Diet-Induced Obesity and Its Differential Impact on Periodontal Bone Loss

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Abstract

Obesity is associated with abnormal lipid metabolism and impaired bone homeostasis. The aim of our study was to investigate the impact of specific elevated fatty acid (FA) levels on alveolar bone loss in a *Porphyromonas gingivalis*-induced model of periodontal disease and to analyze underlying cellular mechanisms in bone-resorbing osteoclasts and bone-forming osteoblasts in mice. Four-week-old male C57BL/6 mice were randomly divided in groups and subjected to a palmitic acid (PA)- or oleic acid (OA)-enriched high-fat diet (HFD) (20% of calories from FA) or a normal caloric diet (C group) (10% of calories from FA) for 16 wk. Starting at week 10, mice were infected orally with *P. gingivalis* (W50) or placebo to induce alveolar bone loss. Animals were sacrificed, and percentage fat, serum inflammation (tumor necrosis factor [TNF]- α), and bone metabolism (osteocalcin [OC], carboxy-terminal collagen crosslinks [CTX], and N-terminal propeptides of type I procollagen [PINP]) markers were measured. Osteoblasts and osteoclasts were cultured in the presence of elevated PA or OA levels and exposed to *P. gingivalis*. Animals on FA-enriched diets weighed significantly more compared with animals on a normal caloric diet ($P < 0.05$). Both obese groups had similar percentages of fat ($P =$ nonsignificant); however, alveolar bone loss was significantly greater in animals that were on the PA-enriched HFD ($P < 0.05$). TNF- α levels were highest in the PA group ($P < 0.001$) and increased in all groups in response to *P. gingivalis* inoculation ($P < 0.01$), whereas bone remodeling markers OC, CTX, and PINP were lowest in the PA group ($P < 0.001$) and highest in the C group. Bacterial challenge decreased bone metabolism markers in all groups ($P < 0.01$). Further, osteoclasts showed an augmented inflammatory response to *P. gingivalis* in the presence of hyperlipidemic PA levels as opposed to OA cultures, which responded similarly to controls. These findings indicate that the specific FA profile of diet rather than weight gain and obesity alone modulates bone metabolism and can therefore influence alveolar bone loss.

Keywords: palmitic acid, oleic acid, high-fat diet, bone metabolism, inflammation, osteoclast

Introduction

Periodontitis is a chronic inflammatory disease of tooth-supporting tissue, characterized by local progressive loss of bone and periodontal attachment (Papapanou et al. 1989). Historically, periodontal disease has been considered primarily to be an infectious disease caused by bacteria in the dental plaque (Theilade 1986; van Winkelhoff et al. 2002). More recently, the inflammatory host response has been shown to be crucial in the development and progression of the disease, and focus has been placed on defining determinants of the local host response to bacteria and bacterial products (Cekici et al. 2014). Changes in bone metabolism and increased proinflammatory mediators have been implicated as factors that can influence local host response. Therefore, increasing interest is focused on systemic conditions associated with the occurrence and progression of periodontal disease (Graves et al. 2011; Hasturk et al. 2012).

Obesity is a systemic heterogeneous disease with a fundamental basis in the imbalance between energy intake and expenditure (Pi-Sunyer et al. 1998). The increasing prevalence of obesity has resulted in a global health problem, with 2.1 billion people classified as overweight or obese in 2013 (Ng et al. 2014). Obesity is associated with an increased risk of

cardiovascular disease, type 2 diabetes, cancer, asthma, and osteoarthritis (Guh et al. 2009). Epidemiological studies have further characterized the association of periodontal disease with obesity (Chaffee and Weston 2010; Suvan et al. 2011) and indicated that obesity is second only to smoking as a risk factor for inflammatory periodontal tissue destruction (Nishida et al. 2005). Animal studies explored the association between obesity and periodontal disease further, showing that bacterial-induced alveolar bone destruction is greater in obese animals compared with normal-weight controls (Amar et al. 2007).

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

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Recently, Li et al. (2015) investigated obesity-associated periodontal bone loss and described an increased local inflammatory response to periodontal injection of bacterial lipopolysaccharides (LPS) in obese animals fed a high-fat diet (HFD).

Nutrition research has highlighted that not all fats are equal in their impact on health (Fernández-Real et al. 2012; Kien et al. 2014). Additionally, our previous study demonstrated that bone-resorbing osteoclasts exhibit differential activity depending on their hyperlipidemic environment. We showed in vitro that with elevated levels of saturated palmitic acid (PA), osteoclastogenesis and production of inflammatory cytokines are up-regulated (Drosatos-Tampakaki et al. 2014), and that obesity-associated bone loss is enhanced in obese animals on a PA-enriched HFD (PA-HFD) compared with an oleic acid (OA)-enriched HFD (OA-HFD) (Drosatos-Tampakaki et al. 2014). These results, combined with the potential role of host inflammatory response in initiation and progression of periodontal disease, led us to investigate whether saturation of fatty acids (FAs) is an important variable promoting alveolar bone loss in a *Porphyromonas gingivalis*-induced model of periodontal disease in obesity.

Materials and Methods

Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Columbia University (New York, NY, USA). Mice were maintained under appropriate barrier conditions in a 12-h light-dark cycle and received food and water ad libitum in accordance with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

FA-HFD to Induce Weight Gain

Four-week-old male C57BL/6 mice were randomly divided into groups ($n = 10$ per group) and put on either PA-HFD or OA-HFD (20% calories from fat) (Research Diets, Inc., New Brunswick, NJ, USA) or normal caloric chow diet (10% calories from fat) for a total of 16 wk (Appendix Table). *P. gingivalis* inoculation was started at week 10 of high-fat feeding.

Experimental Periodontal Disease

P. gingivalis W50 (Baker et al. 2000) (ATCC 53978; American Type Culture Collection, Manassas, VA, USA) was used (see the Appendix).

Osteoclast and Osteoblast Cultures

Osteoclasts and osteoblasts were isolated from bone marrow cells and calvariae, respectively. See the Appendix.

RNA, Gene Expression Analysis

See the Appendix.

Protein Analysis

See the Appendix.

Lipid Analysis

See the Appendix.

Dual-Energy X-Ray Absorptiometry (DEXA) Analysis

See the Appendix.

Statistical Analysis

Comparisons between 2 groups were performed using unpaired 2-tailed Student's *t* tests. Comparisons between more than 2 groups were performed using 1-way analysis of variance (ANOVA), with Tukey-Kramer multiple comparisons testing used for multiple comparison posttests. Pearson correlation coefficient and linear regression analysis was used to check for dependences. All values are presented as mean $\pm$ SD. Differences between groups were considered statistically significant at $P < 0.05$. Analyses were performed in GraphPad/InStat3 (GraphPad Software Inc., San Diego, CA, USA).

Results

PA Increases the Inflammatory Osteoclastic Response to *P. gingivalis*

One major characteristic of obesity is increased circulating free fatty acids (FFAs) due to excessive food intake (Ebbert and Jensen 2013). We selected PA and OA to determine whether the inflammatory response of bone-forming osteoblasts and bone-resorbing osteoclasts to *P. gingivalis* differs at hyperlipidemic FA levels and with differing FA saturation. Specifically, we investigated induction of tumor necrosis factor (TNF)- α and interleukin (IL)-6, proinflammatory cytokines that have been implicated in periodontitis-associated bone loss (Pischon et al. 2007; Pacios et al. 2012).

As shown in Figure 1, presence of *P. gingivalis* significantly increased TNF- α gene expression in osteoclasts that had been cultured with PA (Fig. 1A). Further, *P. gingivalis* induced IL-6 expression in all conditions, with the most pronounced expression seen in osteoclasts cultured with PA (Fig. 1B). Despite being present at hyperlipidemic levels, OA did not attenuate osteoclasts' inflammatory response to *P. gingivalis* infection.

The Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) genes produce proteins involved in the innate immune response that participate in identifying pathogenic substances for destruction. TLR4 is activated by LPS found in the cell membrane of gram-negative bacteria such as *P. gingivalis* (Maglione et al. 2015). Analysis of TLR4 gene expression showed a trend of increased expression in response to *P. gingivalis* in all conditions, with PA eliciting the highest response (Fig. 1C). Although TLR2 is also part of the innate immune response to bacteria, it is involved in recognition of gram-positive bacteria (Maglione et al. 2015). Accordingly, there

was no significant difference in TLR2 gene expression with the addition of *P. gingivalis* in any of the culture conditions (Fig. 1D).

To determine whether the increase in inflammatory gene expression translates into increased secretion of the corresponding gene products, we analyzed TNF- α and IL-6 secretion in cell culture media in response to *P. gingivalis*. We found a significant increase in the concentration of TNF- α secreted into culture medium by osteoclasts with the addition of *P. gingivalis* in all 3 conditions (Fig. 1E). Notably, OA cultures demonstrated significantly lower TNF- α secretion compared with PA cultures in the presence of *P. gingivalis*. IL-6 secretion was significantly increased in all conditions in response to bacterial inoculation but did not differ between OA and PA (Fig. 1F).

Because bone homeostasis is a balance between formation and resorption (Rodan and Martin 2000), we evaluated the impact of OA and PA on osteoblasts. In addition to examining the proinflammatory cytokines TNF- α and IL-6, we analyzed receptor activator of nuclear factor- κ B (RANKL) and osteoprotegerin (OPG), both of which are involved in regulating osteoclasts (Warren et al. 2015). We observed a nonsignificant trend to increased RANKL and reduced OPG gene expression in osteoblasts cultured with PA and *P. gingivalis* (Appendix Fig. 2A, B). IL-6 gene expression showed a trend, being higher in all cultures exposed to *P. gingivalis* and high-fat conditions; however, there was no difference between OA and PA (Appendix Fig. 2C). There was no trend with respect to TNF- α gene expression (Appendix Fig. 2D). These results concur with our previous study, indicating that PA's primary effect on bone homeostasis is through the influence of PA on osteoclasts rather than osteoblasts (Drosatos-Tampakaki et al. 2014).

PA-HFD Increases Alveolar Bone Loss in Obese Mice

The heightened response of osteoclasts to hyperlipidemic PA led us to investigate whether hyperlipidemic PA and OA levels have differential impacts on alveolar bone loss in a *P. gingivalis*-induced model of periodontal disease. Animals fed an isocaloric PA-HFD or OA-HFD had similar weight gain and percentage of total body fat (weight in grams: 47.3 ± 3.6 [OA] vs 44.9 ± 3.6 [PA]; fat as percentage of total: 47.13 ± 3.11 [OA] vs 46.44 ± 5.14 [PA]; $P = \text{nonsignificant [NS]}$, Appendix Fig. 1). There was no difference in the distance between the cemento-enamel junction (CEJ) and alveolar bone crest (ABC) between animals that were not challenged with bacteria,

Figure 1. *Porphyromonas gingivalis* induces increased gene expression and secretion of inflammatory cytokines in osteoclast cell cultures. (A) Tumor necrosis factor (TNF)- α gene expression. (B) Interleukin (IL)-6 gene expression. (C) Toll-like receptor 4 (TLR4) gene expression. (D) Toll-like receptor 2 (TLR2) gene expression. (E) TNF- α secreted into culture medium, measured at day 9 of culture. (F) IL-6 secreted, measured at day 7 of culture. All gene expression levels given as fold change versus control. C, control; PA, palmitic acid; OA, oleic acid. Pg+ indicates cell cultures exposed to *P. gingivalis*; Pg- indicates respective controls with no *P. gingivalis* exposure. * $P < 0.05$. *** $P < 0.001$.

Figure 2. Palmitic acid (PA)-enriched high-fat diet (HFD) results in greater alveolar bone loss after inoculation with *Porphyromonas gingivalis*. (A) Distance between the cemento-enamel junction (CEJ) and alveolar bone crest (ABC) represented as percentage of control, with each diet condition serving as control to its respective Pg+ condition. (B) Representative jaw section of a mouse on PA-HFD depicting area between CEJ-ABC with placebo inoculation. (C) Representative jaw section of a mouse on PA-HFD depicting area between CEJ-ABC with *P. gingivalis* inoculation. Arrow = CEJ; arrowhead = ABC. C, control; OA, oleic acid. Pg+ indicates cell cultures exposed to *P. gingivalis*. * $P < 0.05$.

regardless of control or HFD conditions ($P = \text{NS}$). However, PA-HFD animals exhibited greater alveolar bone loss when inoculated with *P. gingivalis* compared with PA-HFD animals inoculated with control solution, displaying a nearly 20% increase in the distance between the CEJ and the ABC (Fig. 2A–C). Neither the control animals nor the OA-HFD animals

Figure 3. High-fat feeding with specific fatty acid (FA) results in increased circulating levels of the corresponding FA in serum. Feeding with a palmitic acid (PA) high-fat diet (HFD) results in greater free fatty acids (FFAs) in bone than feeding with an oleic acid (OA) HFD. Bacterial inoculation attenuates the increase in serum and reduces FA level in bone in animals on regular diet (C group) or PA-HFD. (A) Serum levels of PA (C16:0). (B) Serum levels of OA (C18:1). (C) Total serum FFA levels. (D) PA in bone. (E) OA in bone. (F) Total FFA in bone. Pg+ indicates cell cultures exposed to *Porphyromonas gingivalis*. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. **** $P < 0.0001$.

showed significant change in CEJ-ABC distance when inoculated with *P. gingivalis* compared with their respective placebo controls.

Bacterial Infection Reduces FFA in Serum and Reduces PA Accumulation in Bone

Studies have shown that high dietary FA intake results in increased FFA in serum as well as increased accumulation of specific FAs in tissues (Lu et al. 2015). Compared with animals on a regular diet, mice fed an HFD exhibited a nonsignificant increase in total FFA in serum (Fig. 3C). However, mice on a PA-HFD exhibited significantly increased PA in serum compared with animals on an OA-HFD or regular diet (Fig. 3A). Similarly, mice on an OA-HFD had significantly higher levels of OA in serum compared with those on a PA-HFD or regular diet (Fig. 3B). In both HFD conditions, inoculation with *P. gingivalis* resulted in significant reduction of FFA levels in serum. This was seen for total FFA levels as well as for specific FFAs (Fig. 3A–C).

Analysis of bone revealed that increased PA in both diet and serum correlated with increased PA in bone (Fig. 3D, $P = 0.0021$, $r = 0.85$). However, in the OA-HFD, there was no correlation between elevated OA in serum and OA in bone (Fig. 3E, $P = 0.12$, $r = -0.56$). Individual and total FFA levels in bone were significantly higher in PA animals than in OA animals (Fig. 3D–F). Inoculation with bacteria significantly reduced both PA and total FFA levels in bone for animals on either the regular diet or PA-HFD. Animals on the OA-HFD showed no significant change in FA level in bone after bacterial inoculation.

Exposure to Bacterial Infection and PA Leads to Increased Expression of TNF- α and RANKL in Gingival Tissue

Inflammatory cytokines such as TNF- α are involved in the host inflammatory response to bacterial infection and have been associated with progression of periodontal disease (Graves et al. 2011; Pacios et al. 2012; Cekici et al. 2014). Further, a recent study indicated that concurrent exposure of macrophages to LPS, a component of gram-negative bacterial membranes, and PA may trigger increased inflammatory cytokine production via a nuclear factor- κ B-mediated pathway (Jin et al. 2013).

Our investigation of gingival tissue overlaying areas of alveolar bony defects revealed that obese animals infected with *P. gingivalis* while on a PA-HFD indeed exhibited higher gingival expression of both TNF- α and RANKL (Fig. 4A, B) compared with control animals, which suggests that PA-HFD promotes local inflammatory activation in response to infection.

Periodontal Infection Increases Systemic TNF- α Levels and Reduces Markers of Bone Metabolism In Vivo

We analyzed bone formation markers type 1 procollagen N-terminal propeptide (P1NP) and osteocalcin (OC), as well as bone resorption marker C-telopeptides of type I collagen (CTX), and TNF- α in serum of all animals. PA-HFD animals exhibited significantly lower levels of all bone metabolism markers and significantly elevated TNF- α production compared with animals receiving OA-HFD and regular diet (Fig. 4C–F). Oral swabbing with *P. gingivalis* reduced serum markers of bone metabolism (Fig. 4C–E) and increased systemic TNF- α levels in all diet groups compared with their respective placebo-swabbed controls (Fig. 4F). The increase in systemic TNF- α was negatively correlated with markers of bone metabolism (OC: -0.55 , $P = 0.002$; P1NP: -0.6 , $P = 0.0005$; CTX: -0.61 , $P = 0.0004$). Further, in obese animals on a PA-HFD, systemic TNF- α was positively correlated with amount of alveolar bone loss ($r = 0.68$, $P < 0.05$).

Discussion

Our study demonstrates that contrary to OA-HFD, PA-HFD has a detrimental impact on bone metabolism and the establishment of periodontal lesions in response to *P. gingivalis*.

inoculation in obese mice. In addition, hyperlipidemic PA levels increase the inflammatory osteoclastic response to *P. gingivalis* infection in vitro.

Recent epidemiological studies and animal experiments describe an association between obesity and periodontal disease (Pischon et al. 2007), and emerging research has highlighted that not all fats are equal in their impact on health. Studies have demonstrated that diets higher in unsaturated fat than in saturated fat are associated with decreased cardiovascular disease risk (Estruch et al. 2013) and decreased circulating low-density lipoprotein (Kien et al. 2014). Specifically, a recent dietary intervention study demonstrated increased bone formation markers (OC and P1NP) in serum of human subjects on an OA-enriched diet (Fernández-Real et al. 2012). We, therefore, focused our study on the impact of PA and OA, the most abundant saturated and monounsaturated FAs in the Western diet and in adipose tissue (Baylin et al. 2002).

Many studies have shown that PA promotes inflammation while OA has anti-inflammatory properties (Vassiliou et al. 2009; Gupta et al. 2012). Given the contribution of inflammation to bone degradation (Azuma et al. 2000) and impaired bone formation (Tomomatsu et al. 2009), the proinflammatory nature of PA may account for its aggravating impact on bone health by promoting osteoclast differentiation and activity via increased TNF- α . This is reflected in our results, as osteoclast cultures exposed to *P. gingivalis* exhibited a significant increase in TNF- α expression when cultured with hyperlipidemic levels of PA. The lack of a similar increase with hyperlipidemic OA suggests that lipid composition plays an integral role in the induction of the inflammatory response. In addition to governing an inflammatory response, FA might also exhibit differential direct antibacterial properties that could be defined in future studies (Huang et al. 2010).

In translating our in vitro model to mice, we found that the periodontal pathogen, *P. gingivalis*, induced significantly greater alveolar bone loss in animals fed a PA-HFD compared with animals fed a regular diet or OA-HFD and inoculated with the same bacterium. Mice on a PA-HFD that were inoculated exhibited a nearly 20% greater distance between the ABC and CEJ than their noninoculated PA-HFD counterparts, suggesting that the presence of hyperlipidemic levels of PA in the diet may impair the host's ability to respond to infection, thereby leading to increased alveolar bone loss. In support of this hypothesis, we found that gingival tissue of animals exposed to *P. gingivalis* exhibited higher expression of TNF- α and RANKL. Additionally, bone metabolism markers were reduced

Figure 4. Mice fed a palmitic acid (PA) high-fat diet (HFD) and inoculated with *Porphyromonas gingivalis* have increased inflammatory gene expression in gingival tissue, decreased serum production of bone metabolism markers, and increased tumor necrosis factor (TNF)- α production in serum. Bacterial inoculation further reduces bone metabolism markers and increases TNF- α production. Parts A and B present gene expression in gingival tissue. Parts C through F present gene product concentration in serum. (A) TNF- α gene expression. (B) Receptor activator of nuclear factor- κ B (RANKL) gene expression; values given as fold change vs PA without *P. gingivalis*: $P = 0.057$. (C) Osteocalcin (OC). (D) N-terminal propeptides of type I procollagen (P1NP). (E) Carboxy-terminal collagen crosslinks (CTX). (F) TNF- α . Pg+ indicates cell cultures exposed to *P. gingivalis*. * $p < 0.01$. *** $p < 0.001$.

and TNF- α levels increased in the serum of animals inoculated with *P. gingivalis*, regardless of diet. Notably, regression analysis revealed that the increase in systemic TNF- α was negatively correlated with bone metabolism markers and positively correlated with alveolar bone loss. These results suggest a link between TNF- α and disruption of bone homeostasis; as TNF- α levels rise, bone formation decreases and bone loss is allowed to progress.

To examine the emerging link between dietary FA and bone metabolism in the presence of infection, we investigated whether high-fat feeding resulted in FA accumulation in serum and bone, and our results confirmed this with respect to an increase of specific FFAs in animals' serum. However, only the PA-HFD induced a subsequent increase of FA in bone, with higher levels of both PA and OA in bone compared with animals fed the OA-HFD. This accumulation of FFA in serum in both HFD conditions, and in bone in the PA condition, was significantly attenuated by inoculation with *P. gingivalis*. One explanation for this could be that the bacteria themselves are oxidizing the lipids, thereby decreasing lipid levels in the surrounding areas (Jurtshuk 1996).

We fed 4-wk-old mice an HFD for 16 wk to create a model of diet-induced obesity that replicates methods used in similar studies and parallels our previous study (Amar et al. 2007; Drosatos-Tampakaki et al. 2014; Li et al. 2015). *P. gingivalis* inoculation was initiated at 14 wk of age. To account for a

potential impact of HFD on skeletal development as opposed to mature bone homeostasis, we compared each diet group to its respective placebo control when analyzing alveolar bone loss. We found no differences in initial bone height when analyzing the placebo animals regardless of diet condition. Human periodontitis is a complex condition associated with multiple pathogens (van Winkelhoff et al. 2002); however, *P. gingivalis* has been strongly implicated in this disease through its central role in creating biofilm dysbiosis and inducing inflammation (Hasturk et al. 2007; Hajishengallis and Lambris 2012). Future research could investigate the impact of additional pathogens under hyperlipidemic conditions and how their presence relates to human periodontal disease.

It has been demonstrated that *P. gingivalis* is able to enter periodontal connective tissue after colonizing epithelial cells (Tribble and Lamont 2010), and it directly invades osteoblasts in a murine periodontal disease model (Zhang et al. 2010). Nevertheless, oral tissue cells such as epithelial cells, and fibroblasts, which are more directly exposed to bacteria, could be evaluated in their response to an HFD enriched with PA or OA combined with bacterial infection, as in a recent study by Li et al. (2015), which showed that PA increases the inflammatory response of macrophages to *P. gingivalis* infection.

The alveolar bone loss in our study was not as pronounced as in a similar study (Li et al. 2015), likely because the test animals used by this group weighed 61% more than controls, whereas ours were only 18% to 25% heavier. However, we intentionally selected a diet with only 20% of calories from fat to overcome the impact of secondary conditions associated with obesity as described in our previous study (Drosatos-Tampakaki et al. 2014). We also used relatively low PA levels and only 6 h of *P. gingivalis* exposure in our cell culture models to complement our animal model and to investigate the early effects of infection. This may explain why we saw minimal differences in our osteoblast cultures, whereas another study demonstrated that osteoblasts from obese mice exhibited significantly reduced proliferation and increased apoptosis compared with those from control mice, when assessed 8 d after bacterial exposure (Dittmann et al. 2015). The osteoclast preparations were not 100% pure, being obtained from bone marrow flushings, which contain stromal cell populations in addition to osteoclasts. This method has been previously used in similar studies (Akiyama et al. 2014) and allowed us to maintain experimental consistency (Drosatos-Tampakaki et al. 2014).

Considering our results showing greater alveolar bone resorption in mice on PA-HFD, we expected to see elevated CTX production in these animals, as CTX is a marker for bone resorption. What we saw, however, was significant reduction. It is possible that while hyperlipidemic conditions reduce markers of both bone formation and resorption, the reduction of formation markers is more substantial, resulting in a net increase in the ratio of resorption to formation markers. Further, samples were only collected when animals were sacrificed, and samples from additional time points might show different dynamics. Our combined data highlight the complexity of the

system governing bone metabolism, and future studies will further define the impact of diet and infection on these bone markers.

In conclusion, our study shows that contrary to OA, PA demonstrates an inflammatory potential that can accelerate alveolar bone loss in experimental periodontal disease in obese mice and affect the inflammatory osteoclastic response to *P. gingivalis* infection in vitro. In concert with other recent works, this highlights pathways through which dietary composition may initiate or accelerate disease processes and indicates mechanisms through which we can potentially prevent disease.

Author Contributions

M. Mulukey, T. Gold, A. Al-Sahli, contributed to data acquisition and analysis, critically revised the manuscript; K. Kiefhaber, contributed to data acquisition and analysis, drafted and critically revised the manuscript; R. Celenti, H. Jiang, contributed to data acquisition, drafted and critically revised the manuscript; S. Cremers, T. Van Dyke, contributed to data interpretation, drafted and critically revised the manuscript; U. Schulze-Späte, contributed to conception, design, and data interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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