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***In vivo* and *ex vivo* actions of a novel *P. gingivalis* inhibitor on multi-species biofilm, inflammatory response and periodontal bone loss**

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SUMMARY

Chronic periodontitis is one of the most common infectious inflammatory diseases worldwide. Current therapeutic options for the disease are only partially and temporarily successful due to periodontal re-emergence of pathogens such as *Porphyromonas gingivalis*, a keystone bacterium in the oral microbial communities, which elicits a dysbiosis between the microbiota and the host. Previously, we reported a peptide inhibitor of *P. gingivalis* (SAPP) that specifically targets *P. gingivalis* and reduces its virulence potential *in vitro*. Here, we show that SAPP can modulate the ability of *P. gingivalis* to suppress the host innate immune system. Using a cytokine array analysis, we found that the levels of several cytokines including IL-6, IL-8, and MCP-1 in the culture media of human oral keratinocytes (HOKs) were significantly diminished in the presence of *P. gingivalis*. Whereas the levels of these cytokines were restored, at least partially, in the culture media of HOKs by SAPP treatment. Furthermore, we also observed in an *ex-vivo* assay that SAPP efficiently inhibited biofilm primed formation by mixed species oral bacteria, and significantly dampened the abnormally innate immune responses induced by these bacteria. We also demonstrated, using a mouse model, that SAPP could prevent alveolar bone loss induced by *P. gingivalis*. Our results suggest that SAPP specifically targets *P. gingivalis* and its associated bacterial communities and could be envisioned as an emerging therapy for periodontitis.

Keywords

P. gingivalis inhibitor; Periodontitis

1. INTRODUCTION

Periodontitis is characterized by an irreversible loss of the periodontal attachment apparatus including the alveolar bone. Ultimately, severe periodontitis leads to increased mobility and

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loss of the tooth. The disease along with the associated oral pathogens, such as *Porphyromonas gingivalis*, have been epidemiologically and mechanistically associated with systemic conditions such as coronary artery disease, rheumatoid arthritis, diabetes (Hajishengallis, 2015; Kumar, 2013; Linden, Lyons, & Scannapieco, 2013), and most recently with Alzheimer's Disease (Dominy et al., 2019). It is recently reported that 743 million people are affected with severe periodontitis worldwide (Tonetti, Jepsen, Jin, & Otomo-Corgel, 2017). According to the Centers for Disease Control and Prevention, chronic periodontitis affects approximately 64.7 million Americans (<https://www.perio.org/newsroom/periodontal-disease-fact-sheet>). The prevalence and economic burden of periodontitis will likely continue to increase with the growing aging population (Kassebaum et al., 2014), and it was estimated that the annual cost of periodontal therapy exceeded \$14 billion in the United States (Brown, Johns, & Wall, 2002). Therefore, prevention and treatment of periodontitis are important for both oral health and overall health of adults.

Our previous study on interspecies communication have led to the discovery of an antagonistic relationship between *Streptococcus cristatus* and *P. gingivalis* (Xie et al., 2000). A *S. cristatus* surface protein, arginine deiminase (ArcA) was identified as the signaling molecule to which *P. gingivalis* responds to by repressing the expression of the *fimA* gene and consequently reducing production of the fimbrial adhesin, FimA (Wu & Xie, 2010; Xie, Lin, Wang, Wu, & Lamont, 2007). These studies led to the discovery of an 11-mer peptide derived from ArcA, which we have designated as streptococcal derived anti-*P. gingivalis* peptide (SAPP). SAPP can bind to the surface of *P. gingivalis* and repress the expression of virulence genes encoding the fimbrial adhesins, FimA and Mfa1, as well as genes encoding gingipain proteinases (Ho, Lamont, & Xie, 2017a). Further SAPP treatment significantly decreased the ability of *P. gingivalis* to form monospecies and heterotypic biofilms, and to invade oral epithelial cells, while SAPP did not affect the viability and growth rate of *P. gingivalis* *in vitro*. The gingipain enzymatic activity of *P. gingivalis* was also greatly reduced by SAPP treatment. (Ho, Lamont, & Xie, 2017b). Moreover, results from an *ex vivo* study revealed that the levels of *P. gingivalis* as well as other periodontitis-associated bacteria, such as *Tannerella forsythia* and *Treponema denticola* in the mixed species biofilms were decreased with a single SAPP treatment (Ho et al., 2018).

In this study, we tested the efficacy of SAPP on elimination of *P. gingivalis*-induced mixed species biofilm formation using *in vitro* and *ex vivo* dental plaque models as well as inflammatory response by measuring the cytokine secretion and alveolar bone loss in a mouse model of periodontitis. Here, we report that SAPP possesses physiological properties to interfere with the virulence potential of *P. gingivalis* in complex environments including human biofilm and experimental periodontitis.

2. MATERIALS AND METHODS

2.1. Conditions of bacterial growth and cell culture

P. gingivalis strains (wild type 33277, *fimA* mutant FAE, and gingipain mutant KDP128) were cultured from frozen stocks in either Trypticase soy broth (TSB) or on TSB blood agar plates supplemented with yeast extract (1 mg/ml), hemin (5 µg/ml), and menadione (1 µg/ml), and incubated at 37°C in an anaerobic chamber (85% N₂, 10% H₂, 5% CO₂).

Streptococcus gordonii DL1 was grown in Trypticase peptone broth (TPB) supplemented with 0.5% glucose at 37°C under aerobic conditions. Human oral keratinocytes (HOKs) were purchased from ScienCell Research Laboratories, Inc. and grown in a medium containing essential and non-essential amino acids, vitamins, organic and inorganic compounds, hormones, growth factors and trace minerals. (ScienCell).

2.2. Peptide synthesis and activity

SAPP (NIFKKNVGFKK) and its derivative P10 (NIAKKNVGFKK) were synthesized by Biomatik and purified using high performance liquid chromatography (HPLC) to achieve 95% purity. The purified peptide was resuspended in nuclease/proteinase-free PBS, aliquoted, and stored at -20°C.

2.3. Ex-vivo assays

To assess the impact of SAPP on biofilm formation of mixed species *ex vivo*, we used dental plaque samples collected from patients with chronic periodontitis. The protocol for biological sampling from human volunteers was reviewed and approved by the Meharry Medical College Institutional Review Board (IRB) (approval number 16-08-584). Patients with chronic periodontitis presenting radiographic periodontal bone loss of 4 mm at multiple teeth were recruited from the Periodontal Clinic at the Meharry Medical College (1005 D. B. Todd, Nashville, TN).

An IRB-approved informed consent was obtained from all patients before sampling. Dental plaque specimens were collected by trained clinicians at the Meharry Medical College dental clinic using paper points as previously described (Wang, Wu, Lamont, Lin, & Xie, 2009). A total of 13 participants, including 7 males and 6 females with a mean age of 50 years (age range of 26 – 62 years), were enrolled.

Dental plaque was collected using paper points inserted in periodontal pockets greater than 4 mm for 30 sec and then transferred to sterile vials containing 2 ml TSB and incubated in an anaerobic chamber for 4 h. To create biofilm formation of native mixed-species bacteria from each sample were then divided to four wells in a six-well plate with and without SAPP (48 µM), respectively, and further cultured with 2 ml of fresh TSB for 24 h. After 24 h culture, another dose of SAPP (48 µM) was added for a repeated treatment of SAPP, thereafter the bacteria were continually cultured for an additional 24 h. Sessile and planktonic bacteria were then collected from both SAPP-treated and untreated (control) wells as pairs. Bacterial DNA was extracted using a DNeasy UltraClean Microbial Kit (Qiagen). For stimulation of epithelial cells, sessile bacteria from the other pair of wells (SAPP-treated and untreated) were collected using a cell culture scraper and resuspended in 500 µl of PBS. Planktonic bacteria were collected by centrifugation and resuspended in PBS (OD₆₀₀ = 1.0). The suspended bacteria (100 µl) were subjected to HOK treatments.

2.4. Cytokine arrays

To determine the changes in inflammatory response to *P. gingivalis* strains treated with or without SAPP *in vitro*, HOKs (2×10⁵ cells/well) in a six-well plate were pre-treated with or without SAPP (3µM) or P10 (a scrambled peptide) and infected with *P. gingivalis* 33277 at

MOI of 10 for 24 h. Untreated and uninfected HOK cells were used as a control. The growth media of HOKs (100 µl) were collected. Quantification of cytokines (IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, MCP-1, INF-γ and TNF-α) released from HOKs were determined by Quantibody™ Human Inflammation Array 1 (RayBiotech) according to manufacturer's instructions. Biotinylated IgG was used as positive control. The arrays were scanned, and the data were extracted and analyzed using Q-Analyzer® (RayBiotech).

2.5. Enzyme-linked immunosorbent assay (ELISA)

To determine the inflammatory response to mixed species bacterial cultures obtained from clinical samples, HOKs (1×10^5 cells) were seeded in each well of a 12-well plate one day before infection. The host cells were then exposed to sessile bacteria (100 µl/well) or the bacterial growth media (100 µl/well) collected from the *ex vivo* assays. After culture for 24 h, the media of HOKs were collected and subjected to ELISA by using Single-Analyte ELISArray Kits for IL-6, IL-8 and MCP-1, according to the manufacturer's instructions (Qiagen). Concentrations of the cytokines were quantitated using their respective standard curves.

2.6. Total DNA isolation and qPCR

To determine the changes in bacterial biofilm profiles in *ex vivo* assays, sessile bacteria and planktonic bacteria from the *ex vivo* assays were suspended in 300 µl of PowerBead solution and subjected to DNA extraction using the DNeasy UltraClean Microbial Kit (Qiagen). DNA concentrations were determined using the NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific). Bacterial cells in biofilms were quantitated using the QuantiTect SYBR green PCR kit with specific *16S rRNA* (TGGGTTTAAAGGGTGC GTAG and CAATCGGAGTTCCTCGTGAT) of *P. gingivalis* or *23S rRNA* of streptococci (TACATAGCCGACCTGAGAGGGT and TCCACTCTCACACCCGTTCTTC) primers. Standards used to determine bacterial cell numbers were prepared using genomic DNA purified from known numbers of bacterial cells were isolated for a qPCR assay to generate standard curves.

2.7. Animal study

To determine the impact of SAPP treatment in periodontal disease, a mouse-model of experimental periodontitis induced by *P. gingivalis* was used, six-week-old specific-pathogen-free BALB/cByJ mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were housed under specific pathogen-free conditions at the animal facility of Meharry Medical College and given regular mouse chow diet and water ad libitum. All animal studies were approved by the animal ethics committee at Meharry Medical College. After a three-day quarantine, the mice were given sulfamethoxazole (800 µg/ml) and trimethoprim (400 µg/ml) in their drinking water for seven days, followed by a seven-day antibiotic-free period. The mice were randomly allocated to different experimental groups (four males and four females/per group). Test groups were infected intraorally by micropipettes with *S. gordonii* DL1 (10^9 cells) in PBS containing 2% carboxymethyl cellulose (PBS-CMC) once daily for 5 days and then infected with *P. gingivalis* 33277 (10^8 cells) grown in the presence or absence of SAPP (30 µM) or P10 (a scrambled peptide) every other day for 14 days (Xie, Hong, Sharma, & Wang, 2012). Mice without any

treatment were included as controls. Eight weeks after the final *P. gingivalis* exposure, mandibles were harvested after the mice were sacrificed by CO₂ inhalation. Formalin-fixed mandibles were scanned in all three spatial planes at a resolution of 14 µm with the vivaCT 80 *in-vivo* preclinical microCT scanner (ScancoMedical). The distance between the cemento-enamel junction and alveolar bone crest was measured at two distolingual sites of the first molars using the three-dimensional images. The measurements were taken twice by two individuals blinded to the study design.

2.8. Statistical analyses

A Student's *t*-test was used to determine the statistical significance of differences for normal distributed data. A Mann Whitney U test was used to compare groups that were not normally distributed. $p < 0.05$ was considered significant. All assays were performed in three biological replicates.

3. RESULTS

3.1. Impact of SAPP on mixed-species biofilm formation

Our previous *in vitro* studies revealed that SAPP inhibits the expression of several major virulence genes in *P. gingivalis* (Ho et al., 2017a, 2017b) and significantly reduces its ability to adhere to and invade host cells such as oral epithelial cells (Ho et al., 2017b). We also demonstrated in an *ex vivo* study that SAPP (48 µM) could inhibit biofilm formation of mixed species oral bacteria by 50% after single treatment during a 48 h culture period (Ho et al., 2018). In the present study, we tested the impact of repeated SAPP treatment to determine optimum effective application on inhibiting *P. gingivalis* attachment. As shown in Fig. 1A and B, concentrations of DNA extracted from sessile bacteria cultured in the presence of SAPP were significantly lower than those extracted from sessile bacteria cultured without SAPP ($1,101 \pm 446$ ng vs $2,717 \pm 1,618$ ng, $P < 0.05$), suggesting that SAPP effectively reduces biofilm formation of the mixed species oral bacteria. Conversely, the differences in DNA concentrations of planktonic bacteria cultured with or without SAPP were not statistically significant ($11,590 \pm 3,086$ ng vs $9,902 \pm 3,252$ ng, $P > 0.05$). Theoretically, higher bacterial mass should be found in the planktonic cultures with SAPP, since bacterial attachment appears to be lower in the presence of SAPP. We speculate that it may be due to rapid growth of planktonic bacteria in the culture media.

Previously, we reported that a single SAPP treatment (48 µM) reduced *P. gingivalis* colonization in heterogeneous biofilms by 50% (Ho et al., 2018). We speculated that a repeated SAPP treatment would further reduce the number of *P. gingivalis* cells in biofilms. To investigate this possibility, dental plaque samples were cultured anaerobically for 52 h and exposed to SAPP twice. As can be seen in Fig. 2A, a repeated SAPP treatment resulted in an approximate 3.7-times decrease in *P. gingivalis* counts in the mixed species biofilms, when compared to the number of *P. gingivalis* cells in the biofilms without any treatment, while twice as many planktonic *P. gingivalis* cells were found in the presence of SAPP compared to those in the culture media without SAPP. However, there is no significant difference observed in numbers of streptococci in either sessile or planktonic statuses, indicating that SAPP is a specifically targeted *P. gingivalis* peptide (Fig. 2B). These results

are consistent with those of our previous viability study suggesting that SAPP does not affect *P. gingivalis* growth, although significantly reduces its virulence potential (Ho et al., 2017b). Our data also demonstrate that repeated treatment with SAPP may eventually eradicate *P. gingivalis* from oral microbial communities by eliminating the attached bacteria.

3.2. modulation of human innate immune responses by SAPP

Previously we have shown the effect of SAPP on dampening the ability of *P. gingivalis* to manipulate IL-8 levels (Ho et al., 2017b). Here, we determined the effect of *P. gingivalis* on a group of cytokines including IL-1 α , IL-1 β , IL-4, IL-6, IL-8, IL-10, IL-13, MCP-1, INF- γ , and INF- β . HOKs were exposed to *P. gingivalis* 33277 continuously cultured in the presence or absence of SAPP (24 μ M) for 22 h. Levels of cytokines, especially IL-6, IL-8 and MCP-1, in the media of HOKs exposed to *P. gingivalis* were significantly decreased, with reduction ranging from 20 to 82-fold, compared to those in the media of unexposed HOKs (Fig. 3A and B). *P. gingivalis* exposed to SAPP only induced the reduction of IL-6 level by 25%, IL-8 level by 17%, and MCP-1 level by 35%. Control peptide, P10 in which a key residue phenylalanine of SAPP was substituted with alanine, had no impact on the ability of *P. gingivalis* to modulate cytokine levels.

To further investigate the effect of SAPP on cytokine levels in the HOK media, we exposed HOKs to *P. gingivalis* mutant strains derived from wild type strain 33277, FAE (a long fimbrial mutant), and KDP128 (a triple gingipain mutant). Similar patterns of these cytokine levels after treatment were observed in the growth media of HOKs in response to *P. gingivalis* 33277 or FAE exposure (Fig. 4 A–C). The levels of IL-6, IL-8, and MCP-1 in the growth media of HOKs were significantly reduced by exposure of 33277 or FAE, and SAPP was able to block action of these *P. gingivalis* strains. In contrast, *P. gingivalis* KDP128 did not have impact on the cytokine levels (Fig. 4 D–F). These data suggest that gingipain activity of *P. gingivalis* is responsible for reduction of the cytokine levels, probably through cytokine degradation.

We also tested the role of SAPP on IL-6, IL-8, and MCP-1 levels in the culture media of HOKs treated with *ex-vivo* samples grown with or without SAPP using an ELISA assay. Consistent with our observations from *in vitro* studies using purified *P. gingivalis*, levels of IL-6, IL-8, and MCP-1 were significantly lower in the media of HOKs exposed to the dental plaque samples than those found in the media of HOKs without any treatment (Fig. 5. A–C). SAPP treatment was also able to partially reverse the cytokine level reduction, suggesting an efficient action of SAPP on protecting periodontal tissues against dysbiosis of immuno-inflammatory status induced by pathogenic oral microbial communities.

3.3. Protection from alveolar bone loss by SAPP in mice

We further investigated the *in vivo* effects of SAPP using a murine model. In this model, BALB/cByJ mice were randomly allocated into seven experimental groups including those exposed to PBS-CMC only, SAPP only, *S. gordonii* only, *S. gordonii*/*P. gingivalis*, *S. gordonii*/*P. gingivalis* with 10 μ M SAPP, *S. gordonii*/*P. gingivalis* with 30 μ M SAPP, and *S. gordonii*/*P. gingivalis* with 30 μ M P10 peptide. As shown in Fig. 6, mice infected with the *S. gordonii*/*P. gingivalis* exhibited greater alveolar bone loss (0.308 ± 0.013 mm) compared

to the control mice treated with PBS-CMC only (0.282 ± 0.017 mm, $p < 0.05$). While, significant difference in bone loss was not found between the untreated mice (0.282 ± 0.017 mm) and mice infected with both *S. gordonii* and *P. gingivalis* grown with 10 or 30 μ M SAPP (0.289 ± 0.022 mm, 0.264 ± 0.030 , respectively, $p > 0.05$), suggesting that *P. gingivalis* lost its ability to induce bone loss after SAPP treatment. We also did not observe significant higher level of bone loss in the group infected with *S. gordonii* and *P. gingivalis* grown with P10 (0.309 ± 0.02 mm, $p < 0.05$) than those in control group. In addition, we found that mice exposed to a much higher concentration of SAPP (2.4 mM), or to *S. gordonii* infection alone did not have a higher level of bone loss compared to that found in the control mice.

We also examined the effect of SAPP exposure and bacterial infection on the growth of mice was also examined by measuring and comparing body weight of eight week- and 18-week-old mice. There were no differential growth rates observed among mice with or without any treatment compared to the control mice exposed to PBS-CMC only (Fig. 7). These findings suggest that any toxicity of SAPP to the experimental mice is minimal.

4. DISCUSSION

Although conventional treatments such as scaling and root planing are capable of suspending periodontal inflammation, re-emergence of periodontal pathogens presents a challenge to successful and stable therapeutic outcome (Uzel et al., 2011). Therefore, a regular maintenance visit is essential for periodontitis patients to prevent progression of the disease. Studies have shown that oral bacteria return within hours after professional plaque removal, and biofilms re-develops in one or two days (Socransky, Manganiello, Propas, Oram, & van Houte, 1977; Teles et al., 2012). It has been recognized that targeting periodontal pathogens, particularly keystone pathogens that define the virulence potentials of the entire microbial communities, is a more efficient approach than eliminating the entire oral microbiota and will likely maintain a host-compatible healthy oral microbiota. *P. gingivalis* has long been known as a periodontitis associated bacterium, and several virulence factors of the microorganism appear to contribute to its attachment, invasion, and immune manipulation (Haffajee & Socransky, 1994; Lamont & Jenkinson, 1998; Zenobia & Hajishengallis, 2015). As a low-abundance resident of the oral microbiota, *P. gingivalis* can influence other members of microbial communities and elevate the ability of the entailed community to initiate dysbiosis and periodontitis (Hajishengallis, Darveau, & Curtis, 2012; Lamont & Hajishengallis, 2015). Our previous studies revealed that SAPP, a short peptide derived from *S. cristatus*, specifically targets *P. gingivalis* and repressed expression of several virulence factors such as fimbrial proteins and gingipains (Ho et al., 2018; Ho et al., 2017a, 2017b). As a result, the ability of *P. gingivalis* to establish infection is significantly reduced. In this study we show that SAPP treatment also resulted in reduced biomass of the microbial biofilms, in addition to the decrease in *P. gingivalis* counts in the biofilms. Previous studies, using mouse or rabbit models, reported that *P. gingivalis*, even as a low abundance biofilm component, facilitated overgrowth of other members of microbial biofilms, subsequently leading to local inflammation (Hajishengallis et al., 2011; Zenobia et al., 2014).

Based on keystone pathogen hypothesis in periodontitis, colonization of *P. gingivalis* in microbial communities elicits loss of balance between the commensal microbiome and the host response and uncontrolled inflammatory/immune response (Lamont & Hajishengallis, 2015; Van Dyke & Sima, 2020). *P. gingivalis* is strongly associated with several systemic inflammatory diseases including cardiovascular disease, Alzheimer's disease due to its ability to manipulate human immune responses¹⁵. We have previously shown the effect of SAPP on dampening ability of *P. gingivalis* to manipulate IL-8 level¹². In this study, we examined the effect of SAPP in maintaining local immune surveillance using *in vitro* and *ex vivo* models. We found that human primary oral keratinocytes produce considerable higher levels of IL-6, IL-8, and MCP-1 compared to those of IL-1, IL-4, IL-10, and IL-13. Levels of IL-6, IL-8, and MCP-1 in the growth media were significantly reduced after exposure to *P. gingivalis* and mixed species of oral bacterial. SAPP treatment reversed the reduction in levels of these cytokines. Effect of SAPP on cytokine recovery most likely results from its inhibitory activity against gingipains of *P. gingivalis* at the transcriptional level, which has previously been demonstrated (Ho et al., 2017b). It is further supported by our data that a gingipain triple mutant lost the ability of wild-type *P. gingivalis* strain to manipulate local cytokine level. Similarly Chang et al. reported that IL-8 level was significantly decreased in the culture media of epithelial cells exposed to *P. gingivalis*, and that approximately 90% of IL-8 was degraded after a 30 min incubation with IL-8 (Zhang, Dong, Kashket, & Duncan, 1999). Moreover, clinical studies demonstrated a lower IL-8 concentration in gingival crevicular fluid of chronic periodontitis patients compared to that found in individuals without periodontitis (Finoti et al., 2017).

Our data raise questions on the role of cytokines in the initiation and progression of periodontitis. Cytokines and chemokines are known for their complex function. Many of them possess functional versatility which suggest that they can play conflicting roles in the pathogenesis of periodontitis. IL-8 and MCP-1 are chemoattractants that specifically recruit polymorphonuclear leukocytes and monocytes, respectively. Primary role of these cells is to eliminate pathogens and to protect the host from infections. However, excessive accumulation of these immune cells can also lead to tissue destruction in periodontitis (Lee, Aitken, Sodek, & McCulloch, 1995). Although the role of cytokines and their concentration in the pathogenesis of periodontitis needs to be further clarified, here, we provide compelling evidence that SAPP is capable of subverting the activity of *P. gingivalis* and its manipulation on microbial species to reduce fitness of dysbiotic microbial communities. Hence, SAPP is a promising therapeutic candidate against biofilms associated with periodontitis and can be used as an adjunct therapy for periodontitis.

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Fig. 1. Effects of SAPP on biofilm formation of oral bacteria.

Biofilm formation was performed in the presence or absence of SAPP using an *ex-vivo* assay. The masses of sessile bacteria (SB) and planktonic bacteria (PB) were quantitated by measuring bacterial DNA level. An asterisk indicates a statistically significant difference between DNA concentrations of bacteria grown with and without SAPP ($P < 0.05$; *t*-test).

Fig. 2. Colonization of *P. gingivalis* in multispecies biofilms.

Multispecies biofilms were formed using dental plaque samples retrieved from 13 patients with chronic periodontitis. The sample from each patient was incubated in the presence or absence of SAPP. The levels of sessile and planktonic *P. gingivalis* (A) and streptococci (B) were determined using qPCR with *P. gingivalis* specific 16S rRNA primers and streptococcal specific 23S rRNA primers, respectively. Mean levels of *P. gingivalis* and streptococci detected are presented, and an asterisk indicates a statistically significant difference between the cultures incubated with and without SAPP (Mann Whitney U test, $P < 0.05$).

Fig. 3. Cytokine levels in the grown media of HOKs exposed to *P. gingivalis*.

Levels of cytokines in the culture media of HOKs exposed to *P. gingivalis* treated with SAPP or P10 were measured using a cytokine array Kit. A) Representative images from cytokine antibody arrays. B) The signal intensity was determined for each antigen-specific antibody spot, and each bar represents the mean of the cytokine concentration. Standard deviations were calculated from three biological replicates. Asterisks indicate statistically significant differences between cytokine concentrations in the culture supernatants of HOKs treated with *P. gingivalis* grown with and without SAPP ($p < 0.05$; *t*-test).

Fig. 4. The effect of *P. gingivalis* mutants on cytokine levels in HOK growth media. Concentrations of IL-6, IL-8, and MCP-1 in the growth media of HOKs exposed to a *fimA* mutant (FAE) (A-C) or a gingipain triple mutant (KDP128) (D-F) were determined using cytokine arrays. Each bar represents the mean of the cytokine concentration. Asterisks indicate statistically significant differences between cytokine concentrations in the culture supernatants of HOKs treated with *P. gingivalis* strains grown with and without SAPP ($p < 0.05$; *t*-test).

Fig. 5. The impact of SAPP on cytokine levels modulated by oral microbial communities.

HOKs were exposed to sessile (Sb) or planktonic (Pb) dental plaque samples grown with or without SAPP (48 μ M) for 24 h. The culture media were collected, and levels of IL-6 (A), IL-8 (B), and MCP-1 (C) were quantitated using ELISAs. Each bar represents the cytokine level relative to that found in the culture media of HOKs without treatment (PBS only, designated as 100). Error bars represent the SEM. * $P < 0.05$.

Fig. 6. Protection from alveolar bone loss by SAPP.

Alveolar bone loss in mice under different growth conditions was determined using an *in-vivo* microCT scanner (vivaCT 80). Mean distances between the cemento-enamel junction and alveolar bone crest are presented in millimeters. Asterisks indicate statistically significant differences among the different groups ($n=8$ for each group; $p < 0.05$; t -test).

Fig. 7. The effect of SAPP in body weights of experimental mice.

Mice were assigned to seven groups (n =8 for each group) and treated with or without SAPP, *P. gingivalis* 33277 (Pg), *P. gingivalis* 33277 and *S. gordonii* DL1 (Pg/Sg). The body weight of each mouse was measured at 8 week and 18-week-old time points. Each bar represents the mean of percentage weight increase at 18-week-old mice compared to 8-week-old mice in each group.