyze captured images and quantify fluorescence corresponding to intracellular *P. gingivalis*. A2a receptor-specific agonist CGS-21680 treatment of *P. gingivalis*-infected GECs resulted in twice the amount of

intracellular *P. gingivalis* as compared to untreated controls (significant at $P < 0.001$) (Figure 2). 100 μ M NECA, an established broad-spectrum adenosine receptor agonist was also used to determine the effects of general adenosine receptor stimulation on *P. gingivalis* infection. Treatment of *P. gingivalis*-infected GECs at 1 h and 3 h post-infection with broad spectrum adenosine receptor agonist NECA resulted in minimal effects on level of intracellular *P. gingivalis* compared to untreated control [Supplemental figure 1a–d].

To better evaluate the role of A2a receptor in the intracellular proliferation of *P. gingivalis*, we performed an antibiotic protection assay comparing the amount of live recoverable bacteria in A2a receptor-specific agonist CGS-21680-treated and un-treated infected GECs (Figure 3). The results of this experiment further confirmed our earlier findings, with twice as many live *P. gingivalis* recovered from GECs that had been treated with A2a receptor-specific agonist CGS-21680, compared to un-treated control. Thus, A2a receptor activation likely plays a key role in promoting elevated levels of intracellular *P. gingivalis* in primary GECs.

Inhibition and depletion of A2a receptor suppresses *P. gingivalis* infection

While our findings presented above indicate that stimulation of the A2a receptor appears to be important for *P. gingivalis* infection, our examinations led us to inquire about what effect preventing A2a receptor activation would have on *P. gingivalis* infection. SCH-58261, an A2a receptor-specific antagonist, was used to assess the impact A2a receptor blockade has on infection. Infected GECs were treated with 25 μ M SCH-58261 at 3 h post-infection and *P. gingivalis* levels were determined at 24 h total infection time using fluorescence microscopy. Quantification of *P. gingivalis* fluorescence levels revealed a significant ($P < 0.001$) suppression of infection compared to control and CGS-21680 treatment conditions (Figure 4A and B). These findings prompted us to further examine the relationship between A2a receptor and *P. gingivalis* interaction by using RNA interference. We first determined that A2a siRNA did not induce cell death in uninfected GECs (data not shown). A2a gene silencing significantly ($P < 0.001$) hindered intracellular numbers of *P. gingivalis* at 24 h, with infection levels ~50% of infected controls (Figure 4A and B). Thus, a functional A2a receptor appears to be important for successful colonization of *P. gingivalis* in primary GECs.

P. gingivalis infection elevates intracellular cAMP levels via A2a receptor

Adenosine receptors, including A2a, are GPCRs that act through adenylyl cyclase to alter the concentration of intracellular cAMP (Jacobson & Gao, 2006). Recent studies of other intracellular organisms indicate an important role for cAMP during infection that contributes to persistence of infection (Macdonald *et al.*, 2013). Cytosolic cAMP levels during infection or CGS-21680, SCH-58261 treatments were measured to verify the effect of adenosine via A2a receptors over a time course ranging from 30 m to 24 h (Figure 5). cAMP levels in GECs showed two-fold increase at 6 h following A2a-selective CGS-21680 treatment and infection with *P. gingivalis* showed a similar trend. The increase in cAMP level was inhibited when A2a receptor-stimulated GECs were treated with the A2a receptor-specific antagonist SCH-58261. An initial increase was observed in *P. gingivalis* infected samples, but began receding to normal levels at 8 h post infection in the presence of SCH-58261. This data indicates that during *P. gingivalis* infection, cAMP levels are elevated in GECs, and the A2a receptor contributes to this observed effect (Figure 5 and 6).

DISCUSSION

Small molecules including ATP and adenosine act as DS in tissues and help prime the innate and adaptive immune arms to respond to tissue insult via purinergic signaling (Sitovsky &

Ohta, 2005; Bours *et al.*, 2006; Ishii & Akira, 2008). Purinergic signaling through ATP has been shown to be strongly involved in mediating inflammatory responses, including upregulation of pro-inflammatory cytokines, production of ROS, and cell death. Conversely, adenosine is associated with reduction in inflammation and immunosuppressive actions including inhibition of neutrophil migration through vascular walls and preventing T-cell-mediated tumor destruction (Ohta *et al.*, 2006; Karmouty-Quintana *et al.*, 2013). Thus, the extracellular environment containing potent DS ATP and adenosine can act through their respective receptors (e.g. P2X7 or A2a receptors) to modulate the inflammatory status of tissues infected by opportunistic pathogens.

The complex nature of the relationship between intracellular pathogens and their host cells involves inside-out and outside-in signaling. Adenosine receptor activation appears to be a conserved theme for bacterial pathogens, with several reports highlighting the importance of adenosine receptors in bacterial infections. For example, persistent infections of epithelial cells by *Chlamydia trachomatis* was found to be dependent upon A2b receptor activation (Pettengill *et al.*, 2009) and A2b receptors were found to be responsible for *Clostridium difficile*-mediated inflammation and disease in murine gut epithelium models (Warren *et al.*, 2012). Further studies of the A2b receptor reveals enhanced clearance of *K. pneumonia* in A2b-deficient mice lung tissues, suggesting that this adenosine receptor may provide protection for bacterial infection (Barletta *et al.*, 2012). In addition, Popov *et al.* (2011) reported stimulation of the A3 receptor contributes to resolution of *B. anthracis* infections, suggesting a potential role for adenosine receptors in cutaneous infections by this pathogen. The role of A2a receptor in bacterial infections on the other hand, remains yet to be fully characterized, with few studies directly examining the effects of A2a receptor on infected tissues. Despite this, there is a real need to characterize the interaction between A2a receptor and opportunistic persistent bacteria because the anti-inflammatory nature of adenosine signaling makes it a target for chronic pathogens. To date, studies have only examined the A2a receptor-infection relationship in the context of sepsis models (Sullivan *et al.*, 2004; Németh *et al.*, 2006; Warren *et al.*, 2012) and bacterial toxin-challenged macrophages or monocytes (Souza *et al.*, 2009; Sun *et al.*, 2010). Only Alam *et al.* (2009) directly examined the impact of A2a receptor activation on bacterial survival during infection, with the results showing increased *H. pylori* present in tissue sections of gut mucosa. Our findings on the interaction between the A2a receptor and the opportunistic infection represent a first direct visualization of colonizing bacteria inside the host cells using the A2a receptor-specific agonist and a stable analog of adenosine, CGS-21680. Additionally, using A2a selective agonist CGS-21680 to stimulate *P. gingivalis*-infected GECs induced recovery of more live bacteria determined by antibiotic protection assay. This finding indicates that there are more *P. gingivalis* within the host cell upon stimulation by the agonist treatment and the bacteria are replicating at significantly high rates. The observed increase numbers of intracellular *P. gingivalis* was abolished when *P. gingivalis*-infected GECs were treated with A2a receptor-specific antagonist SCH-58261, implicating A2a receptor as the key contributor to observed elevation in bacterial number. The treatment of the *P. gingivalis*-infected GECs with adenosine, which is unstable *in-vitro*, at 10 μ M also enhanced the intracellular infection by ~50% (data not shown) Furthermore, the use of self-fluorescing *P. gingivalis* transformant strain (PgFbFP) enabled us to demonstrate that A2a receptor-specific agonist CGS-21680 significantly increased the number of metabolically active *P. gingivalis* within the host cells and the depletion of A2a receptor by siRNA substantially reduced the metabolically active / live *P. gingivalis*. These results suggest that not only does intracellular *P. gingivalis* respond to activation of A2a receptor, but that the bacteria become more active and proliferate at higher rates.

Previous literature demonstrates that adenosine directly enhances growth of pathogenic strains of Enteropathogenic *Escherichia coli* (Crane *et al.*, 2009). Our own data indicates

that directly supplying adenosine to *P. gingivalis* cultures has a detrimental effect on TSB cultured-*P. gingivalis* growth (supplemental figure 2a), suggesting *P. gingivalis* perhaps requires a concerted interaction between host cell machinery in order to utilize adenosine signaling for proliferation. However, elevated cAMP production by adenylyl cyclase after A2a receptor activation could explain the observed increase in bacterial number in *P. gingivalis*-infected GECs by dampening and/or evading innate immune response. A2a engagement has been shown to play a nonredundant role in downregulating inflammation *in-vivo*, and adenosine was demonstrated to inhibit IL-8 secretion by intestinal epithelial cells through its ability to prevent NF- κ B activation. This is consistent with *P. gingivalis*' ability to inhibit IL-8 production and suppress IL-1 secretion in GECs (Yilmaz *et al.*, 2010, Takeuchi *et al.*, 2013). On the other hand, elevated intracellular cAMP has been shown to promote growth of *Mycobacterium* species in macrophages (Bai *et al.*, 2009), thus it is plausible that cAMP could serve as an energy source for the replicating *P. gingivalis* in the A2a receptor-activated GECs (Figure 5 and 6).

Recent studies from our laboratory indicate that *P. gingivalis* effector enzyme, nucleoside-diphosphate-kinase (Ndk) is secreted extracellularly from *P. gingivalis*-infected GECs. This nucleotide converting enzyme homologue catalytically depletes extracellular ATP, thereby resulting in inhibition of P2X₇ receptor-mediated cellular ROS production and host cell apoptosis (Yilmaz *et al.*, 2008; Choi *et al.*, 2013). The inside-out effect we observed suggests that *P. gingivalis* has the capacity to reduce extracellular nucleotide concentrations of ATP, thereby acting as a generator of adenosine. It remains to be determined if *P. gingivalis* Ndk facilitates adenosine receptor activation, however it is tempting to speculate that there could be a connection between inhibiting pro-inflammatory ATP while simultaneously generating anti-inflammatory adenosine. Interestingly, alteration of adenosine concentrations appears to be an important survival mechanism for some opportunistic pathogens. *Staphylococcus aureus* and *B. anthracis* were found to possess an enzyme, "Adenosine synthase", which aids in evasion of host immune defenses (Thammavongsa *et al.*, 2009). The study found that adenosine synthase homologues have 5' nucleotidase activity, which synthesizes adenosine from ATP, resulting in enhanced survival in blood and reduced phagocytic clearance of these opportunistic pathogens. The same study also found that other important oral cavity bacteria including *Enterococcus faecalis* and *Streptococcus mutans* possess uncharacterized homologues of adenosine synthase. This line of inquiry has not been fully explored to date, however preliminary investigations we have carried out in our laboratory agree that *P. gingivalis* also possesses a putative Adenosine synthase homologue (PGN 0282, 2',3'-cyclic-nucleotide 2'-phosphodiesterase, *P. gingivalis* ATCC 33277) that has yet to be characterized.

In summary, this study identified a novel mechanism, specifically the A2a adenosine receptor, which may be utilized by *P. gingivalis* to sustain successful persistent infections in the oral epithelium. However, subsequent studies are strongly needed to determine the precise mechanisms of A2a ligation on modulation of *P. gingivalis* infection in GECs. We also recognize that the presence of *P. gingivalis* in the subgingival crevice is not a monoinfection, but rather a prominent component of a complex and diverse microbial community. Other microbial species might regulate the pathogenic potential of *P. gingivalis* via differential modulation of a number of purinoreceptors in the gingival epithelium. Taken together, these results indicate an important role for A2a receptor in promoting intracellular infection by *P. gingivalis* and the adenosine receptors could represent a potential target for therapeutic intervention in chronic periodontitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Primary GECs express Adenosine receptors. (A) RT-PCR analysis of adenosine receptor expression profile in primary GECs. Amplicons of expected sizes were detected for A1 (384 bp), A2a (430 bp), A2b (121 bp), and A3 (801 bp) receptors. The results are representative of at least three different GEC lineages (B) Detection of A2a receptor expression at the cell membrane of GECs via immunofluorescence microscopy. Cells were fixed and subsequently incubated with A2a receptor primary antibody. Staining of cells with conjugated Alexa-Fluor 594 secondary antibody (red) and DAPI (blue) was used to visualize expression pattern of A2a receptor. The staining revealed a diffuse expression of the A2a receptor throughout the cell membrane.

Figure 2. A2a receptor activation results in elevated number of intracellular *P. gingivalis*. GECs were infected with *P. gingivalis* at moi 100 for 24 h total infection time. Samples were fixed and stained with *P. gingivalis* primary antibody and subsequently stained with Oregon Green 488 secondary antibody (green) and DAPI (blue) to visualize infection. (A) i. Infected untreated GECs, ii. Infected GECs treated 1 h post-infection with A2a receptor selective

agonist CGS-21680, iii. Infected GECs treated 3 h post-infection with CGS-21680. Images presented here are representative of at least 175 fields studied in experiments that were performed two separate times in duplicate. (B) Analysis of fluorescent levels using ImageJ software revealed elevated levels of *P. gingivalis* in both treatment conditions compared to control. At 1 h post-infection *P. gingivalis* levels were ~2.5 times that of control and were ~3 times higher at 3 h post-infection. Asterisks (*) denote statistical significance ($P < 0.001$ Student *t*-test).

Figure 3.

Antibiotic protection assay of *P. gingivalis*-infected GECs yields more live bacteria recovered after stimulation of A2a receptor. Stimulation of A2a receptor in *P. gingivalis*-infected GECs resulted in greater amounts of live bacteria recovered after host cell lysis and plating of intracellular contents on Blood agar plates. There was greater than twice the amount of *P. gingivalis* recovered after A2a activation compared to un-stimulated infected control. Asterisk (*) indicates statistical significance ($P < 0.001$ Student *t*-test) between untreated infected controls and A2a receptor activated infected GECs.

Figure 4.

Suppression of *P. gingivalis* infection via pharmacological inhibition or RNAi knockdown of A2a receptor. (A) Primary GECs were infected with the PgFbFP strain at moi 100 for 24 h, fixed and stained with DAPI (blue) to visualize nuclei. The relative amount of intracellular bacteria determined by ImageJ software analysis of images detected using fluorescence microscopy. Treatment of infected GECs with A2a receptor specific agonist CGS-21680 resulted in a ~100% increase in levels of metabolically active bacteria compared to untreated infected control. A2a receptor specific antagonist SCH-58261 treated infected GECs yielded ~30% less infection compared to control. A2a receptor knockdown GECs were infected with the PgFbFP strain and the intracellular bacteria levels at 24 h post-infection were quantified and compared to wild-GEC controls. Asterisks (*) indicate statistical significance ($P < 0.001$ Student *t*-test) between CGS-21680 and control, SCH-58261 and control, A2a knockdown condition and control (B) The representative images of the conditions described above i. PGFbFP-infected GECs, ii. PGFbFP-infected GECs were treated with A2a receptor-specific agonist CGS-21680, iii. PGFbFP-infected GECs were treated with A2a receptor-specific antagonist SCH-58261, iv. PGFbFP-infected A2a receptor knockdown GECs. Images presented here are representative of at least 175 fields studied in experiments that were performed two separate times in duplicate.

Figure 5. Activation of A2a receptor during *P. gingivalis* infection increases intracellular cAMP levels. Un-infected and non-treated GECs were used as control (CON). GECs infected with *P. gingivalis*, A2a receptor-specific agonist CGS-21680 treated GECs (CGS), A2a receptor-specific antagonist SCH-58261-treated GECs (SCH), A2a receptor stimulated and infected GECs (CGS + *P.gingivalis*), A2a receptor stimulated and subsequently inhibited GECs (CGS + SCH), and A2a receptor antagonist treatment followed by infected GECs (SCH + *P. gingivalis*) were utilized as experimental conditions. Intracellular cAMP levels were measured using an ELISA-based cAMP detection kit (Assay Designs) at 30 m, 2 h, 6h, 12 h, and 24 h post-infection. Results represent normalized levels of cAMP compared to un-infected non-treated controls and were obtained from experiments performed in triplicate.

Figure 6.

Anti-inflammatory adenosine signaling network contributes to persistence of *P. gingivalis* infection in primary GECs. Exogenous sources of adenosine stimulate A2a receptor, resulting in cAMP formation via adenylyl cyclase activity. cAMP may indirectly serve as a potential energy source to promote proliferation of intracellular *P. gingivalis*. Additional activation of anti-inflammatory signaling networks such as protein kinase A (PKA), may also downregulate inflammation of infected tissues. Adenosine-mediated reduction in inflammatory state of *P. gingivalis* infected tissue and inadvertent energy source supplementation may contribute to persistent infections by *P. gingivalis* in the oral mucosa.

Table 1

RT-PCR primers for adenosine receptors with optimized annealing temperatures and expected amplified product size.

Subtype	Forward (5'-3')	Reverse (3'-5')	Expected size	Optimized annealing temperature (C)
A1	CCACAGACCTACTTCCACAC	GTAGATGAGGACCATGAGGA	384	56
A2a	AACGTCACCACTACTTTGT	AGTTGAAGTACACCATGTAG	430	61
A2b	GCTCCATCTTCAGCCTTCTG	ACCCAGAGGACAGCAATGAC	121	66
A3	CTGCTTGAGTCCTGAGTCAC	CCACACCTCAGAGACTGATT	801	61