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## Activation of Notch-1 in oral epithelial cells by *P. gingivalis* triggers the expression of the antimicrobial protein PLA<sub>2</sub>-IIA

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### Abstract

*P. gingivalis* (*Pg*) is an oral pathogen with the ability to induce oral dysbiosis and periodontal disease. Nevertheless, the mechanisms by which mucosal responses to the oral microbiota in the presence of specific pathogens such as *Pg* could abrogate the host-microbe symbiotic relationship leading to periodontitis remain unclear. Herein, we identified the Notch-1/PLA<sub>2</sub>-IIA axis as a new molecular pathway through which *Pg* could be specifically modulating oral epithelial antimicrobial and inflammatory responses. *Pg* activated Notch-1, and inhibition or silencing of Notch-1 completely abrogated *Pg*-induced PLA<sub>2</sub>-IIA in oral epithelial cells (OECs). Activation of Notch-1 and PLA<sub>2</sub>-IIA production were associated with *Pg*-produced gingipains. Other oral Gram-positive and Gram-negative species failed to induce similar responses. *Pg* enhanced OEC antimicrobial activity through PLA<sub>2</sub>-IIA. Increased Notch-1 activation correlated with higher PLA<sub>2</sub>-IIA gingival expression and changes in the abundance of specific oral bacteria phyla during periodontal disease. Oral bacterial species exhibited differential antimicrobial susceptibility to

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### DISCLOSURE

The authors declare no conflict of interest.

### AUTOR CONTRIBUTIONS

AA, YA, SK designed and performed *in vitro* experiments, analyzed data and wrote manuscript; AK assisted *in vitro* experiments; MJN, LO, JG, MM, and JLE contributed designing and conducting the nonhuman primate experiments as well as analyzing the data; AJS supported the experimental design and statistical analysis; OA design the study, supervised and performed experiments, analyzed data and wrote the manuscript.

PLA<sub>2</sub>-IIA. These findings support previous evidence suggesting an important role for epithelial Notch-1 activation and PLA<sub>2</sub>-IIA production during health and disease at mucosal surfaces, and provide new mechanistic information concerning the regulation of epithelial antimicrobial and pro-inflammatory responses modulated by oral pathogenic bacteria associated with periodontal disease.

### Keywords

Notch-1; PLA<sub>2</sub>-IIA; oral epithelial cells; *P. gingivalis*; antimicrobial; periodontal disease

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## INTRODUCTION

Although specific oral pathogenic bacterial species such as *Porphyromonas gingivalis* (*Pg*) have been related to established periodontal lesions and can recapitulate periodontal disease in animal models, the early events associated with epithelial-oral pathogen interactions in a complex microbial ecology that creates a microenvironment enabling the initiation of this chronic inflammatory disease remain unclear.

*Pg* is an oral Gram-negative (G<sup>-</sup>), anaerobic bacterium that has been broadly associated with periodontal disease<sup>1</sup> and systemic chronic inflammatory disorders such as atherosclerotic cardiovascular disease, diabetes, and rheumatoid arthritis.<sup>2</sup> Paradoxical results indicate that *Pg* has subdued pro-inflammatory effects in immune and non-immune cells (*i.e.*, cytokines/chemokines production) when compared with the effect of other oral bacterial species.<sup>3, 4</sup> This response, has been linked to several *Pg* virulence factors such as proteases (gingipains), and Lipopolysaccharide (LPS) structural differences compared to other G<sup>-</sup> species.<sup>4, 5</sup> The breadth of mechanisms by which *Pg* could be driving inflammation remains undetermined. Although, recent evidence suggests that *Pg*, rather than directly being inducing inflammation, it could be indirectly orchestrating changes in the host-oral microbiota interactions (*i.e.*, dysbiosis), which will ultimately drive a chronic inflammatory response leading to further destruction of the periodontal tissues.<sup>6</sup> This new model of *Pg*-driven dysbiosis/periodontitis implies that there could be important variations in both the host response and the oral microbial ecology elicited by *Pg* that can affect their symbiotic relationships; nonetheless, the mechanisms involved in *Pg*-induced dysbiosis remain undefined.

Epithelial cells rather than being simply a passive barrier play a critical and active role in maintaining mucosal homeostasis through a constant interaction with the microbiota that lead to the production of an array of antimicrobial and immunoinflammatory factors.<sup>7</sup> Therefore, we hypothesized that oral epithelial antimicrobial and immunoinflammatory responses are specifically modulated by oral pathogens such as *Pg* when compared with oral commensals.

PLA<sub>2</sub>-IIA is a highly cationic lipolytic enzyme produced by multiple cells (*e.g.*, hepatocytes, platelets, macrophages, and Paneth cells) that belong to a subfamily of 10 secreted PLA<sub>2</sub> enzymes. PLA<sub>2</sub>-IIA plays a critical role in infection and inflammation through its potent antimicrobial properties, as well as its role in the pathway for lipid mediators' production

with activation of inflammatory cells such as neutrophils, macrophages and platelets.<sup>8-10</sup> Specifically, PLA<sub>2</sub>-IIA exhibits potent antimicrobial activity due to its high affinity for the phosphatidylethanolamine and phosphatidylglycerol, which are enriched in the membrane of bacteria.<sup>11</sup> Consistent with its antimicrobial properties, PLA<sub>2</sub>-IIA has been recently shown to be an important candidate for host genes that strongly influence the gut microbiota composition<sup>12</sup>, as well as a host molecule that can be used by bacterial complexes to regulate species survival in the lungs.<sup>13</sup> In addition to its antimicrobial properties, PLA<sub>2</sub>-IIA induces inflammation by activating neutrophils and macrophages through an M-type receptor (C-type lectin family), enhancing the expression of prostaglandin E2, as well as inducing the production of fatty acids, phospholipids, and release of mitochondrial DNA through its enzymatic activity on extracellular mitochondria expelled from activated platelets.<sup>9, 10</sup>

Herein, using transcriptomic analysis of immunoinflammatory genes in human oral epithelial cells (OECs) we have identified PLA<sub>2</sub>-IIA as a new molecule through which *Pg* could be specifically modulating OEC antimicrobial and inflammatory responses. Specifically, we demonstrated that PLA<sub>2</sub>-IIA expression involves the activation of Notch-1 receptor in OECs by *Pg* gingipains and the antimicrobial activity of OEC is modulated by *Pg* through PLA<sub>2</sub>-IIA. Interestingly, both PLA<sub>2</sub>-IIA expression and Notch-1 activation showed significant elevations in gingival tissues early during initiation and progression of periodontal disease concurrently with oral dysbiosis, and oral bacterial species showed differential antimicrobial susceptibility to PLA<sub>2</sub>-IIA. Our findings suggest that a significant increase of PLA<sub>2</sub>-IIA induced by *Pg* in OECs may affect the growth and survival of specific bacterial species of the oral microbiome.

## RESULTS

### PLA<sub>2</sub>-IIA induction by *Pg* in oral epithelial cells

To identify specific OEC responses elicited by *Pg* that could potentially be associated with disease, transcriptional activity of 520 immunoinflammatory genes with antimicrobial and pro-inflammatory properties were evaluated in OECs exposed to *Pg* for 24 hours using nanostring, and compared with the responses enhanced by the oral commensal *S. gordonii* (*Sg*). Interestingly, *Pg* induced a remarkable increase in the transcription of PLA<sub>2</sub>-IIA when compared with the response induced by *Sg*, with expression levels exceeding about 50 times those of classical immunoinflammatory mediators (*e.g.*, cytokines/chemokines and classical antimicrobial peptides such as human beta-defensins) produced by OECs. PLA2G2A was the most up-regulated gene (~3,900-fold) elicited by *Pg* in OECs (Figure 1). These results were further validated by qRT-PCR where *Pg* induced about a 100- and 500-fold increase in mRNA levels of PLA2G2A at 24 and 48h after bacterial challenge (Figure 2A). Consistently with the observed transcriptional response, protein levels of PLA<sub>2</sub>-IIA were significantly elevated in *Pg*-exposed OEC lysates (Figure 2B). Interestingly, strong up-regulation of PLA<sub>2</sub>-IIA was specifically induced by *Pg* in OECs. All other oral Gram-positive and Gram-negative bacterial species tested, failed to induce similar responses, while enhancing IL-8 production a standard response to Gram-negative periodontopathogens [*e.g.*, *F. nucleatum* (*Fn*), *A. actinomycetemcomitans* (*Aa*), and *T. forsythia* (*Tf*)] (Figure 2C).

### Notch-1 activation is involved in *Pg*-induced PLA<sub>2</sub>-IIA expression

The unique ability of *Pg* for inducing PLA<sub>2</sub>-IIA expression in OECs, could not be simply explained, by the engagement of pattern recognition receptors (PRRs) such as Toll-like receptors 2 and 4 (TLR2 or TLR4), since most oral bacteria can engage these receptors.<sup>14, 15</sup> Evidence indicates that Notch-1 signaling is associated with PLA<sub>2</sub>-IIA production in intestinal epithelial cells *in vivo*, which appears to play a critical role in gut homeostasis<sup>16</sup> and epithelial Notch-1 is a critical regulator of these mucosal immune responses.<sup>17, 18</sup> In addition, it has been demonstrated that *Pg* stimulates Notch-1 expression in aortic smooth muscle cells (AoSMCs) and osteoblast progenitor cells (OBPCs).<sup>19, 20</sup> Based on this evidence, we hypothesized that Notch-1 activation in OECs could be a possible target for *Pg* upregulating PLA<sub>2</sub>-IIA expression. To explore this, the surface and intracellular expression levels of Notch-1 in OECs cells were first evaluated by flow cytometry. Both, OKF6 and TIGK cells were positive for surface and intracellular Notch-1 expression, with OKF6 cells exhibiting higher mean fluorescence intensity levels than TIGK cells (Supplementary Figure. 1).

The ability of *Pg*, *S. gordonii* (*Sg*), and *F. nucleatum* (*Fn*) to activate Notch-1 in OECs was further evaluated by determination of mRNA levels for Hairy and enhancer of split 1 (HES-1), a gene specifically transcribed upon Notch-1 activation.<sup>21, 22</sup> *Pg* increased the expression of HES-1 by 3- to 9-fold in a dose-dependent manner (Figure 3A). However, *Fn* and *Sg* did not affect HES-1 expression although both increased IL-8 mRNA levels (Figure 3B). Consistently, nuclear levels of Notch-1 intracellular domain (NICD) were significantly higher in OKF6 cells exposed to *Pg* but not *Fn* or *Sg* compared to un-stimulated cells (Figure 3C–3D). Further, to determine whether Notch-1 activation was involved in *Pg*-induced PLA<sub>2</sub>-IIA, the Notch-1/gamma secretase inhibitors DAPT and JLK6 were used. Protein levels of *Pg*-induced PLA<sub>2</sub>-IIA were completely abrogated by both inhibitors at nanomolar (DAPT) and micromolar (JLK6) concentrations without changes in cell viability (Figure 4A and Supplementary Figure 2). Consistent with these findings, OKF6 cells expressing lower levels of Notch-1 after transfection with a specific siRNA for Notch-1 (Figure 4B and 4C), exhibited a significant reduction in the *Pg*-induced PLA<sub>2</sub>-IIA mRNA (25-fold less) (Figure 4D). These findings were consistent with a reduction in *Pg*-induced PLA<sub>2</sub>-IIA protein levels, which were almost completely abrogated when compared with cells transfected with the siRNA negative control. (Figure 4D). In contrast, Notch-1 silencing enhanced both constitutive and *Pg*-induced IL-8 expression (Supplementary Figure 3).

Although *Pg* can be sensed by TLR2 and TLR4<sup>23</sup>, expression and neutralization experiments suggested no effect of these TLRs in *Pg*-induced Notch-1 activation or PLA<sub>2</sub>-IIA production by OECs (Supplementary Figure. 4)

### *Pg* gingipains are involved in Notch-1 activation and PLA<sub>2</sub>-IIA production by oral epithelial cells

Among several virulence factors, *Pg* has the unique property to produce three cysteine proteases, Arginine- (rgpA and rgpB) and Lysine- (kgp) gingipains, which degrade many inflammatory mediators such as cytokines/chemokines.<sup>24</sup> Gingipains also affect the

production of epithelial innate factors (*e.g.*, beta-defensins).<sup>25</sup> Since Notch-1 activation involves sequential enzymatic cleavages including the release of the extracellular domain from the cell membrane by the enzyme TNF $\alpha$ -converting enzyme (TACE), followed by a further enzymatic cleavage of the Notch-1 intracellular domain by the gamma-secretase complex, we hypothesized that *Pg* gingipains could be involved in PLA<sub>2</sub>-IIA production by OECs through Notch-1 activation. Accordingly, Notch-1 activation was observed only in OECs exposed to *Pg* wild type (WT) but not in cells challenged with the *Pg* mutant for all gingipains (Figure 5A). Nevertheless, both *Pg* WT and mutant strains increased IL-8 transcription, which was used as a positive control for cell activation. Consistent with these results, significant increase in PLA<sub>2</sub>-IIA protein levels was observed in OECs exposed to *Pg* WT but not the *Pg* mutant (Figure 5B). Importantly, increased protein levels of IL-8 in OEC supernatants from cells exposed to the *Pg* triple mutant confirmed its biological activity (Figure 5C), and as expected, IL-8 levels in supernatants from OECs exposed to *Pg* WT were not detectable due to degradation associated with *Pg* gingipains.

### **Release of *Pg*-induced PLA<sub>2</sub>-IIA into the supernatants is enhanced by *F. nucleatum***

Since oral bacterial species have shown differential ability to induce production and release of pro-inflammatory mediators, we hypothesized that other oral bacterial species could induce release of *Pg*-induced PLA<sub>2</sub>-IIA. Detectable levels of PLA<sub>2</sub>-IIA were found in supernatants from *Pg*-primed OKF6 cells exposed to *Fn* but not *Sg*. Elevated PLA<sub>2</sub>-IIA levels were observed only in cell lysates from *Pg*-exposed OECs. (Figure 6).

### ***P. gingivalis* modulates antimicrobial oral epithelial cell responses through PLA<sub>2</sub>-IIA**

Since the antimicrobial properties of PLA<sub>2</sub>-IIA have been broadly described<sup>11</sup> we sought to determine if the ability for bacterial killing of OECs could be modulated by *Pg* through upregulation of PLA<sub>2</sub>-IIA using *L. monocytogenes* (*Lm*) as a susceptible PLA<sub>2</sub>-IIA target. *Pg*-challenged OEC extracts significantly reduced the number of *Lm* colonies forming units (CFUs), whereas unchallenged OECs extracts did not affect bacterial growth. Pre-incubation of *Pg*-challenged OEC extracts with an anti-PLA<sub>2</sub>-IIA antibody decreased their ability to reduce the number of CFUs when compared with the effect of its corresponding isotype control (Figure 7). These *Pg*-induced OEC antimicrobial responses were similarly induced when rPLA<sub>2</sub>-IIA was used as a control.

### **PLA<sub>2</sub>G<sub>2</sub>A expression, Notch-1 activation and oral microbiome changes during periodontal disease in non-human primates (NHPs)**

To determine whether Notch-1 activation and PLA<sub>2</sub>-IIA expression are up regulated *in vivo* during the pathogenesis of periodontal disease, the ligature induced-periodontitis model in non-human primates was used (Figure 8A). Levels of PLA<sub>2</sub>G<sub>2</sub>A were elevated during initiation and progression of disease; however, significant increases in particular at 2 weeks (100-fold) and 1 month (8-fold) after inducing disease with respect to base line levels were seen (Figure 8B). Interestingly, a similar pattern of Notch-1 activation determined by expression levels of Hairy/enhancer-of-split related YRPW motif protein 1 (HEY-1, gene specifically transcribed upon Notch-1 activation)<sup>26</sup> was observed at 2 weeks (2.5-fold) and 1 month (1.9-fold) (Figure 8C). The expression levels of both genes were comparable with base line levels during the clinical resolution phase of the disease. Noteworthy, variations in

the abundance of the main oral microbiome phyla were observed during the process of disease, such as reduction in actinobacteria, fusobacteria, and spirochaetes, and increase in the frequency of bacteroidetes and firmicutes as examples (Figure 8D). *Pg* abundance increased with disease at 2 weeks (Figure 8E). Selected oral bacterial species from some Phyla showing reduced numbers during disease (e.g., Actinobacteria: *Actinomyces naeslundii*, and Fusobacteria: *F. nucleatum*), also exhibited antimicrobial susceptibility to recombinant human PLA<sub>2</sub>-IIA (rPLA<sub>2</sub>-IIA). However, oral bacteria showing antimicrobial resistance belong to phyla showing no changes/increase in abundance (e.g., Firmicutes: *Streptococcus gordonii* and *Veillonella parvula*, and Bacteroidetes: *Capnocytophaga sputigena*). *Pg* was resistant to the anti-microbial activity of rPLA<sub>2</sub>-IIA (Figure 8F).

## DISCUSSION

*Pg* is an oral Gram-negative anaerobe bacterium with the ability to induce dysbiosis and periodontal disease.<sup>6</sup> Nevertheless, the mechanisms by which mucosal responses to the oral microbiota in the presence of specific oral pathogenic bacterial species such as *Pg* could abrogate the host-microbe symbiotic relationship leading to dysbiosis and later to periodontitis remain not fully understood.

Possible mechanisms associated with the onset of dysbiosis could be a direct impact of certain bacterial species competing or killing other members of the bacterial communities through the production of antimicrobial molecules such as bacteriocins<sup>27</sup>, or the modulation of host mucosal antimicrobial and inflammatory responses that can impact the growth or persistence of susceptible species within these bacterial communities.<sup>28</sup> Indeed, previous studies indicated that subversion of neutrophil responses by *Pg* that involve C5aR and TLR2 receptor activation and the serine protease (Ser B) that decreased IL-8 expression by oral epithelial cells can play a role in oral dysbiosis.<sup>29</sup> Nevertheless, whether or not *Pg* could specifically modulate antimicrobial and immunoinflammatory responses in oral epithelial cells with potential to impact the abundance of certain oral bacterial species remains unknown.

A transcriptome analysis of antimicrobial and inflammatory genes in OECs indicated that *Pg* is a strong inducer of PLA<sub>2</sub>-IIA expression and this response seems to be specific, since other bacterial species failed to induce similar responses. Production of PLA<sub>2</sub>-IIA is induced by other bacterial species such as *Neisseria meningitides* in macrophages and *Pseudomonas aeruginosa* in human bronchial epithelial cells through mechanisms that involve the pili and a type III secretion system respectively.<sup>13, 30</sup> Nevertheless, the cellular and molecular mechanisms by which PLA<sub>2</sub>-IIA expression is modulated in mammalian cells by bacterial components remain unclear. Herein, we demonstrated that *Pg* is able to induce the production of PLA<sub>2</sub>-IIA in a mechanism that involves Notch-1 receptor activation in OECs. To our knowledge, *Pg* could be one of the few microorganisms reported to date, including Hepatitis B and C viruses and Kaposi's sarcoma herpesvirus<sup>31, 32</sup> that have the ability to activate the Notch-1 receptor. Notch-1 activation regulates different cell responses such as proliferation, differentiation and apoptosis as well as seems to be a critical regulator of the mucosal immune responses.<sup>33</sup> As a consequence, activation of Notch-1 specifically by *Pg*

could be contributing in the pathogenesis of diseases in which this oral pathogen has been previously linked, such as periodontal disease, atherosclerosis, and oral cancer.

Although the precise molecular mechanisms by which *Pg* is activating Notch-1 remain to be elucidated, we showed that gingipains produced by *Pg* seem to be involved. In particular, the sequence of the Notch-1 extracellular domain (NECD) that is in close proximity with the cell membrane and interacts non-covalently with the NICD has a sequence of four Arginine amino acids that could be targeted by *Pg* rgp-A and rgp-B gingipains.<sup>34</sup> Thus, it is likely that gingipains and most specifically Arginine-gingipains could be inducing a non-canonical Notch-1 activation, enhancing an initial cleavage of the NECD, with the consequent activation of the gamma-secretase complex and further release and nuclear translocation of the NICD, whereby it can start transcription of specific genes including PLA<sub>2</sub>-IIA. Expression of HES-1 and HEY-1 have been shown to be regulated through Notch-1-independent pathways such as c-jun N-terminal kinase (JNK) signaling, which is also activated by *Pg* in oral epithelial cells.<sup>35, 36</sup> Nevertheless, the use of additional approaches (e.g., nuclear levels of NICD or siRNA Notch-1 silencing) reinforce the observation of a *Pg*-induced Notch-1 activation using these markers.

Previous studies have shown that TLR2 activation by *Pg* induces the expression of other inflammatory and antimicrobial mediators by OECs.<sup>23</sup> The lack of a role for TLR-2 in *Pg*-induced PLA<sub>2</sub>-IIA is consistent with the specificity of these oral epithelial responses induced by *Pg*, while other oral commensal and pathogenic bacteria, which also activate TLR-2, failed to induce similar PLA<sub>2</sub>-IIA responses. OECs have been shown to respond to the TLR4 ligand (i.e., purified *E. coli* LPS), and blocking TLR4 reduced the expression of the CCR5 receptor in OECs.<sup>37</sup> In this study, flow cytometry analysis showed that neither OKF6 nor TIGKs constitutively express TLR4. These findings are consistent with low/undetectable TLR4 mRNA expression levels in primary OECs compared with hematopoietic cells such as macrophages.<sup>38</sup> Although the potential role of TLR4 in *Pg*-induced PLA<sub>2</sub>-IIA could not be completely ruled out at this point (given that TLR4 expression may be induced after bacterial challenge), the contribution of this pathway remains questionable, given that other oral Gram-negative bacteria such as *F. nucleatum*, *T. forsythia* and *A. actinomycetemcomitans* also failed to induce PLA<sub>2</sub>-IIA in OECs.

The anti-microbial properties of PLA<sub>2</sub>-IIA have been described both in *vitro* and *in vivo*.<sup>11-13</sup> Herein, as a proof of concept, we demonstrated *in vitro* that *Pg* enhanced the antimicrobial properties of OECs stimulating the production of PLA<sub>2</sub>-IIA, and oral bacterial species have differential anti-microbial susceptibility to rPLA<sub>2</sub>-IIA. These findings suggest that modulation of PLA<sub>2</sub>-IIA levels in OECs by *Pg* could be contributing to modify the abundance of specific bacterial species within the oral microbiome. Nevertheless, this needs to be mechanistically demonstrated in future *in vivo* studies.

Even though PLA<sub>2</sub>-IIA is an important component in gastrointestinal and respiratory mucosal inflammatory disorders,<sup>10, 39</sup> there are surprisingly few reports demonstrating any variations in the expression levels of PLA<sub>2</sub>-IIA in oral mucosa or gingival crevicular fluid during health or periodontitis.<sup>40, 41</sup> Using the ligature-induced periodontitis model in nonhuman primates we found that significant increases in the gingival expression of PLA<sub>2</sub>-

IIA occur early during the initiation phase of the disease. Accordingly, a similar expression pattern was observed for Notch-1 activation with significant elevations occurring very rapidly after disease induction. These findings suggest that up-regulation of the Notch-1/PLA<sub>2</sub>-IIA axis in gingival tissues could be involved in early events of the pathogenesis of periodontitis. These observations are consistent with previous studies showing elevated levels of PLA<sub>2</sub>-IIA associated with several chronic inflammatory disorders such as rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, as well as diet-induced metabolic syndrome.<sup>42, 43</sup>

While the role of PLA<sub>2</sub>-IIA in the pathogenesis of periodontal disease *in vivo* remains to be determined, previous evidence supports the hypothesis that this enzyme may play a significant role. For example, mouse strains (*e.g.*, C57BL/6, 129/J) that have a natural mutation in PLA2G2A gene (leading to the production of an inactive enzyme) are more resistant to *Pg*-induced periodontitis, than mouse strains (*e.g.*, BALB/c, DBA/2J) that express a functional PLA<sub>2</sub>-IIA enzyme.<sup>44, 45</sup> Therefore, a non-functional PLA<sub>2</sub>-IIA in resistant mice strains may be preventing potential *Pg*-induced responses mediated by PLA<sub>2</sub>-IIA that could lead to dysbiosis and further periodontitis. Future mechanistic studies using different mice strains and PLA<sub>2</sub>-IIA transgenic mice are warranted to test and validate *in vivo* the potential role of this enzyme in oral dysbiosis and periodontal disease.

Overall, we have demonstrated that the oral pathogen *Pg* is a strong activator of PLA<sub>2</sub>-IIA in oral epithelial cells through a mechanism that involves Notch-1 activation by gingipains, and activation of PLA<sub>2</sub>-IIA increases the antimicrobial properties of OECs. Moreover, the Notch-1/PLA<sub>2</sub>-IIA axis is upregulated early in the periodontal disease process, and oral bacterial species exhibit differential antimicrobial susceptibility to PLA<sub>2</sub>-IIA, which seems to correlate with disease-associated oral microbiome changes. These findings support previous studies reporting a positive correlation between Notch-1 activation and PLA<sub>2</sub>-IIA expression in intestinal epithelial cells<sup>16</sup>, and provide new mechanistic information about the regulation of epithelial antimicrobial and pro-inflammatory responses modulated by oral pathogenic bacteria through the Notch-1/PLA<sub>2</sub>-IIA axis that could be possibly contributing to disease.

PLA<sub>2</sub>-IIA with its targeted antimicrobial activity and ability to modulate immunoinflammatory responses, combined with *P. gingivalis* commandeering the Notch-1 receptor pathway, makes this an interesting and novel potential strategy used by this oral pathogen to modify the local ecology that triggers early changes in the bacteria-host homeostatic interactions leading to disease.

## METHODS

### Human cell types and bacteria

The immortalized keratinocyte cell line OKF6, established by ectopic expression of the telomerase catalytic subunit (hTERT) in cells from normal oral mucosal epithelium<sup>46</sup> and the telomerase immortalized human gingival epithelial cells (TIGK)<sup>47</sup> were used in these studies. Both oral epithelial cell lines were cultured in antibiotic-free, serum-free keratinocyte medium (Ker-SFM, Gibco, Carlsbad, CA) supplemented with 0.2 ng/ml human



recombinant epidermal growth factor and 25µg/ml bovine pituitary extract. THP-1 cells were grown in RPMI 1640 (Sigma) with 2 mM L-glutamine and 10% FBS (Seradigm, Radnor, PA). All cells were grown in 5% CO<sub>2</sub> in air at 37°C.

The following bacterial strains were used in these studies: *Porphyromonas gingivalis* 381 (*Pg*), *Streptococcus gordonii* ATCC10558 (*Sg*), *Streptococcus sanguinis* ATCC10556 (*Ss*), *Actinomyces naeslundii* ATCC49340 (*An*), *Aggregatibacter actinomycetemcomitans* JP2 (*Aa*), *Tannerella forsythia* ATCC43037 (*Tf*), *Fusobacterium nucleatum* ATCC25586 (*Fn*), *Capnocytophaga sputigena* ATCC33612, *Veillonella parvula* ATCC10790, and *Listeria monocytogenes* EDGe strain. All bacterial strains, were initially grown on blood agar plates (BBL, Becton Dickinson, Sparks, MD, USA) from a frozen stock and incubated in appropriate aerobic or anaerobic conditions during 24 hours or 3 days, respectively. Further, a liquid culture was started for each strain in 3ml of Brain Heart Infusion Broth alone or supplemented with 5µg/ml Hemin and 1µg/ml Menadione in the case of *Pg*. Cells were incubated for 24h and then overnight stationary cultures were inoculated in fresh BHI broth and allowed to reach logarithmic phase for 3–4h. Finally, bacteria were counted in a hemocytometer by microscopy, and a cell suspension adjusted to 1×10<sup>7</sup> cells/ml in Ker-SFM with supplements for further experiments challenging OECs. *T. forsythia* was grown in 2.5ml of ATCC medium 1921 supplemented with 10 µg/ml of N-acetyl muramic acid (NAM). The *Pg* mutant for gingipains (rgpA-rgpB-kgp) created under the *Pg*381 background was grown in blood agar plates and Trypticase Soy broth supplemented with Yeast extract, Hemin 5µg/ml and Vitamin K 1µg/ml in presence of Erythromycin 10µg/ml and Tetracycline 2µg/ml.

### Immunoinflammatory transcriptome analysis by nanostring

Immunoinflammatory transcriptome analysis of OECs exposed to the oral pathogen *Pg* or the oral commensal *S. gordonii* [MOI-1:100 for 24h] was performed using a set of 520 genes defined by the nCounter Human Immunology Kit panel (Nanostring, Seattle, WA). Briefly, after bacterial exposure, total mRNA was isolated using the PureLink RNA Mini kit (Life Technologies, NY, USA). Further, 100ng of RNA were hybridized with the reporter code set beads for a final volume of 30µl at 65°C for 12 hours. Finally, the samples were processed on the Nanostring Cell Prep Station and data collected using the Nanostring Digital Analyzer version (Nanostring Technologies, Seattle, WA, USA).

### Quantitative RT-PCR for Human PLA2-IIA, HES-1, IL-8

Transcriptional activation of PLA2G2A, HES-1, and IL-8 was evaluated by RT-qPCR in OKF6 cells after different treatments with oral bacteria. RNA was extracted using PureLink™ RNA Mini Kit (Invitrogen) and measured on a NanoDrop (Thermo, Waltham, MA) for concentration and quality. Genomic DNA was removed using PerfeCTa® DNase I (Quanta, Beverly, MA). RNA was then reverse-transcribed using qScript™ cDNA Synthesis Kit (Quanta). qPCR assays were run on 96 well plates using PerfeCTa® SYBR® Green SuperMix (Quanta) and read on LightCycler480 (Roche, Basel, Switzerland) using the following cycling conditions: Initial denaturation: 95°C, 3 min. PCR cycling 45 cycles (95°C, 15 seconds, 60°C, 30 seconds). The sequences for all used primers are shown in

Table 1. Relative quantification method (based on  $2^{-CT}$ ) was used to calculate fold differences using GAPDH as an endogenous control.

### Enzyme linked immunosorbent assays

Protein levels of PLA<sub>2</sub>-IIA and IL-8 either in cell lysates or supernatants were determined by ELISA using the PLA<sub>2</sub> (human type IIA EIA) immunometric assay kit (Cayman Chemicals Company, Ann Arbor, MI, USA) and an IL-8 ELISA MAX standard kit (BioLegend, San Diego, CA). For cell lysates, after different treatments cell monolayers were washed with 1X PBS and lysed with 300µl RIPA buffer for 30 minutes on ice followed by centrifugation at 14,000rpm at 4°C. Supernatants were stored at -20°C for ELISA analysis. Determination of total protein was performed using the DC protein assay kit (BIO-RAD, Hercules, CA, USA) and PLA<sub>2</sub>-IIA amounts obtained by ELISA were normalized to a constant amount of protein for all samples.

### Flow cytometry analysis and Immunofluorescence

For analysis of Notch-1 expression, confluent cell cultures of OKF6 and TIGK cells grown in 75cm<sup>2</sup> flasks were dissociated using Trypsin-EDTA solution for 5 minutes, and washed at 1200rpm for 5 min. Further,  $1 \times 10^5$  cells in 100µL FACS buffer were then either incubated with 5µL monoclonal IgG antibody against Notch-1 conjugated with phycoerythrin (anti-Notch-1-PE) (recognizes the Notch-1 extracellular domain) or an IgG isotype control (R&D Systems, Minneapolis, MN) undiluted for 30 minutes on ice. Cells were then washed twice with PBS (1200rpm, 5 minutes), and resuspended in 100µL FACS buffer for further analysis. For intracellular Notch-1 staining, a similar number of cells were fixed for 15 minutes with 2% paraformaldehyde solution at room temperature and then washed and permeabilized using 1X intracellular staining permeabilization wash buffer (BioLegend, San Diego, CA) before adding the corresponding antibodies.

OKF6 ( $1 \times 10^5$ ) cells grown on coverslips and exposed to *Pg* [1:100] for 24h were fixed, permeabilized and stained with anti-cleaved Notch-1 (NICD) (ab8925; abcam, Cambridge, UK) dilution 1:200 for 1h, followed by goat anti-rabbit AF467 (Invitrogen, Carlsbad, CA) at 1:500 dilution for 1h. DAPI was used to stain the nuclei. Images were captured, and percentage of NICD positive nuclei from three random regions of interest per slide analyzed using the Nikon TiS fluorescent microscope, and NIS-Elements Basic Research software (Tokyo, Japan).

### Notch-1 inhibitors and siRNA silencing

OKF6 cells ( $2 \times 10^5$  cells/well) were seeded in 24-well plates in 1 ml of media and pre-incubated with the gamma-secretase/Notch-1 inhibitors DAPT (EMD Millipore, Billerica, MA) or JLK6 (Tocris Bioscience, Bristol, UK) diluted in DMSO for 1h before challenge with *Pg* [1:50] for 48h. Supernatants were harvested for further analysis and cells lysed for PLA<sub>2</sub>-IIA analysis by ELISA.

For transfection experiments, OKF6 cells ( $1 \times 10^5$ ) were seeded on 12 well plates in 1mL Ker-SFM overnight. Then, cells were transfected with either Notch 1 small interfering RNA (siRNA), or a negative control siRNA (Invitrogen, Carlsbad, CA). Briefly, 2µL per well

Lipofectamine RNAiMAX Reagent (Invitrogen) or 2µl of each siRNA were independently diluted in Opti-MEM Medium. Each diluted siRNA was added to diluted Lipofectamine RNAiMAX Reagent (1:1 ratio) and incubated for 5 minutes at room temperature. The siRNA-Lipid complexes were then added dropwise to the cells (final concentration of siRNA = 20nM) and cells were incubated for 24h at 37°C before being harvested for Notch-1 expression analysis.

### Antimicrobial effect of rPLA<sub>2</sub>-IIA and *Pg*-induced PLA<sub>2</sub>-IIA

Oral bacterial species ( $10^6$ ) growing exponentially in BHI broth were individually exposed to human recombinant PLA<sub>2</sub>-IIA (1µg/ml) (R&D Systems, Minneapolis, MN, USA) or its vehicle (CaCl<sub>2</sub> buffer) for 2h at 37°C in appropriate aerobic or anaerobic conditions and further seeded and incubated in blood agar plates to determine colony forming units (CFUs). Bacteria exposed to 0.1mg/ml penicillin/streptomycin was used as a positive control and *L. monocytogenes* (susceptible target for PLA<sub>2</sub>-IIA) used as control for anti-microbial activity of PLA<sub>2</sub>-IIA.

To test the antimicrobial effect of *Pg*-induced PLA<sub>2</sub>-IIA, OKF6 cells ( $10^6$ ) were exposed or not to *Pg* [1:50] for 48h and cell lysates obtained with 0.1% Triton in water, in presence of protease inhibitors (Complete Mini-EDTA-free, Roche Diagnostics, Indianapolis, IN, USA). Then, 40 µl of OKF6 cell lysates [1 µg/µl total protein] or 10 µg/ml of rPLA<sub>2</sub>-IIA resuspended in CaCl<sub>2</sub> buffer were pre-incubated with anti-PLA<sub>2</sub>-IIA antibody or its corresponding isotype control (30µg/ml) for 30 minutes on ice. Further *Lm* ( $10^6$  in 90 µl of broth) was incubated with 10 µl of either OKF6 cell lysates or rPLA<sub>2</sub>-IIA after different treatments for 2h. *Lm* incubated only with media, CaCl<sub>2</sub> buffer (vehicle for rPLA<sub>2</sub>-IIA) and Triton + PI (OKF6 cell lysis buffer) were used as controls. Finally, 25µl of *Lm* (1:1000 dilution) were seeded on blood agar plates and CFUs quantified after 24h.

### Ligature-induced periodontal disease, gingival tissue and plaque sample analysis

Rhesus monkeys (*Macaca mulatta*) (n=9, 6 females and 3 males) housed at the Caribbean Primate Research Center (CPRC) at Sabana Seca, Puerto Rico, were used in these studies. The range of age was 12–14 years old (Mean=12.6±0.9). Following a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico, a single investigator periodontally examined anesthetized animals as we have previously described.<sup>48, 49</sup> At the initiation of the study, all animals were evaluated clinically for periodontal disease, and only animals with mean bleeding on probing (BOP) of 1 and mean pocket depths (PD) <3 mm were entered into the study.

After taking the baseline gingival samples, ligatures were placed on the second premolar and first and second molars of both maxillary quadrants. Further, clinical evaluation for ligated sites was obtained and a buccal gingival papilla from each animal was taken using a standard gingivectomy technique at each time point as we and others have previously reported.<sup>50,49</sup> Samples were maintained frozen at –80°C in RNAlater solution until real time RT-PCR analysis.<sup>49</sup> Isolated RNA from 3 different animals was pooled (total of 3 groups of three pooled samples/time point) and a total of 1µg was used for each cDNA synthesis reaction. Sequences for the forward and reverse primers for PLA2G2A, HEY-1 and GAPDH are

shown in Table 1. The relative quantification analysis was performed with the LightCycler 480 software (Roche, IN). The concentration ratios for the target genes were calculated by normalizing to the housekeeping gene GAPDH. Oral subgingival plaque samples were also analyzed by 16S rRNA sequencing using Miseq Illumina system (Illumina, San Diego, CA, USA).

### Statistical Analysis

For the *in vitro* experiments, ANOVA was used for comparisons among experimental groups, and unpaired student's *t* test for comparisons between two groups. Significant variation of gene expression in gingival tissues from nonhuman primates at different time was evaluated using a one way ANOVA and a two sample *t*-tests.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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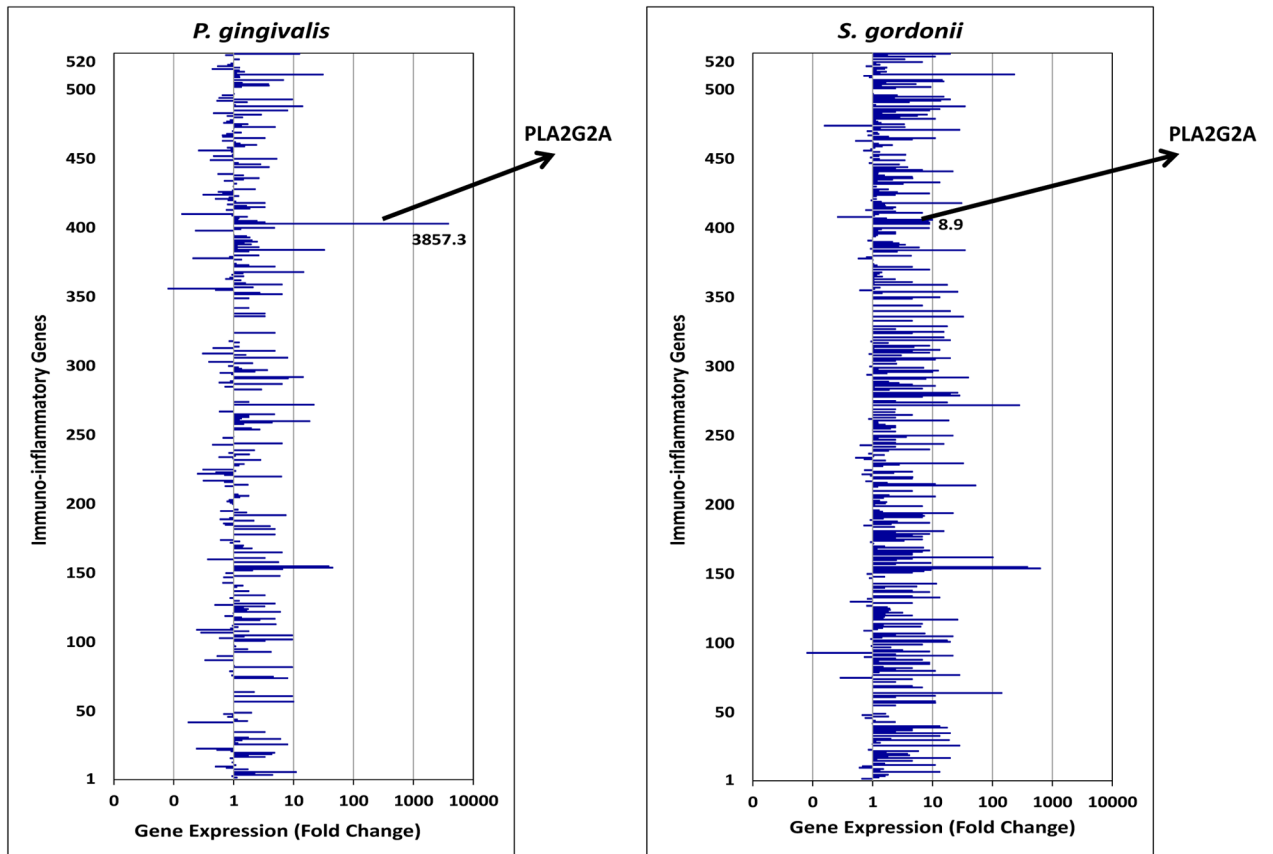
### References

1. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol.* 1998; 25(2):134–144. [PubMed: 9495612]
2. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol.* 2015; 15(1):30–44. [PubMed: 25534621]
3. Darveau RP, Belton CM, Reife RA, Lamont RJ. Local chemokine paralysis, a novel pathogenic mechanism for *Porphyromonas gingivalis*. *Infect Immun.* 1998; 66(4):1660–1665. [PubMed: 9529095]
4. Darveau RP, Arbabi S, Garcia I, Bainbridge B, Maier RV. *Porphyromonas gingivalis* lipopolysaccharide is both agonist and antagonist for p38 mitogen-activated protein kinase activation. *Infect Immun.* 2002; 70(4):1867–1873. [PubMed: 11895949]
5. Imamura T. The role of gingipains in the pathogenesis of periodontal disease. *J Periodontol.* 2003; 74(1):111–118. [PubMed: 12593605]
6. Hajishengallis G, Liang S, Payne MA, Hashim A, Jotwani R, Eskandari MA, et al. Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. *Cell Host Microbe.* 10(5):497–506.
7. Peterson LW, Artis D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol.* 2014; 14(3):141–153. [PubMed: 24566914]
8. Murakami M, Sato H, Miki Y, Yamamoto K, Taketomi Y. A new era of secreted phospholipase A(2). *J Lipid Res.* 2015; 56(7):1248–1261. [PubMed: 25805806]
9. Duchez AC, Boudreau LH, Naika GS, Bollinger J, Belleannee C, Cloutier N, et al. Platelet microparticles are internalized in neutrophils via the concerted activity of 12-lipoxygenase and secreted phospholipase A2-IIA. *Proc Natl Acad Sci U S A.* 2015; 112(27):E3564–3573. [PubMed: 26106157]
10. Bidgood MJ, Jamal OS, Cunningham AM, Brooks PM, Scott KF. Type IIA secretory phospholipase A2 up-regulates cyclooxygenase-2 and amplifies cytokine-mediated prostaglandin

- production in human rheumatoid synoviocytes. *J Immunol.* 2000; 165(5):2790–2797. [PubMed: 10946311]
11. Nevalainen TJ, Graham GG, Scott KF. Antibacterial actions of secreted phospholipases A2. Review. *Biochim Biophys Acta.* 2008; 1781(1–2):1–9. [PubMed: 18177747]
  12. Brodziak F, Meharg C, Blaut M, Loh G. Differences in mucosal gene expression in the colon of two inbred mouse strains after colonization with commensal gut bacteria. *PloS one.* 2013; 8(8):e72317. [PubMed: 23951309]
  13. Pernet E, Guillemot L, Burgel PR, Martin C, Lambeau G, Sermet-Gaudelus I, et al. *Pseudomonas aeruginosa* eradicates *Staphylococcus aureus* by manipulating the host immunity. *Nat Commun.* 2014; 5:5105. [PubMed: 25290234]
  14. Peyret-Lacombe A, Brunel G, Watts M, Charveron M, Duplan H. TLR2 sensing of *F. nucleatum* and *S. sanguinis* distinctly triggered gingival innate response. *Cytokine.* 2009; 46(2):201–210. [PubMed: 19299164]
  15. Gonzalez OA, Li M, Ebersole JL, Huang CB. HIV-1 reactivation induced by the periodontal pathogens *Fusobacterium nucleatum* and *Porphyromonas gingivalis* involves Toll-like receptor 2 [corrected] and 9 activation in monocytes/macrophages. *Clin Vaccine Immunol.* 2010; 17(9):1417–1427. [PubMed: 20610663]
  16. Okamoto R, Tsuchiya K, Nemoto Y, Akiyama J, Nakamura T, Kanai T, et al. Requirement of Notch activation during regeneration of the intestinal epithelia. *Am J Physiol Gastrointest Liver Physiol.* 2009; 296(1):G23–35. [PubMed: 19023031]
  17. Mathern DR, Laitman LE, Hovhannisyann Z, Dunkin D, Farsio S, Malik TJ, et al. Mouse and human Notch-1 regulate mucosal immune responses. *Mucosal Immunol.* 2014; 7(4):995–1005. [PubMed: 24424521]
  18. Hu X, Chung AY, Wu I, Foldi J, Chen J, Ji JD, et al. Integrated regulation of Toll-like receptor responses by Notch and interferon-gamma pathways. *Immunity.* 2008; 29(5):691–703. [PubMed: 18976936]
  19. Zhang B, Elmabsout AA, Khalaf H, Basic VT, Jayaprakash K, Kruse R, et al. The periodontal pathogen *Porphyromonas gingivalis* changes the gene expression in vascular smooth muscle cells involving the TGFbeta/Notch signalling pathway and increased cell proliferation. *BMC genomics.* 2013; 14:770. [PubMed: 24209892]
  20. Xing Q, Ye Q, Fan M, Zhou Y, Xu Q, Sandham A. *Porphyromonas gingivalis* lipopolysaccharide inhibits the osteoblastic differentiation of preosteoblasts by activating Notch1 signaling. *J Cell Physiol.* 2010; 225(1):106–114. [PubMed: 20648628]
  21. Guruharsha KG, Kankel MW, Artavanis-Tsakonas S. The Notch signalling system: recent insights into the complexity of a conserved pathway. *Nat Rev Genet.* 2012; 13(9):654–666. [PubMed: 22868267]
  22. Jarriault S, Le Bail O, Hirsinger E, Pourquie O, Logeat F, Strong CF, et al. Delta-1 activation of notch-1 signaling results in HES-1 transactivation. *Mol Cell Biol.* 1998; 18(12):7423–7431. [PubMed: 9819428]
  23. Darveau RP, Pham TT, Lemley K, Reife RA, Bainbridge BW, Coats SR, et al. *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infect Immun.* 2004; 72(9):5041–5051. [PubMed: 15321997]
  24. Potempa J, Sroka A, Imamura T, Travis J. Gingipains, the major cysteine proteinases and virulence factors of *Porphyromonas gingivalis*: structure, function and assembly of multidomain protein complexes. *Current protein & peptide science.* 2003; 4(6):397–407. [PubMed: 14683426]
  25. Chung WO, Hansen SR, Rao D, Dale BA. Protease-activated receptor signaling increases epithelial antimicrobial peptide expression. *J Immunol.* 2004; 173(8):5165–5170. [PubMed: 15470061]
  26. Maier MM, Gessler M. Comparative analysis of the human and mouse *Hey1* promoter: *Hey* genes are new Notch target genes. *Biochem Biophys Res Commun.* 2000; 275(2):652–660. [PubMed: 10964718]
  27. Sana TG, Flaugnatti N, Lugo KA, Lam LH, Jacobson A, Baylot V, et al. *Salmonella Typhimurium* utilizes a T6SS-mediated antibacterial weapon to establish in the host gut. *Proc Natl Acad Sci USA.* 2016; 113(34):E5044–5051. [PubMed: 27503894]

28. Zenewicz LA, Yin X, Wang G, Elinav E, Hao L, Zhao L, et al. IL-22 deficiency alters colonic microbiota to be transmissible and colitogenic. *J Immunol*. 2013; 190(10):5306–5312. [PubMed: 23585682]
29. Lamont RJ, Hajishengallis G. Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends Mol Med*. 2015; 21(3):172–183. [PubMed: 25498392]
30. Touqui L, Paya M, Thouron F, Guiyoule A, Zarantonelli ML, Leduc D, et al. Neisseria meningitidis pili induce type-IIA phospholipase A2 expression in alveolar macrophages. *FEBS letters*. 2005; 579(22):4923–4927. [PubMed: 16115633]
31. Kongkavitoon P, Tangkijvanich P, Hirankarn N, Palaga T. Hepatitis B Virus HBx Activates Notch Signaling via Delta-Like 4/Notch1 in Hepatocellular Carcinoma. *PloS one*. 2016; 11(1):e0146696. [PubMed: 26766040]
32. Lan K, Choudhuri T, Murakami M, Kuppers DA, Robertson ES. Intracellular activated Notch1 is critical for proliferation of Kaposi's sarcoma-associated herpesvirus-associated B-lymphoma cell lines in vitro. *J Virol*. 2006; 80(13):6411–6419. [PubMed: 16775329]
33. Mathern DR, Laitman LE, Hovhannisyan Z, Dunkin D, Farsio S, Malik TJ, et al. Mouse and human Notch-1 regulate mucosal immune responses. *Mucosal Immunol*. 2014; 7(4):995–1005. [PubMed: 24424521]
34. Ranganathan P, Weaver KL, Capobianco AJ. Notch signalling in solid tumours: a little bit of everything but not all the time. *Nat Rev Cancer*. 2011; 11(5):338–351. [PubMed: 21508972]
35. Curry CL, Reed LL, Nickoloff BJ, Miele L, Foreman KE. Notch-independent regulation of Hes-1 expression by c-Jun N-terminal kinase signaling in human endothelial cells. *Lab Invest*. 2006; 86(8):842–852. [PubMed: 16732296]
36. Wang Q, Sztukowska M, Ojo A, Scott DA, Wang H, Lamont RJ. FOXO responses to *Porphyromonas gingivalis* in epithelial cells. *Cell Microbiol*. 2015; 17(11):1605–1617. [PubMed: 25958948]
37. Giacaman RA, Asrani AC, Gebhard KH, Dietrich EA, Vacharaksa A, Ross KF, et al. *Porphyromonas gingivalis* induces CCR5-dependent transfer of infectious HIV-1 from oral keratinocytes to permissive cells. *Retrovirology*. 2008; 5:29. [PubMed: 18371227]
38. Neiva KG, Calderon NL, Alonso TR, Panagakos F, Walle SM. Type 1 diabetes-associated TLR responsiveness of oral epithelial cells. *J Dent Res*. 2014; 93(2):169–174. [PubMed: 24334435]
39. Yamaguchi O, Sugimura K, Ishizuka K, Suzuki K, Hasegawa K, Ohtsuka K, et al. Correlation between serum phospholipase A(2) IIA levels and histological activity in patients with ulcerative colitis. *Int J Colorectal Dis*. 2002; 17(5):311–316. [PubMed: 12172924]
40. Ishida H, Shinohara H, Nagata T, Nishikawa S, Wakano Y. Phospholipase A(2) Activity in Gingival Crevicular Fluid from Patients with Periodontal Disease: A possible Marker of Disease Activity. *Mediators Inflamm*. 1994; 3(1):17–21. [PubMed: 18472918]
41. Shinohara H, Komatsubara T, Tojo H, Okamoto M, Nishikawa S, Nagata T, et al. Group II phospholipase A(2) in human gingiva with periodontal disease. *Mediators Inflamm*. 1995; 4(2):95–97. [PubMed: 18475622]
42. Ibeas E, Fuentes L, Martin R, Hernandez M, Nieto ML. Secreted phospholipase A2 type IIA as a mediator connecting innate and adaptive immunity: new role in atherosclerosis. *Cardiovascular Research*. 2009; 81(1):54–63. [PubMed: 18755682]
43. Iyer A, Lim J, Poudyal H, Reid RC, Suen JY, Webster J, et al. An inhibitor of phospholipase A2 group IIA modulates adipocyte signaling and protects against diet-induced metabolic syndrome in rats. *Diabetes*. 2012; 61(9):2320–2329. [PubMed: 22923652]
44. Baker PJ, Dixon M, Roopenian DC. Genetic control of susceptibility to *Porphyromonas gingivalis*-induced alveolar bone loss in mice. *Infect Immun*. 2000; 68(10):5864–5868. [PubMed: 10992496]
45. Kennedy BP, Payette P, Mudgett J, Vadas P, Pruzanski W, Kwan M, et al. A natural disruption of the secretory group II phospholipase A2 gene in inbred mouse strains. *J Biol Chem*. 1995; 270(38):22378–22385. [PubMed: 7673223]
46. Dickson MA, Hahn WC, Ino Y, Ronfard V, Wu JY, Weinberg RA, et al. Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol Cell Biol*. 2000; 20(4):1436–1447. [PubMed: 10648628]

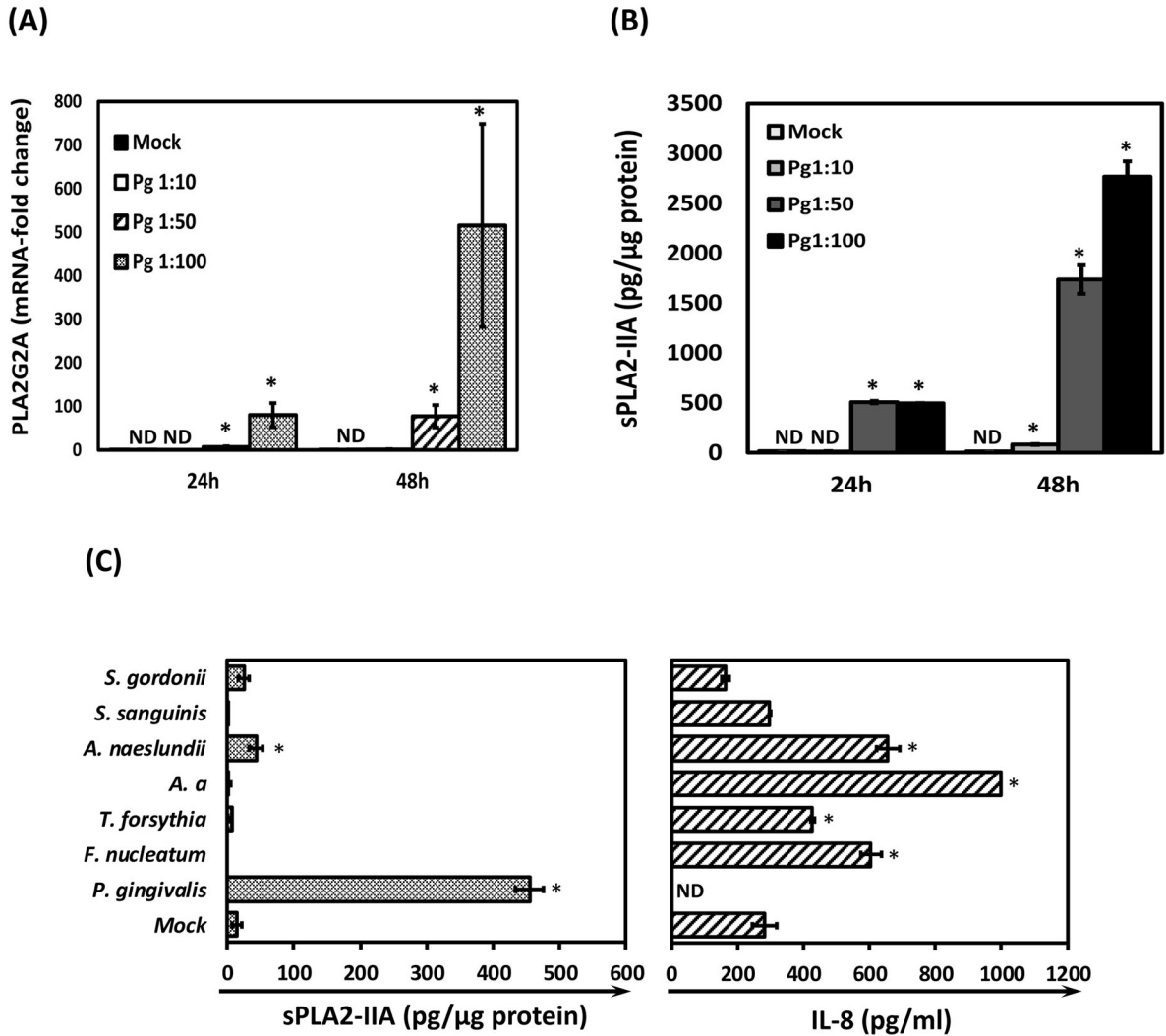
47. Moffatt-Jauregui CE, Robinson B, de Moya AV, Brockman RD, Roman AV, Cash MN, et al. Establishment and characterization of a telomerase immortalized human gingival epithelial cell line. *Journal of periodontal research*. 2013; 48(6):713–721. [PubMed: 23441958]
48. Ebersole JL, Steffen MJ, Gonzalez-Martinez J, Novak MJ. Effects of age and oral disease on systemic inflammatory and immune parameters in nonhuman primates. *Clin Vaccine Immunol*. 2008; 15(7):1067–1075. [PubMed: 18448617]
49. Ebersole JL, Kirakodu S, Novak MJ, Stromberg AJ, Shen S, Orraca L, et al. Cytokine gene expression profiles during initiation, progression and resolution of periodontitis. *J Clin Periodontol*. 2014; 41(9):853–861. [PubMed: 24975876]
50. Moritz AJ, Cappelli D, Lantz MS, Holt SC, Ebersole JL. Immunization with *Porphyromonas gingivalis* cysteine protease: effects on experimental gingivitis and ligature-induced periodontitis in *Macaca fascicularis*. *J Periodontol*. 1998; 69(6):686–697. [PubMed: 9660338]



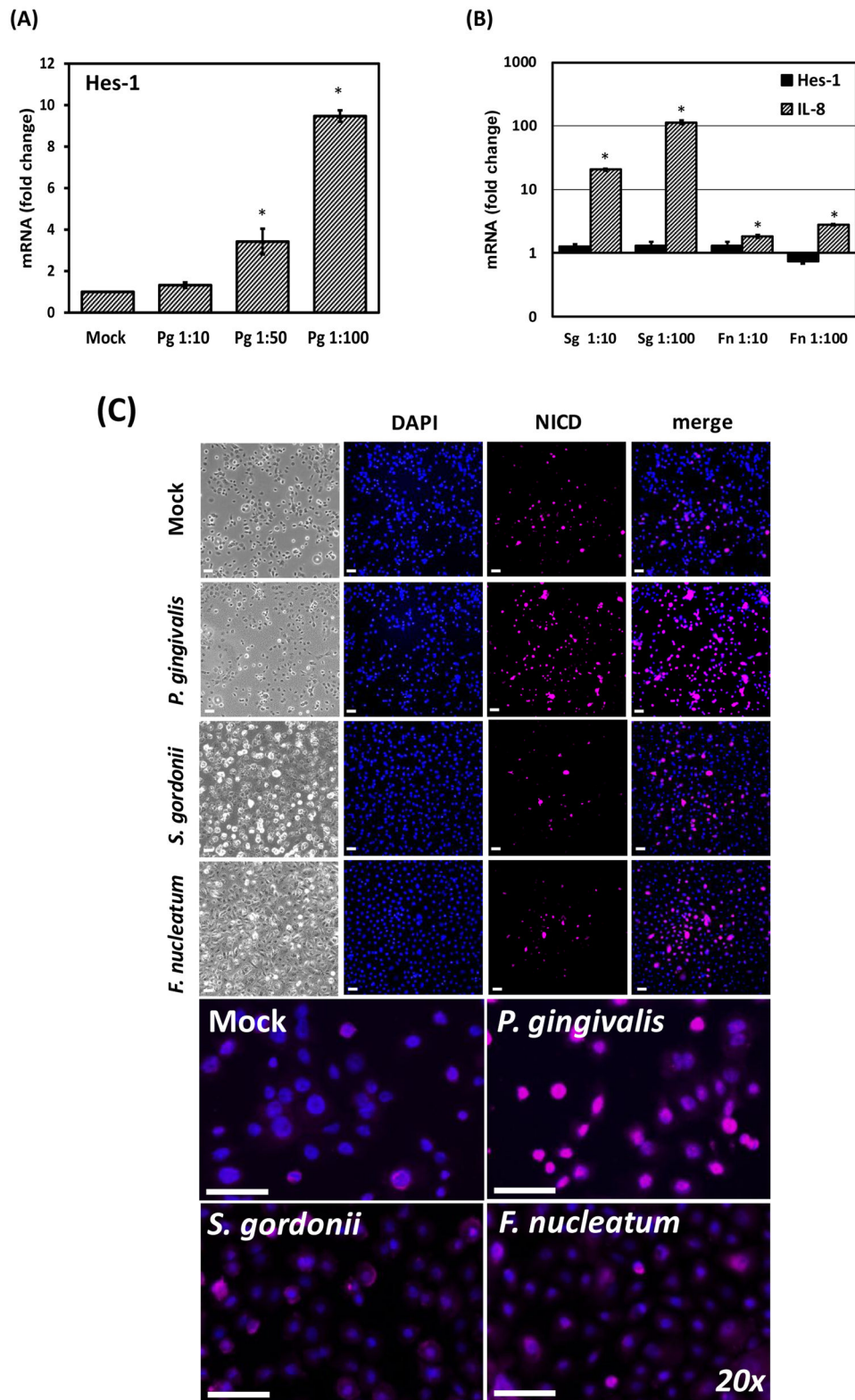
**Figure 1. Transcriptional activation of PLA2G2A is strongly induced by *P. gingivalis* in oral epithelial cells**

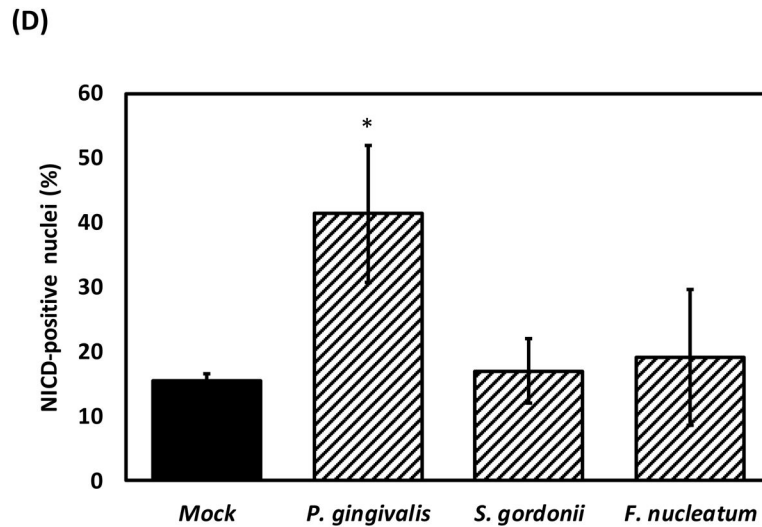
Immunoinflammatory transcriptome analysis (520 genes) of oral epithelial cells ( $2.5 \times 10^5$  OKF6 cells/well) exposed to the oral pathogen *P. gingivalis* or the oral commensal *S. gordonii* [MOI-1:100 for 24h] was obtained using Nanostring. Arrows indicate location of PLA2G2A and values of fold-change in expression compared with un-stimulated cells are shown.





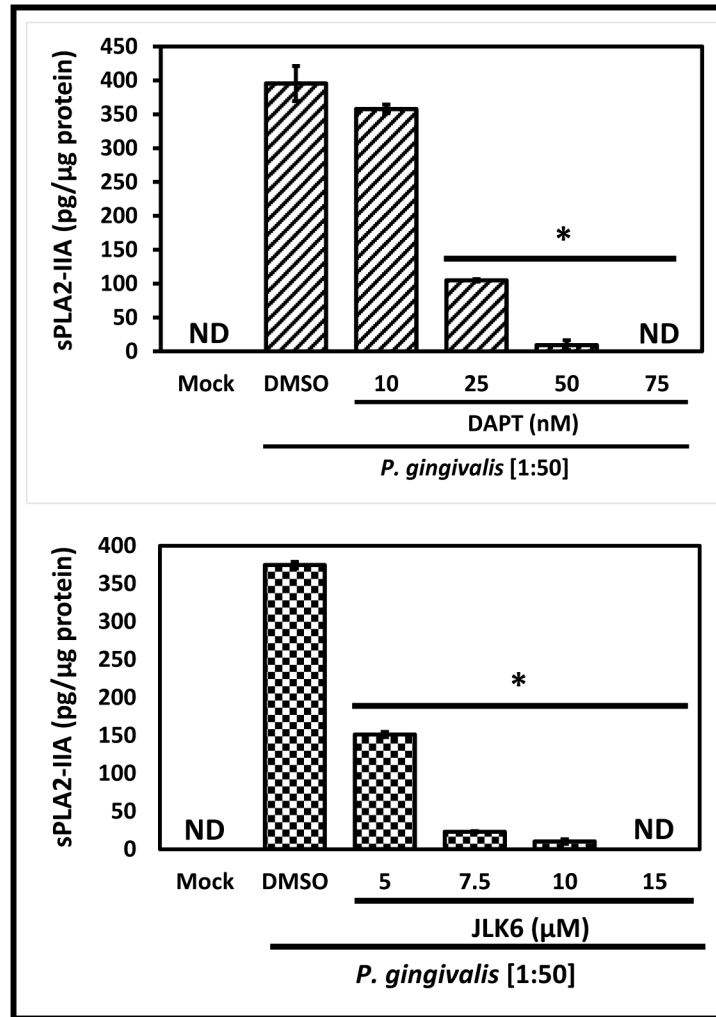
**Figure 2. Expression of PLA<sub>2</sub>-IIA is specifically induced by *P. gingivalis* in oral epithelial cells**  
**(A)** Transcriptional activation of PLA<sub>2</sub>G2A was evaluated by qRT-PCR in OKF6 cells exposed to different multiplicities of infection (MOIs) of *P. gingivalis* (*Pg*) for 24 and 48 hours. **(B)** Protein levels of PLA<sub>2</sub>-IIA induced by *P. gingivalis* at different MOIs for 24 and 48 hours were determined in OKF6 cell lysates by ELISA as described in methods. Normalized amounts of PLA<sub>2</sub>-IIA in cell lysates per 100μg of total protein are shown **(C)** Protein levels of PLA<sub>2</sub>-IIA in cell lysates and IL-8 in supernatants induced by different oral bacterial species at MOI= 1:50 for 48 hours in OKF6 cells were determined by ELISA as described in methods. Normalized amounts of PLA<sub>2</sub>-IIA in cell lysates per 100μg of total protein are shown. The mean ± SD of six replicates from each treatment group from a representative experiment of at least 2 independent experiments are shown. *ND*=Non-detectable. \*p < 0.01 when bacteria treated groups were compared with unstimulated (Mock) cells at 24 or 48 hours. *Aa*: *Aggregatibacter actinomycetemcomitans*.

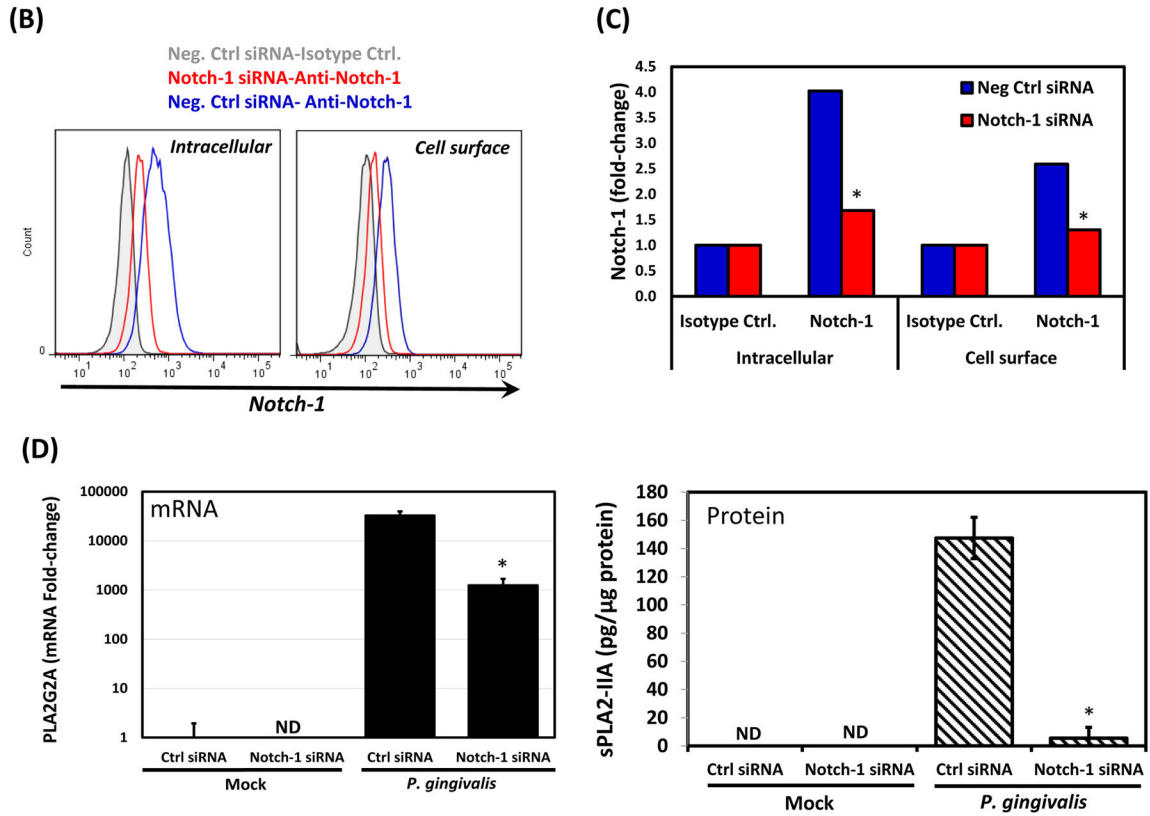




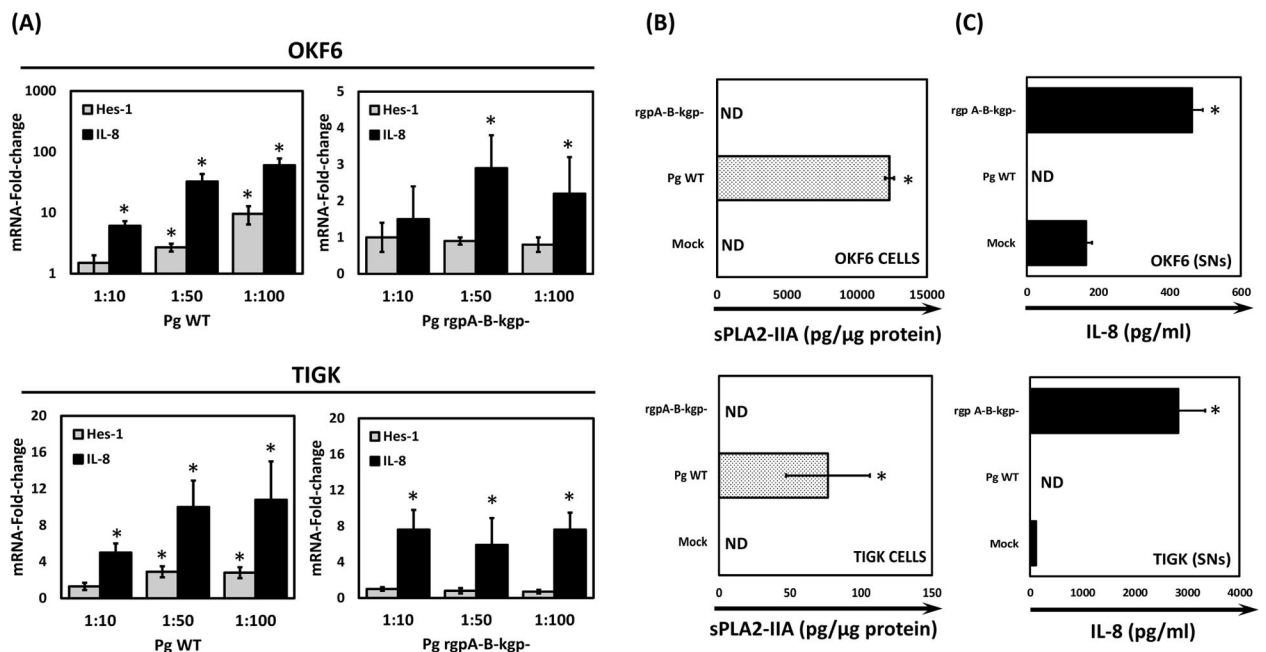
**Figure 3. Notch-1 receptor is activated by *P. gingivalis* in oral epithelial cells**

Notch-1 activation was evaluated by quantifying mRNA levels of Hes-1 by qRT-PCR in OKF6 cells exposed to (A) *P. gingivalis* or (B) *F. nucleatum* (*Fn*) and *S. gordonii* (*Sg*) for 48h. IL-8 expression was used as a control. (C) Nuclear levels of Notch-1 intracellular domain (NICD) in OKF6 cells exposed or not to bacteria, were evaluated and quantified by immunofluorescence as described in methods. Micrographs are showing mock-treated (top) and bacteria-treated OKF6 cells (Bottom) for bright field, DAPI, NICD and DAPI/NICD merge. Higher magnification (20X) of DAPI-NICD merged images is also shown. Scale = 50µm. (D) Percentage of cells positive for nuclear NICD from panel (C) was determined as described in methods. The mean ± SD of triplicates from each treatment group from a representative experiment of at least 2 independent experiments are shown. \*p 0.01 when bacteria-challenged cells were compared with un-stimulated (Mock) cells.

**(A)**

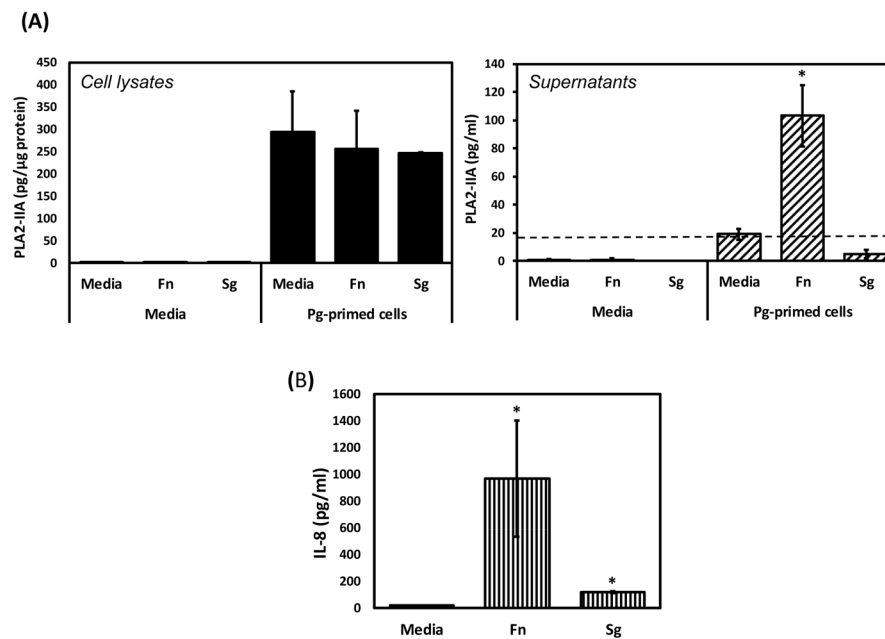


**Figure 4. Notch-1 activation in oral epithelial cells is involved in *P. gingivalis*-induced PLA<sub>2</sub>-IIA**  
**A)** Effect of specific Notch-1/gamma-secretase inhibitors DAPT and JLK6 on regulating the expression of PLA<sub>2</sub>-IIA induced by *P. gingivalis* in oral epithelial cells (OKF6). *Pg*-challenged cells incubated with the solvent for Notch-1 inhibitors (DMSO) were used as control. Normalized amounts of PLA<sub>2</sub>-IIA in cell lysates per 100µg of total protein are shown. **(B)** Representative histograms depicting cell surface and intracellular Notch-1 expression levels determined by flow cytometry in OKF6 cells transfected with 20nM of an siRNA specific for Notch-1 (red line) or its correspondent negative siRNA control (blue line). The gray histogram represents OKF6 cells transfected with a negative siRNA and incubated with an IgG1 isotype control **(C)** Fold-change of cell surface and intracellular Notch-1 expression levels (MFI) determined by flow cytometry and normalized to the MFI from cells incubated with an isotype IgG1 control are shown. **(D)** Levels of PLA<sub>2</sub>-IIA (mRNA and protein) induced by *P. gingivalis* [1:100] for 48h in OKF6 cells transfected with siRNA-Notch-1 or the siRNA-negative control. The mean ± SD of triplicates from each treatment group from a representative experiment reproduced at least twice are shown. ND= Non-detectable. \*p 0.01 DAPT/JLK6 treated cells vs. DMSO-treated cells, and when *P. gingivalis* challenge was done in cells transfected with Notch-1 siRNA vs. cells transfected with a negative siRNA control. ND= Non-detectable.



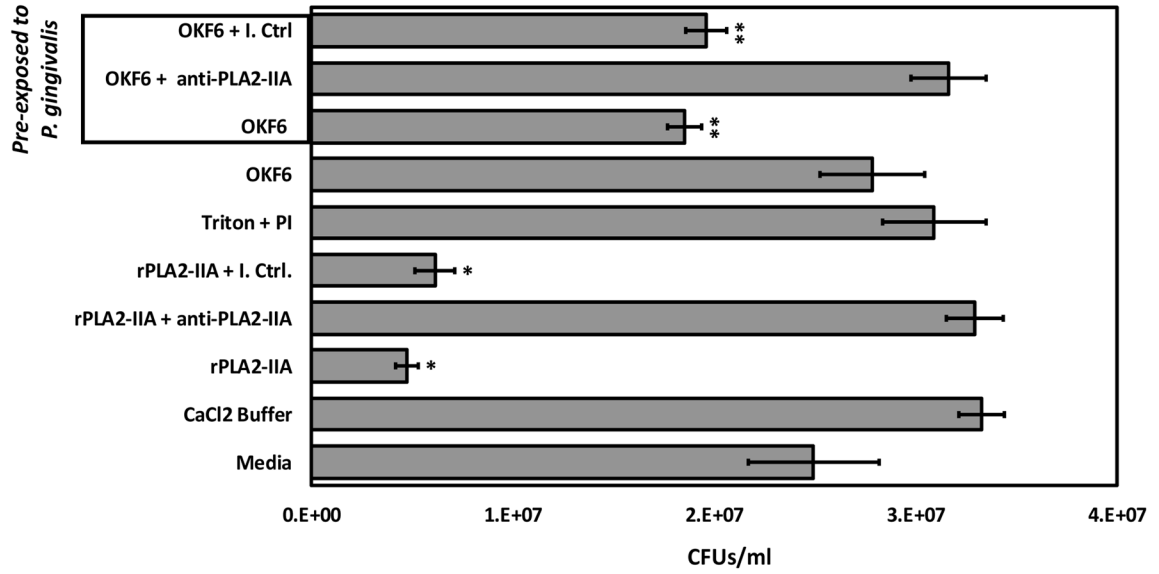
**Figure 5. Gingipains produced by *P. gingivalis* are involved in Notch-1 activation and PLA<sub>2</sub>-IIA production in oral epithelial cells**

(A) Notch-1 activation was determined in OKF6 and TIGK cells by measuring Hes-1 mRNA levels induced by *P. gingivalis* wild type (WT) or the *P. gingivalis* mutant strain for all gingipains (rgpA-B-kgp-) after 48h of stimulation by RT-qPCR. Fold changes were calculated with respect to gene expression levels in cells that were not challenged with bacteria (B) PLA<sub>2</sub>-IIA protein levels produced by OKF6 and TIGK cells in response to *P. gingivalis*-WT and *P. gingivalis*-mutant for gingipains [MOI=1:50] for 48h were determined by ELISA in cell lysates. Normalized amounts of PLA<sub>2</sub>-IIA in cell lysates per 100μg of total protein are shown. (C) Determination of IL-8 levels by ELISA in cell supernatants (SNs) from either OKF6 or TIGK cells was performed as a control for the biological activity of the *P. gingivalis* mutant strain. The mean ± SD of triplicates from each treatment group from a representative experiment are shown. \*p 0.05 when bacteria treated groups were compared with unstimulated (Mock) cells. ND=Non-detectable.



**Figure 6. Release of *P. gingivalis*-induced PLA<sub>2</sub>-IIA into the supernatants is enhanced by *F. nucleatum***

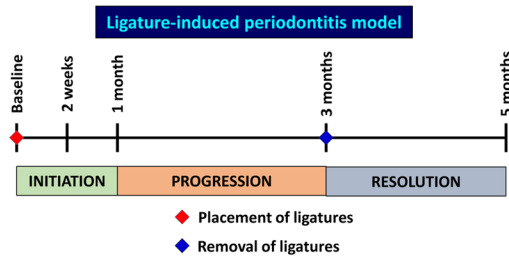
**(A)** PLA<sub>2</sub>-IIA levels were determined by ELISA in cell lysates normalized to total protein (100 µg) and in supernatants from OKF6 cells exposed to other oral bacteria (*Fn-F. nucleatum*, *Sg-S. gordonii*) at MOI=1:50 for 24h, after priming cells with *P. gingivalis* [1:50 for 24h]. **(B)** Determination of IL-8 levels by ELISA in supernatants was performed as control for biological activity of *Fn* and *Sg*. The mean ± SD from two independent experiments by duplicate/triplicate are shown. Interrupted line denotes minimal level of PLA<sub>2</sub>-IIA detection by ELISA (17 pg/ml). \*p 0.01 when bacteria treated groups were compared with unstimulated (Media) cells.



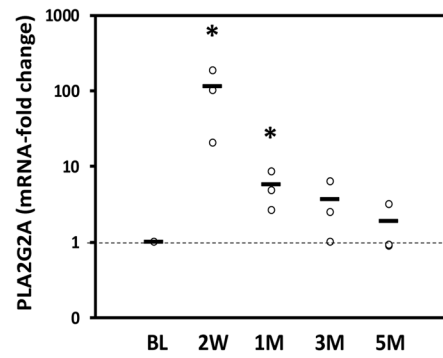
**Figure 7. *P. gingivalis* modulates oral epithelial antimicrobial cell responses through PLA<sub>2</sub>-IIA** OKF6 cells ( $1 \times 10^6$ ) were exposed or not to *P. gingivalis* [1:50] for 48h and cell lysates obtained with 0.1% Triton in water, in presence of protease inhibitors (PI). Further 40  $\mu$ l of either OKF6 cell lysates [1  $\mu$ g/ $\mu$ l total protein] or 10  $\mu$ g/ml of rPLA<sub>2</sub>-IIA resuspended in CaCl<sub>2</sub> buffer were pre-incubated with either 30 $\mu$ g/ml of anti-PLA<sub>2</sub>-IIA antibody or its corresponding isotype control (I. Ctrl.) for 30 minutes on ice before testing their antimicrobial activity against *L. monocytogenes* (Lm). Lm ( $10^6$  in 90  $\mu$ l broth) was incubated with 10  $\mu$ l of OKF6 cell lysates or rPLA<sub>2</sub>-IIA after different treatments for 2h. Lm CaCl<sub>2</sub> buffer (vehicle for rPLA<sub>2</sub>-IIA) incubated only with media, and Triton + PI (OKF6 cell lysis buffer) were used as controls. Further 25 $\mu$ l of Lm (1:1000 dilution) were seeded on blood agar plates and colony forming units (CFUs) quantified after 24h. The mean  $\pm$  SD of triplicates from each treatment group from a representative experiment of 2 independent experiments are shown. \*p 0.01, \*\* p 0.05 denotes significant reduction in CFUs/ml when different treatments were compared with bacterial growth only with media.



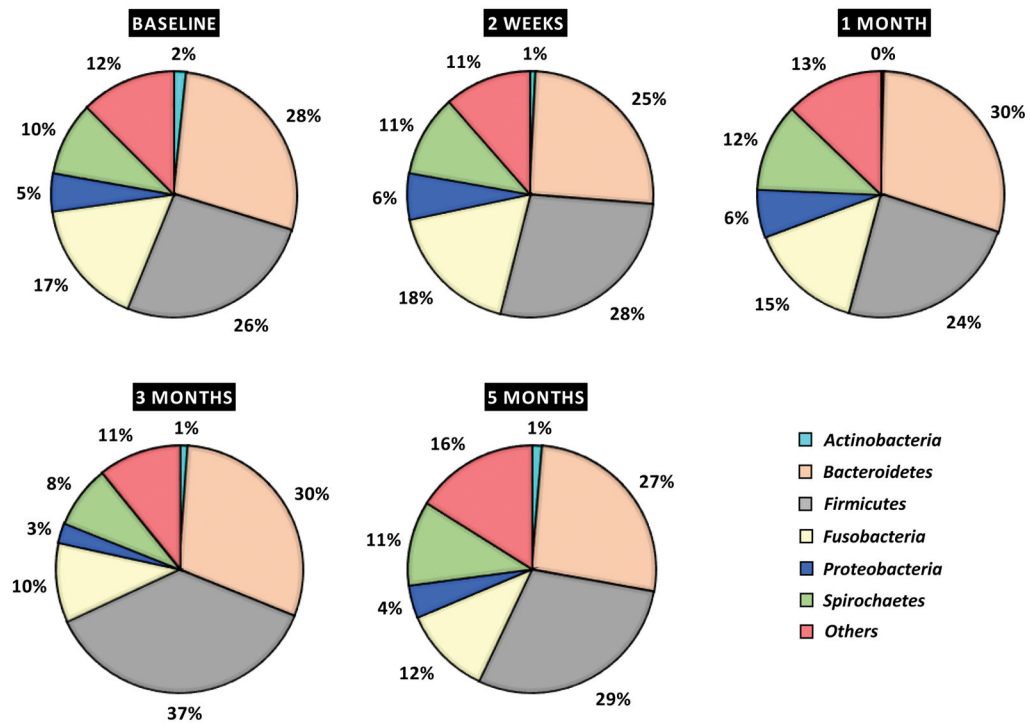
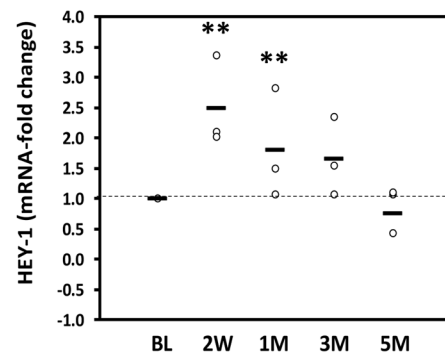
(A)



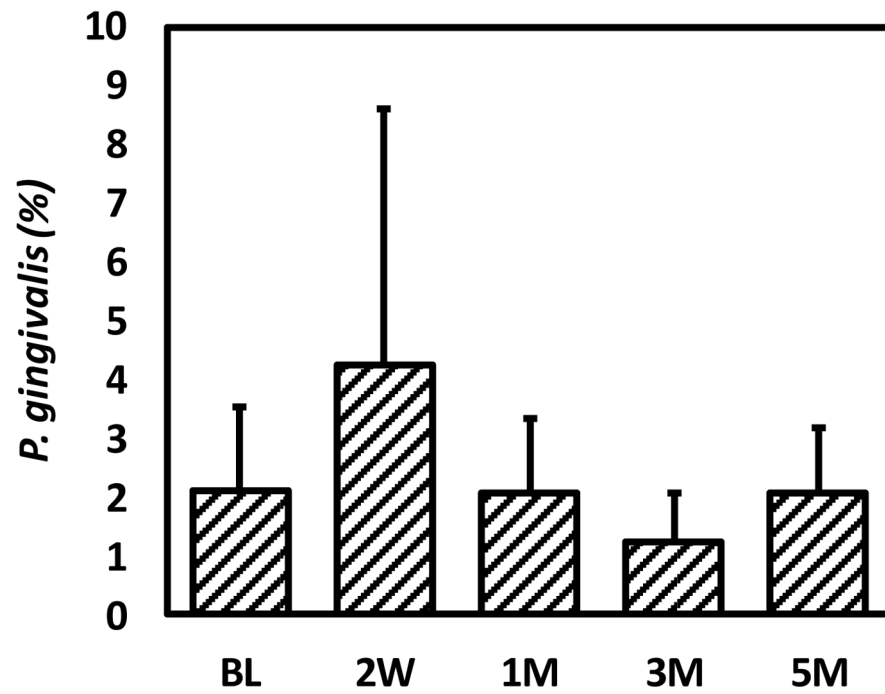
(B)



(C)



(E)



(F)

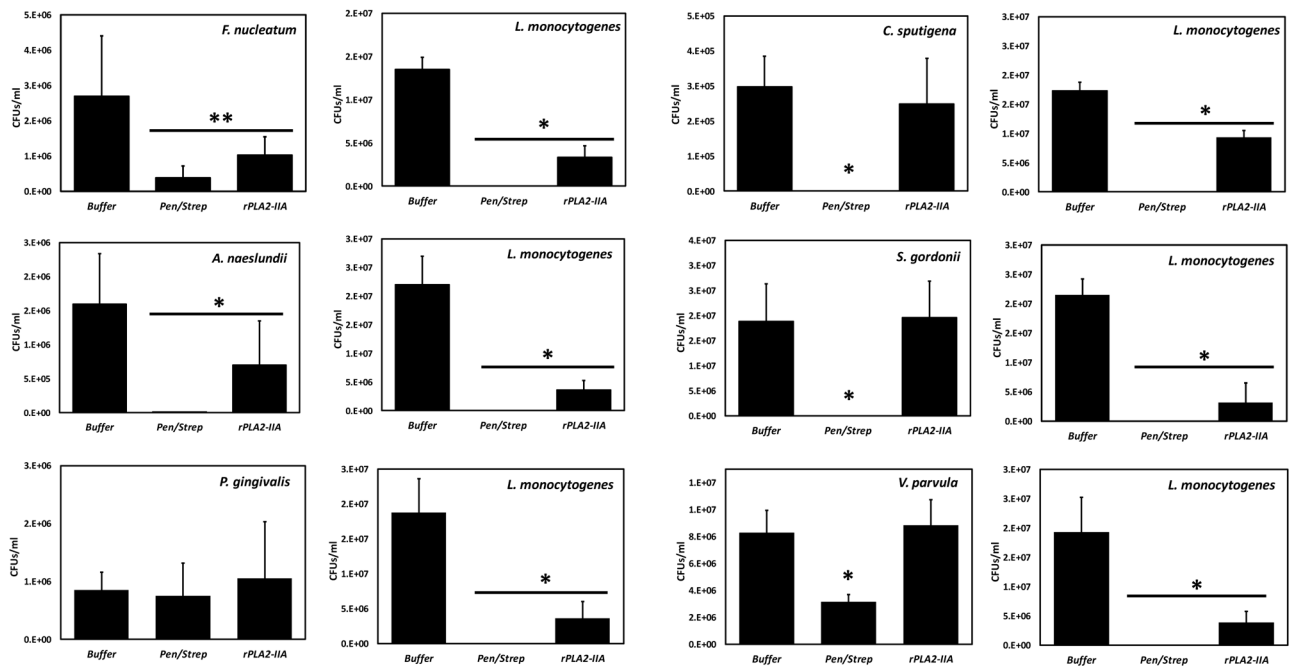


Figure 8. PLA2G2A expression, Notch-1 activation and oral microbiome changes during initiation, progression and resolution of periodontal disease in non-human primates (NHPs)

(A) The ligature-induced periodontitis model was used in nonhuman primates *M. mulatta* (n=9) as described in methods to determine variations in gingival gene expression by qRT-PCR during initiation (2 weeks and 1 month), progression (3 months) and resolution (2 months after removing ligatures) of periodontal disease. Fold changes in gene expression with respect to baseline levels are shown for (B) PLA2G2A and (C) HEY-1 (a marker for Notch-1 activation). Gingival biopsies (n=9) were pooled in three groups of 3 samples/each for qRT-PCR analysis. The mean of gene expression is depicted by horizontal bars in each time point. (D) Variation in abundance of the main oral bacterial Phyla and (E) *P. gingivalis*, was determined by 16S rRNA sequencing in oral subgingival plaque samples from NHPs. DNA from plaque samples was isolated (Magpure LC DNA isolation kit III, Roche), barcoded and amplified using 16S universal primers for V4 region (254bp amplicon). Amplicons for each sample were purified and pooled in equimolar concentrations and sequenced using Miseq (Illumina). Sequences were clustered into phylotypes based on their sequence similarity and these binned phylotypes were assigned to their respective taxonomic classification using the Human Oral Microbiome Database (HOMD V13). (F) Antimicrobial effect of recombinant human PLA<sub>2</sub>-IIA (1µg/ml for 2h) in selected oral bacterial species (10<sup>6</sup>) was evaluated by determination of colony forming units (CFUs). Bacteria exposed to 0.1 mg/ml penicillin/streptomycin (Pen/Strep) was used as a positive control and *L. monocytogenes* (susceptible target for PLA<sub>2</sub>-IIA) was used as control for the anti-microbial activity of PLA<sub>2</sub>-IIA in each experiment. The mean ± SD of triplicates from each treatment group from 2–3 independent experiments are shown \*p < 0.01 \*\*p < 0.05 when gene expression was compared with baseline levels, and when number of CFUs/ml of Pen/Strep or rPLA<sub>2</sub>-IIA-treated groups compared vs. Buffer.

**TABLE 1**

Sequence of primers used for human and non-human primate genes.

<i>Human Genes</i>	SEQUENCE	Tm	Amplicon
PLA2G2A	Forward 5'-AGT CAA CTG TGT GAG TGT GAT AAG -3' Reverse 5'-TCC ACA GGC GAG GAG TAG -3'	62 62	108
HES-1	Forward 5'-GCC AGT TTG CTT TCC TCA TTC -3' Reverse 5'-AAG GTG ACA CTG CGT TGG -3'	62 62	111
IL-8	Forward 5' - CTG CGC CAA CAC AGA AAT TAT -3' Reverse 5' - AAA CTT CTC CAC AAC CCT CTG	62 62	97
GAPDH	Forward 5'-CTC TGA CTT CAA CAG CGA CA -3' Reverse 5'-GTA GCC AAA TTC GTT GTC ATA CC -3'	62 62	112
<i>Non-human Primate Genes</i>	SEQUENCE	Tm	Amplicon
PLA2G2A	Forward 5'-CGG CAC CAA ATT TCT GAG CTA-3' Reverse 5'-AGC AGC CTT ATC GCA TTC AC-3'	63 63	106
HEY-1	Forward 5'-GAC CGT GGA TCA CCT GAA A-3' Reverse 5'-CCC AAA CTC CGA TAG TCC ATA G-3'	62 62	96
GAPDH	Forward 5'-CAC CCA CTC TTC CAC CTT C-3' Reverse 5'-CCT GTT GCT GTA GCC AAA TTC-3'	62 62	103