RESEARCH REPORTS
Biological

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J Dent Res 91(9):877-882, 2012

ASC-dependent RIP2 Kinase Regulates Reduced PGE_2 Production in Chronic Periodontitis

ABSTRACT

Levels of prostaglandin E_2 (PGE_2) and its processing enzyme, prostaglandin-endoperoxide-synthase-2/cyclooxygenase-2 (PTGS2/COX-2), are elevated in actively progressing periodontal lesions, but suppressed in chronic disease. *COX-2* expression is regulated through inflammatory signaling that converges on the mitogen-activated protein kinase (MAPK) pathway. Emerging evidence suggests a role for the inflammatory adaptor protein, ASC/Pycard, in MAPK activation. We postulated that ASC may represent a mediator of the MAPK-mediated regulatory network of PGE_2 production. Using RNAi-mediated gene silencing, we demonstrated that ASC regulates *COX-2* expression and PGE_2 production in THP1 monocytic cells following infection with *Porphyromonas gingivalis (Pg)*. Production of PGE_2 did not require the inflammasome adaptor function of ASC, but was dependent on MAPK activation. Furthermore, the MAP kinase kinase kinase CARD domain-containing protein RIPK2 was induced by *Pg* in an ASC-dependent manner. Reduced ASC and RIPK2 levels were revealed by orthogonal comparison of the expression of the RIPK family in ASC-deficient THP1 cells with that in chronic periodontitis patients. We show that pharmacological inhibition of RIPK2 represses PGE_2 secretion, and RNAi-mediated silencing of *RIPK2* leads to diminished MAPK activation and PGE_2 secretion. These findings identify a novel ASC-RIPK2 axis in the generation of PGE_2 that is repressed in patients diagnosed with chronic adult periodontitis.

KEY WORDS: periodontal disease, COX-2, PTGS2, *Porphyromonas gingivalis*, ASC, RIPK2.

DOI: 10.1177/0022034512454541

Received January 9, 2012; Last revision June 18, 2012; Accepted June 19, 2012

A supplemental appendix to this article is published electronically only at http://jdr.sagepub.com/supplemental.

INTRODUCTION

From 5 to 20% of the population is affected by severe periodontitis (Petersen *et al*., 2005), a condition that is the leading cause of tooth loss in adults and increases risks for several major systemic diseases, including coronary heart disease, atherosclerosis, rheumatoid arthritis, cancer, and preterm birth (Lamster *et al*., 2008; Hayashi *et al*., 2010). In this study, we abided by the 1999 World Workshop for the Classification of Periodontal Diseases and Conditions to define the chronic periodontitis condition (Caton and Armitage, 1999). The establishment of chronic periodontitis entails multiple cycles of progression and remission mediated by the modulation of pro-inflammatory signaling activities (Demmer and Papapanou, 2010). Identification of underlying inflammatory mechanisms of periodontal disease holds promise for the development of novel targets of periodontal treatment. One inflammatory mediator that has spanned the interface between bacterial infection and periodontal tissue damage is prostaglandin E_2 (PGE_2; Offenbacher *et al*., 1993; Noguchi and Ishikawa, 2007). Although the correlation between PGE_2 levels in gingival crevicular fluid (GCF) and clinical parameters of periodontitis such as periodontal attachment loss and signs of bleeding on probing has been documented (Noguchi and Ishikawa, 2007; Zhong *et al*., 2007; Zhang *et al*., 2011), it is critical to interpret the value of PGE_2 levels in periodontitis progression in a stage-sensitive fashion. For example, acute challenge of rat periodontal tissue with lipopolysaccharide (LPS) induces the expression of prostaglandin-endoperoxide synthase-2/cyclooxygenase-2 (PTGS2, or COX-2), which is responsible for the biosynthesis of PGE_2 (Miyauchi *et al*., 2004). However, cumulative damage of periodontal tissue is negatively associated with PGE_2 levels in GCF (Zhong *et al*., 2007). One possible mechanism for the reduced PGE_2 expression during chronic disease involves epigenetic modification leading to decreased *COX-2* mRNA synthesis. Recently, Zhang *et al.* reported hypermethylation at the *COX-2* promoter, which was associated with squelched PGE_2 mRNA expression and lowered PGE_2 production in chronic inflamed tissues (Zhang *et al.*, 2011). The expression of *COX-2* is known to be regulated by extracellular stimuli through a variety of inflammatory signaling pathways that converge on the mitogen-activated protein kinase (MAPK) pathway (Tsatsanis *et al*., 2006). However, the molecular machinery that is involved in MAPK-mediated regulation of PGE_2 generation remains poorly defined.

Emerging evidence suggests that ASC (apoptosis-associated speck-like protein containing a CARD), an important mediator of host pro-inflammatory

responses, is pivotal in mediating NF-κB and MAPK signaling, and cytokine and chemokine expression in THP1 monocytic cells following infection with *Porphyromonas gingivalis (Pg)* (Taxman *et al.*, 2006b, 2011). *Pg* is a bacterial pathogen that has been unequivocally shown to mediate tissue destruction and trigger host inflammatory responses in chronic periodontitis (Hayashi *et al.*, 2010). We postulated that PGE_2 production may be regulated through ASC-dependent activation of MAPK. A MAPK pathway regulator that associates with ASC *via* its CARD (caspase activation and recruit domain), RIPK2, belongs to the RIP family of kinases (Receptor-Interacting serine/threonine-Protein Kinase); (Navas *et al.*, 1999; Sarkar *et al*., 2006). The RIPK family acts as integrators of cellular stress responses for a diverse array of stimuli, including pathogenic infection, inflammation, and DNA damage (Meylan and Tschopp, 2005). We hypothesized that RIPK2 may mediate ASC-dependent MAPK and PGE_2 activation.

MATERIALS & METHODS

Bacterial Culture

We used the A7436 strain, because of its established immunostimulatory effect on monocytic cells (Taxman *et al*., 2006b, 2011; Huang *et al*., 2009) and virulence properties (Genco *et al*., 1991). *Pg* strain A7436 was grown anaerobically in Wilkins-Chalgren broth to late-exponential phase (OD 0.8 to 1.2 at 600 nm). Frozen aliquots were stored in media containing 10% glycerol at -80°C and thawed immediately prior to use. The enumeration and viability of bacteria were verified by serial plating.

Cell Culture and Generation of Knockdown Cell Lines

THP1 monocytic cells (ATCC, Manassas, VA, USA) were cultured in RPMI, 10% FCS. THP1 cell lines bearing a short hairpin RNA (shRNA) for RNA interference (RNAi)-mediated gene silencing of ASC (shASC#1 and shASC#2) or a control, non-silencing shRNA (shCTRL) were generated with retroviral vectors and have been described previously (Taxman *et al.*, 2006a,b, 2011). Lentiviral shRNA vectors for gene silencing of RIPK2 and GFP were from the TRC Collection (Open Biosystems, Lafayette, CO, USA). Lentivirus was packaged in 293T cells with vectors pMD2.G and psPAX2 (Addgene plasmids 12259 and 12260, Cambridge, MA, USA). Cells were selected in 1 μg/mL puromycin for 2 wks, and JRed marker expression was confirmed by FACS. Bacterial infections (10^6 cells/mL in 10% FCS) were at 10 MOI unless otherwise indicated. Where indicated, cells were pretreated for 1 hr with DMSO solvent control (Sigma-Aldrich, St. Louis, MO, USA), 10 μg/mL Anakinra/Kineret® (Amgen, Thousand Oaks, CA, USA), 10 μM YVAD-cmk (Clontech, Mountain View, CA, USA), 10 μM U0126 (Promega Corporation, Madison, WI, USA), 1-10 μM PP2, SB202190, or SB203580 (LC Laboratories, Woburn, MA, USA).

Patient Information

Demographic information for the 17 participants included in the microarray studies and 30 participants included in the real-time PCR analyses are detailed in the Results section. The study was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill. Subjects were excluded if they: (1) used either antibiotics or non-steroidal anti-inflammatory medications within 1 mo of the periodontal treatment; or (2) had a medical history significant for systemic diseases and were actively participating in the treatment thereof. Periodontal evaluation included probing depths, clinical attachment level, and bleeding on probing at 6 sites *per* tooth. Gingival tissue was sampled at the interproximal region. Specimens were treated with RNA*later* (Qiagen, Valencia, CA, USA) overnight at 4ºC and stored at -80ºC.

Microarray Analysis

Microarray analysis of triplicate samples of *Pg*-infected shCTRL and shASC#1 THP1 cells was performed at the Duke Microarray facility with 34,000 spot custom chips based on the version 3 Human oligo set (Operon Biotechnologies, Inc., Huntsville, AL, USA), and has been described previously (Taxman *et al*., 2011). Microarray analyses of gingival biopsies of the 17 individual study participants were performed individually with the GeneChip® Human Gene 1.0 ST Array covering 28,869 genes (Affymetrix, Santa Clara, CA, USA). Scanning was performed with the GeneChip Scanner 3000 7G platform (Affymetrix, Santa Clara, CA, USA). Gene expression was analyzed in GeneSpring 11 for interpretation of fold changes and statistical validation.

Real-time PCR

ASC mRNA levels were quantified with Taqman Assays on Demand® (Applied Biosystems, Carlsbad, CA, USA). *COX-2* and *RIP2* mRNA were measured by SYBR-based quantitative real-time PCR as described previously (Taxman *et al*., 2006a,b). The primer sequences were as follows: *COX-2*-GCCTGCT TGTCTGGAACAAC; TGGTGCCTGGTCTGATGATG; *RIP2*-CTGGCACTGTGTCGTCCGCC;TCTTTCACTGTCGAGCA GCGGAG, *18s*-CGGCTACCACATCCAAGG; and GCTG CTGGCACCAGACTT. All values were standardized to *18s* rRNA expression.

Western Blot Analysis

Western blot analysis was performed as described previously (Taxman *et al.*, 2006b), with antibodies against RIP2 (Cell Signaling Technology #4982, Danvers, MA, USA), pERK (Santa Cruz Biotechnology, Inc. #sc-7383, Santa Cruz, CA, USA), pJNK (Cell Signaling Technology #4668, Danvers, MA, USA), and GAPDH (Millipore # MAB374, Billerica, MA, USA). Results are representative of at least 3 experiments.

PGE_2 ELISA Analysis

Supernatants were collected 18 to 24 hrs following infection with *Pg*. Quantification of PGE_2 levels was performed by ELISA (R&D Systems #KGE004B, Minneapolis, MN, USA).

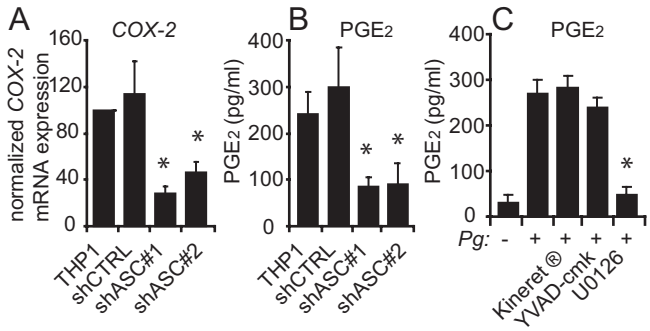

Figure 1. Activation of ASC-dependent PGE_2 in THP1 monocytic cells following infection with *Pg*. **(A)** Expression of *COX-2* mRNA in non-transduced THP1 cells and THP1 cells expressing a control shRNA (shCTRL) or 1 of 2 shRNA directed against *ASC* (shASC#1 and shASC#2). Expression levels were determined by real-time PCR of RNA isolated 2 hrs following treatment of cells with 10 MOI *Pg*. Results are normalized to the expression of 18s rRNA and expressed as averages with standard deviation for duplicates. Results are representative of 3 independent experiments. *$p < 0.05$. **(B)** Production of PGE_2 following 18-hour infection of control and ASC knockdown THP1 cell lines with 10 MOI *Pg*. PGE_2 levels were measured by ELISA. Results are expressed as averages and standard deviation of 3 specimens, and represent 3 independent experiments. *$p < 0.05$. **(C)** ELISA analysis for PGE_2 in supernatants from THP1 cell cultures prior to or following 18-hour incubation with *Pg*. Where indicated, cells were pre-treated with the IL-1β receptor antagonist Kineret®, the caspase-1 inhibitor YVAD-cmk, or the ERK inhibitor U0126. * indicates statistical difference from *Pg*-infected THP1 control sample as determined by *t* test ($p < 0.05$). Results represent 3 independent experiments.

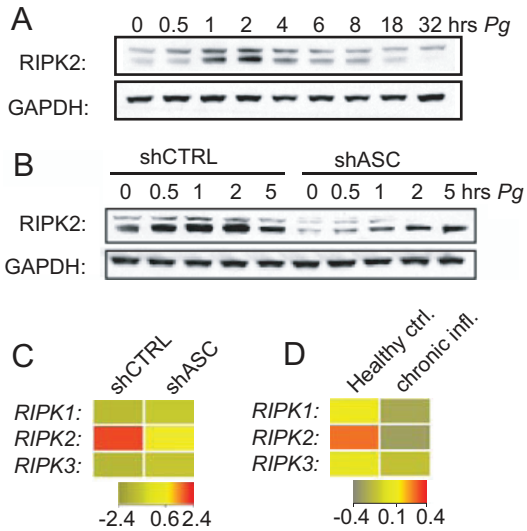

Figure 2. Orthogonal comparison of RIP kinase expression levels in ASC-deficient THP1 cells and in chronic periodontitis patients. **(A)** Western blot of RIPK2 protein levels in THP1 cells following an extended time-course of infection with *Pg*. **(B)** Western blot of RIPK2 protein levels in shCTRL and shASC#1 THP1 cells following a time-course of infection with *Pg*. GAPDH is shown as an internal control. Results are representative of 3 independent experiments. **(C)** Levels of *RIPK1*, *RIPK2*, and *RIPK3* mRNA in control *vs.* ASC knockdown THP1 cells 2 hrs following infection with *Pg*. *RIPK2* was reduced 2.4-fold in ASC knockdown cells (p value = 1.092×10^{-4}; Q value = 3.276×10^{-4}), as determined by microarray analysis. **(D)** Levels of *RIP1*, *RIP2*, and *RIP3* mRNA in chronic periodontitis patients *vs.* healthy control individuals, as determined by microarray analysis. *RIP2* was 1.5-fold lower in the patient sample population (p value = 0.011; Q value = 0.032).

RESULTS

ASC Regulates *COX-2* Expression and PGE_2 Production in THP1 Monocytic Cells following Infection with *Pg*

Monocytes are one of the primary contributors of cytokines and chemokines in periodontal degeneration (Page, 1992). We have established the THP1 monocytic cell line as a useful model to study the interaction between the host and *Pg* infection (Taxman *et al*., 2006b, 2011; Huang *et al*., 2009). To directly assess the function of ASC in regulating PGE_2 production, we constructed THP1 cell lines with reduced *ASC* expression using 2 different short hairpin RNAs: shRNA#1 (> 90% knockdown) and shASC#2 (~70% knockdown; Taxman *et al*., 2006a). Levels of *COX-2* mRNA and secreted PGE_2 were significantly reduced following *Pg* infection in both of the ASC knockdown cell lines as compared with either non-transduced cells (THP1) or cells bearing an empty vector (shCTRL) (Figs. 1A, 1B).

ASC is known as an adaptor in the inflammasome complex, which is critical in the processing of IL-1β following pathogenic or environmental challenges (Broz and Monack, 2011). To test whether IL-1β signaling is required for the induction of PGE_2, we pre-treated THP1 cells with the IL-1β receptor antagonist Kineret® at a dose shown to obliterate signaling (Taxman *et al.*, 2006b). Induction of PGE_2 was unaffected by Kineret®, suggesting that PGE_2 induction is IL-1β-independent (Fig. 1C). PGE_2 induction was also unaffected by YVAD-cmk, a specific inhibitor of caspase-1, the activation of which is absolutely required for IL-1β maturation in THP1 cells following infection with *Pg* (Taxman *et al*., 2006b; Huang *et al*., 2009).

We and others recently demonstrated that ASC mediates the activation of MAPK independent of the inflammasome (Hasegawa *et al*., 2009; Taxman *et al*., 2011). Therefore, we postulated that the ASC-dependent activation of PGE_2 may be regulated by MAPK activity. To test this possibility, we treated THP1 cells with U0126, an inhibitor of the ERK pathway, prior to infection with *Pg* (Fig. 1C, last lane). U0126 nearly abolished PGE_2 protein production. Collectively, these results suggest that ASC is essential for *Pg*-induced PGE_2 production, and that its function in activating PGE_2 involves MAPK activation independent of its role in the inflammasome.

ASC Modulates the Expression of the MAP Kinase kinase kinase RIPK2

Homotypic CARD:CARD associations are essential for a host of inflammatory signaling cascades (Bouchier-Hayes and Martin, 2004). A search for CARD-containing proteins with signaling activity upstream of the MAPKs led us to consider a possible role for RIPK2 in PGE_2 regulation. RIPK2 directly associates with ASC upon activation with LPS (Sarkar *et al*., 2006). As one of the primary pathogens that triggers periodontal inflammation and tissue degeneration, *Pg* potently induced

A Clinical Parameters of Study Participants (n=30)

Demographic/ Clinical Parameters	Periodontal Health Group (n=13)	Chronic Periodontitis Group (n=17)	p value
Age (yrs) (mean ±SD)	39.38 ±14.05	46.53 ±12.01	0.145
Gender			
Males/Females	3/10	8/9	0.26
Probing Depth (mm) (mean ±SD)	2.23 ±0.93	5.71 ±1.21	<0.0001
Clinical Attachment Loss (mm) (mean ±SD)	0.85 ±0.90	4.53 ±1.74	<0.0001
Smoking status (Never/Ex/Current smoker)	9/4/0	8/3/6	0.056
Alveolar bone loss	No	Yes	

B

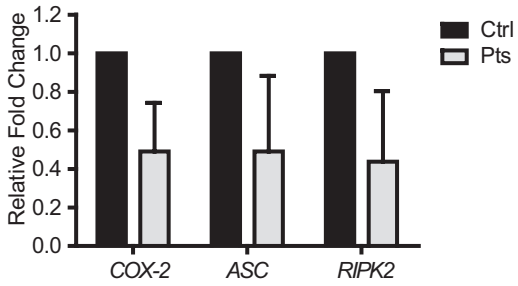

Figure 3. *ASC* and *RIPK2* are repressed in the gingival tissue of chronic adult periodontitis patients. **(A)** Demographic profile of age-matched 13 healthy volunteers and 17 patients. All patients demonstrated elevated probing depth, clinical attachment loss, and moderate to severe bone loss. **(B)** Relative mRNA fold change of *COX-2*, *ASC*, and *RIPK2* in gingival tissues from 13 healthy volunteers and 17 chronic adult periodontitis patients.

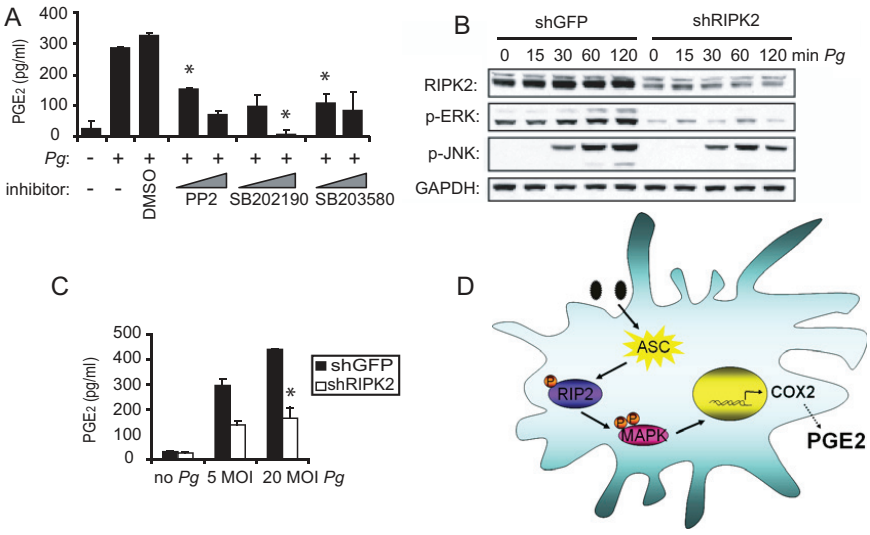

Figure 4. RIPK2 is required for the activation of PGE_2 production following infection with *Pg*. **(A)** Pharmacological inhibition of RIPK2 leads to reduced PGE_2 production. ELISA analysis of supernatants was performed prior to or following 18-hour infection with *Pg*. Where indicated, cells were pre-treated with the carrier DMSO or the RIPK2 inhibitors PP2, SB202190, or SB203580 at a concentration of 1 and 10 μM. Results are expressed as average with standard deviation of duplicates, and represent 2 independent experiments.* $p < 0.05$. **(B)** Western analysis of RIPK2, p-ERK, and p-JNK protein levels in control GFP knockdown cells (shGFP) and RIPK2 knockdown cells (shRIP2) following a time-course of infection with *Pg*. Results are representative of 3 independent experiments. **(C)** ELISA of PGE_2 levels in supernatants of shGFP and shRIP2 cells following 18-hour infection with *Pg* at 5 or 20 MOI. Results are expressed as average with standard deviation of duplicates, and represent 3 independent experiments. * $p < 0.05$. **(D)** Model for ASC and RIPK2-dependent activation of PGE_2 production following *Pg* infection.

RIPK2 protein expression (Fig 2A). Furthermore, repression of ASC strongly mitigated this induction (Fig. 2B). To assess the specificity of this repression for RIPK2, we performed microarray analysis using RNA from *Pg*-infected shCTRL and shASC THP1 cells. *RIPK2* expression was reduced 2.4-fold in *Pg*-infected ASC knockdown cells (p value = 1.092×10^{-4}; corrected p value = 3.276×10^{-4}) (Fig. 2C), whereas 2 other members of the RIPK family of MAPK-activating proteins (Meylan and Tschopp, 2005), *RIPK1* and *RIPK3*, were minimally changed.

ASC and *RIPK2* Expression Is Reduced in Gingival Tissues from Individuals with Chronic Periodontitis

We performed microarray analysis to assess whether levels of *RIPK2* may be regulated *in vivo*. RNA was collected from gingival biopsies from seven patients with chronic adult periodontitis and ten healthy control individuals (Appendix Fig.). Similar to our findings from *Pg*-infected THP1 ASC knockdown cells (Fig. 2C), *RIPK2* was reduced 1.5-fold in chronic periodontitis gingival tissue (p value = 0.0108; corrected p value = 0.0324), while *RIPK1* and *RIPK3* showed minimal change (Fig. 2D).

To verify these findings, we recruited 17 chronic periodontitis patients and 13 healthy volunteers. Patients and control individuals were age-matched ($p > 0.1$). Those with chronic periodontitis demonstrated marked increases in probing depth, clinical attachment loss, and bone loss as compared with healthy control individuals (Fig. 3A). Consistent with our previous study (Zhang *et al.*, 2011), *COX-2* expression was repressed in chronically inflamed gingival tissue (Fig. 3B). Furthermore, *ASC* and *RIPK2* were also reduced. These findings are consistent with a potential role for ASC in modulating *RIPK2* expression both *in vitro* and *in vivo*.

RIPK2 Is Required for MAPK Activation and PGE_2 Induction by *Pg*

We utilized two approaches to determine directly whether RIPK2 regulates PGE_2 induction. First, THP1 cells were treated with a panel of pharmacological agents

known to inhibit RIPK2 kinase function (Bain *et al.*, 2007) prior to infection with *Pg*. A dose-dependent reduction in PGE_2 was observed for each of these inhibitors (Fig. 4A). Second, *RIPK2* expression was inhibited by RNAi-mediated gene silencing. The activation of the MAPKs, ERK, and JNK in RIPK2-deficient cells was markedly reduced, verifying the role of RIPK2 as an upstream activator of these signaling molecules (Fig 4B). *RIPK2* gene silencing also led to the reduced production of PGE_2 following infection with *Pg* at 2 different MOIs (Fig 4C). The findings collectively demonstrate that RIPK2 mediates PGE_2 production in response to a pathogen mediating periodontal destruction.

DISCUSSION

The progression of chronic adult periodontitis is featured by cycles of exacerbation and remission, eventually leading to irreversible periodontal structure destruction. A key to the development of novel treatment is the identification of critical inflammatory mediators that are regulated during these cycles and targeting of these mediators with small bioactive pharmacologic molecules. Initial studies suggested that the up-regulation of PGE_2 is associated with bone loss in humans and in animal models and that COX-2 inhibitors may reduce bone loss (Offenbacher *et al.*, 1993; Noguchi and Ishikawa, 2007). However, the ligature-induced periodontitis model features rapidly progressing periodontal inflammation, which more aptly reflects disease exacerbation, but not remission stages associated with human chronic adult periodontitis. In addition, for affected individuals, shallow sites are more likely to be responsible for the progression of periodontitis, whereas deep sites display lower levels of PGE_2 (Beck *et al.*, 1997). Indeed, previous studies have suggested that *COX-2* levels are lower in chronically inflamed gingival tissue (Zhong *et al*., 2007; Zhang *et al*., 2011).

The regulation of COX-2 is dependent upon both genetic and epigenetic mechanisms. In addition to a hypermethylation pattern at the *COX-2* promoter region that dampens the production of *COX-2* mRNA (Zhang *et al*., 2011), the present study identified a novel mechanism controlling the production of PGE_2 that could have relevance in the pathogenesis of periodontitis (Fig. 4D). When a periodontal pathogen such as *Pg* engages the host monocytic cells, ASC translates the insult into MAPK activation in a RIPK2-dependent fashion. Despite the high structural similarity among RIPKs, our study suggested a specificity of RIPK2 in mediating the inflammatory responses of periodontal tissue. Recent evidence reveals a central role of RIPK2 in mediating innate immune activation through association with NOD2 (Tigno-Aranjuez *et al*., 2010). NOD2 is an NLR (nucleotide-binding, leucine-rich repeats) protein that is associated with Crohn's and other chronic auto-inflammatory diseases (Borzutzky *et al*., 2010). Notably, NOD2 has also been reported to have a critical role in the sensing of periodontal pathogens (Okugawa *et al*., 2009).

The pattern of ASC-mediated, RIPK2-dependent PGE2 production in THP-1 cells in response to *Pg* challenge in our study conceptually agrees with that observed in gingival tissues from patients with chronic adult periodontitis. The establishment of chronic periodontitis, however, is the cumulative outcome of the interaction between the host and multiple pathogens; thus, we chose *Pg* as a simplified model pathogen to highlight the significance of the ASC-RIPK2 axis. Consistent with our findings, a prior study showed reduced ASC levels upon *Pg* infection in Mono-Mac-6, a monocytic cell line (Bostanci *et al.*, 2009); however, in the same study, differences were not observed in *ASC* in healthy *vs*. diseased gingival tissues. Given that PGE_2 is differentially regulated during disease remission/exacerbation (Zhong *et al*., 2007; Zhang *et al*., 2011), differences in disease stage could explain this discrepancy between our studies. In addition, the expression profiles of apoptotic and inflammatory signaling genes for gingival tissues are known to be highly age-dependent (Gonzalez *et al*., 2011). Finally, differences in the timing of biopsies, *i.e*., before or after non-surgical periodontal treatment, could contribute to the observed discrepancy. It is widely known that microarray can squelch differences between samples; however, our results were additionally verified by real-time PCR. Future study is necessary to delineate the role of RIPK2 in the progression stage of periodontitis development and to evaluate whether RIPK2 inhibitors might prevent chronic disease progression by stabilizing PGE_2 levels during periods of exacerbation.

ACKNOWLEDGMENTS

This study was supported by NIH grant R01-DE016326 (to J.T.) and grant RR-00046, now UL1RR025747 (to S.O.). The author(s) declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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