INTRODUCTION

The human oral cavity harbors a substantial and continuously evolving load of microbial species. Many infectious diseases, including periodontal diseases, appear to be infections mediated by the overgrowth of commensal organisms, rather than by the acquisition of an exogenous pathogen. As microorganisms evolve more rapidly than their hosts, immune mechanisms that determine the ecological balance of commensal organisms also need to change to preserve homeostasis. The immune system involvement will depend on the

Background and Objective: Soluble epoxide hydrolase (sEH) is an enzyme in the arachidonate cascade which converts epoxy fatty acids (EpFAs), such as epoxyeicosatrienoic acids (EETs) produced by cytochrome P450 enzymes, to dihydroxyeicosatrienoic acids. In the last 20 years with the development of inhibitors to sEH it has been possible to increase the levels of EETs and other EpFAs in vivo models. Recently, studies have shown that EETs play a key role in blocking inflammation in a bone resorption process, but the mechanism is not clear. In the current study we used the sEH inhibitor (1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU) to investigate the immunomodulatory effects in a mouse periodontitis model.

Material and Methods: Mice were infected on days 0, 2, and 4 with Aggregatibacter actinomycetemcomitans and divided into groups (n = 6) that were treated orally, daily for 15 days, with 1 mg/kg of TPPU. Then, the mice were killed and their jaws were analyzed for bone resorption using morphometry. Immunoinflammatory markers in the gingival tissue were analyzed by microarray PCR or western blotting.

Results: Infected mice treated with TPPU showed lower bone resorption than infected mice without treatment. Interestingly, infected mice showed increased expression of sEH; however, mice treated with TPPU had a reduction in expression of sEH. Besides, several proinflammatory cytokines and molecular markers were down-regulated in the gingival tissue in the group treated with 1 mg/kg of TPPU.

Conclusions: The sEH inhibitor, TPPU, showed immunomodulatory effects, decreasing bone resorption and inflammatory responses in a bone resorption mouse model.

KEYWORDS
bone, epoxyeicosatrienoic acid, inflammation, periodontitis, soluble epoxide hydrolase, TPPU
severity and progression of periodontal disease and both the innate and the adaptive immune responses may be involved. The knowledge of how immune mechanisms and inflammatory responses are regulated is important for understanding the pathogenesis of complex diseases, such as periodontitis; in addition, it is important to continue considering mechanisms that regulate inflammatory responses because they may open up novel therapeutic targets.

Epoxyeicosatrienoic acids (EETs) are biologically active metabolites of arachidonic acid that are generated by the activity of cytochrome p450 (CYP) enzymes. Once formed, EETs are unstable, being rapidly converted into less active or inactive dihydroxyeicosatrienoic acids by soluble epoxide hydrolase (sEH). This conversion may be prevented by inhibitors of sEH (sEH), so EETs can be stabilized, prolonging their biological effects. Studies on these effects emphasize the role of EETs in vasodilatation of systemic vessels and control of blood pressure, grounding their promise in the treatment of vascular diseases, such as atherosclerosis and hypertension. Emerging additional benefits have been described for EETs, which are currently recognized as potent anti-inflammatory agents. They can inhibit cytokine-induced inflammation signaling mediated by nuclear factor-kappaB, thus reducing the expression of proinflammatory mediators and cell adhesion molecules. Systemic overexpression of human CYP2J2, a CYP epoxygenase isoform that produces EETs, led to a significant reduction in the tumor necrosis factor-alpha (TNF-α)-induced high plasma levels of adhesion molecules and inflammatory cytokines in rats, suggesting that EETs and other epoxy fatty acids act as anti-inflammatory mediators. In addition, sEH pharmacological inhibitors, such as 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU), have been the focus of in-depth investigation because, by reducing the metabolism of EETs, these inhibitors were demonstrated to alleviate inflammatory and fibrotic diseases in animal models. Therefore, sEH may be an important target for the modulation of periodontal disease and bone resorption.

In this study, using an animal model of periodontitis, we explored the molecular mechanisms by which TPPU may modulate the immunological response to avoid disease-related bone loss.

2 | MATERIAL AND METHODS

2.1 | Animals

Male mice were maintained in standard conditions, under a 12-hour light-dark cycle with food and water ad libitum. Six mice per group, weighing 20-25 g, were age-matched. All procedures were carried out in agreement with the standards for the care of laboratory animals, as outlined in the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Resource Services of the University (University of California).

2.2 | Periodontitis model and treatments

Aggregatibacter actinomycetemcomitans (JP2) was cultured under anaerobic conditions to ensure viability and virulence. The mice received 1 × 10^9 colony-forming units/mL of A. actinomycetemcomitans, diluted in 100 μL of phosphate-buffered saline containing 2% carboxymethylcellulose, orally. Oral colonization of A. actinomycetemcomitans was performed before, at day 0, and at the end of the experiment, as previously described. The solution was placed in the oral cavity, and the procedure was repeated 2 and 4 days after the first inoculation. Treatment with TPPU, which was dissolved in PEG400, was initiated after the last inoculation of bacteria by oral gavage. The dose of TPPU used was 1 mg/kg/d for 15 days, as previously tested. The negative control group consisted of uninfected mice (sham-infected), and mice in the positive control group were infected with A. actinomycetemcomitans and received vehicle only (PEG400). The mice were continuously weighed every 2 or 3 days after TPPU treatment begin. The day after the treatment period ended, the mice were killed by cardiac puncture after anesthesia with xylazine/ketamine. The whole buccal and palatal periodontal tissues of the upper and lower molars were collected, weighed, and the portions of gingival tissue were separated into two halves: one half was used for extraction of protein and the other half was used for extraction of mRNA. The amount of gingival tissue for protein extraction was triturated and homogenized in 300 μL of the appropriate buffer containing protease inhibitors (Sigma-Aldrich, St Louis, MO, USA), followed by centrifugation for 10 minutes at 10 000 g. The supernatant was rapidly frozen and stored at −70°C for further gene expression analyses or western blotting.

2.3 | Quantification of alveolar bone loss

Evaluation of alveolar bone loss was performed as described previously. The jaws were removed and defleshed, then immersed overnight in 3% hydrogen peroxide, washed in phosphate-buffered saline, and stained with 1% methylene blue. Horizontal bone loss was assessed morphometrically by measuring the distance between the cement–enamel junction and the alveolar bone crest of the first and second molars. Measurements were made at 14 buccal sites per mouse, under a microscope, and bone measurements were analyzed using the Image J software (Bethesda, MD, USA). Random and blinded measurements were made by the same calibrated person, achieving intra-examiner reproducibility of >90%.

2.4 | Western blotting

Tissues were lysed and clarified by centrifugation, and protein concentrations were determined using the bicinchoninic acid protein assay kit (ThermoFisher Scientific, Waltham, MA, USA). Equal amounts of protein (20 μg) from the gingival tissue were resolved by SDS-PAGE and transferred to poly(vinylidene difluoride) membranes, and then incubated with rabbit anti-mouse sEH lg (1:1000; Dr Hammock, University of California, Davis) or α-tubulin (1:1000; Santa Cruz Biotechnology; Santa Cruz, CA, USA). After incubation with the goat anti-rabbit secondary antibodies, proteins were visualized using Luminata™ Fort Western HRP substrate (Millipore, Burlington, MA, USA). Pixel intensities of immunoreactive bands were measured using the Image J software (Bethesda, MD, USA).
were quantitated using FluorChem Q Imaging software (Alpha Innotech, San Leandro, CA, USA). Data are presented as total protein expression normalized to α-tubulin.\textsuperscript{12}

\subsection*{2.5 | PCR Array}

The RNeasy Micro Kit (Qiagen) was used for total RNA extraction, and 300 ng was retrotranscribed using the RT\textsuperscript{2} First Strand Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. For the study of gingival tissue gene expression, the 96-well Qiagen PCR Array Mouse Innate & Adaptive Immune Responses was used in combination with the RT\textsuperscript{2} SYBR Green qPCR Mastermix (Qiagen), using 10 ng of cDNA per well. Results for each assay were normalized to the average of all five housekeeping genes. Gene expression changes were reported if a 3.0-fold threshold was detected.

\subsection*{2.6 | Statistical analysis}

Results were expressed as mean ± SD. Data were analyzed using the Shapiro-Wilk test and were found to be normally distributed. One-way ANOVA followed by Tukey’s test was applied for post hoc analysis. Significance was considered at a level of 0.05.

\section*{3 | RESULTS}

The effect of oral administration of TPPU on \textit{A. actinomycetemcomitans}-induced periodontal bone resorption was assessed by quantifying, in all mice, the distance between the cement-enamel junction and the alveolar bone crest during the experimental period. Sham-infected mice showed no bone resorption during the experimental period (Figure 1). On the other hand, animals orally infected with \textit{A. actinomycetemcomitans} showed high bone resorption, which was completely abrogated in the group treated orally with TPPU (Figure 1). Mice were constantly weighed every 2 or 3 days after starting treatment with TPPU during the experimental period, and there was no statistical variation among the groups (Figure 2, $P > .05$).

Western blot analysis of gingival tissue demonstrated that mice orally infected with \textit{A. actinomycetemcomitans} showed increased expression of sEH, which was abrogated in the infected animals treated with sEH inhibitor ($P < .05$) as demonstrated in Figure 3A. The data from band density are expressed as arbitrary units (Figure 3B).

A PCR array analysis was performed to evaluate the the gene expression profile of 84 genes involved in the host response to bacterial infection. Applying a 3-fold change cut-off value for the differential expression, we identified 6 genes that were upregulated in the mice infected and treated with TPPU in comparison with infected animals: interleukin-13 (\textit{Il13}), interleukin-23 (\textit{Il23}), serum amyloid P-component (\textit{Apcs}), interferon-beta (\textit{Ifnb}), mannose-binding lectin (protein C) 2 (\textit{Mbl2}), myeloperoxidase (\textit{Mpo}). On the other hand, 18 genes were downregulated in the group treated with TPPU in comparison with the infected, but not treated, group: CD8 antigen (\textit{Cd8}), CD40 antigen, Ig (\textit{Cd40lg}), chemokine (C-C motif) receptor 6 (\textit{Ccr6}), CD4 antigen (\textit{Cd4}), chemokine (C-C motif) receptor 8 (\textit{Ccr8}), lysozyme 2 (\textit{Lyz2}), toll-like receptor 1 (\textit{Tlr1}), signal transducer and activator of transcription 4 (\textit{Stat4}), interferon-gamma (\textit{IFN\gamma}), toll-like receptor 9 (\textit{Tlr9}), chemokine (C-C motif) receptor 4 (\textit{Ccr4}), interleukin-5 (\textit{Il5}), interferon-alpha2 (\textit{Ifna2}), CD40 antigen (\textit{Cd40}), chemokine (C-X-C motif) receptor 3 (\textit{Cxcr3}), GATA binding protein...
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![Figure 3](image3.png)

**Figure 3** Expression of soluble epoxide hydrolase (sEH) protein in gingival tissues. Density of the sEH bands was normalized to that of α-tubulin. Protein band intensity is represented as arbitrary units (mean ± SD). *P < .05 among the infected mice; the noninfected and infected groups were treated with 1 mg/kg of 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU). A.a, Aggregatibacter actinomycetemcomitans

![Figure 4](image4.png)

**Figure 4** Effects of treatment with 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU) on the expression of a panel of inflammatory genes on gingival tissue. Values are expressed as fold change in gene expression between Aggregatibacter actinomycetemcomitans-induced periodontal bone resorption in mice and those treated with TPPU. Negative values represent gene downregulation, and positive values represent gene upregulation. A 3-fold difference cut-off point was set. Apcs, serum amyloid P component; Ccr4, chemokine (C-C motif) receptor 4; Ccr6, chemokine (C-C motif) receptor 6; Ccr8, chemokine (C-C motif) receptor 8; Cd4, CD4 antigen; Cxcr3, chemokine (C-X-C motif) receptor 3; Gata3, GATA binding protein 3; Ifnα2, interferon-α2; Ifnβ, interferon-β; IFNγ, interferon-γ; Il5, interleukin-5; Il13, interleukin-13; Il23, interleukin-23; Lyz2, lysozyme 2; Mbl2, mannos-binding lectin (protein C) 2; Mpo, myeloperoxidase; Mx1, MX dynamin-like GTPase 1; Rag1, recombination activating gene 1; Stat4, signal transducer and activator of transcription 4; Tlr1, toll-like receptor 1; Tlr9, toll-like receptor 9

3 (Gata3), recombination activating gene 1 (Rag1), and MX dynamin-like GTPase 1 (Mx1). Figure 4 summarizes the genes that were affected by TPPU. It is worth highlighting that although expression of forkhead box P3 (Foxp3) was downregulated (by ~2.8-fold), this was only borderline statistically significant.

## 4 | DISCUSSION

Participation of the immune system in response to the aggressive growth and tissue damage stimulated by microorganisms is important because many of the immunological factors involved in response to the bacteria can induce a change in homeostasis and affect the alveolar bone.13

Studies have shown that EETs play a key role in the anti-inflammatory process and would have a beneficial effect in preventing bone loss caused by periodontal disease.10,14,15 EETs are rapidly metabolized by sEH and thus EETs have a short half-life, making natural EETs difficult to use pharmacologically. Therefore, TPPU was developed to increase the half-life of EETs and thus enhance their biological effects.16 As demonstrated, the A. actinomycetemcomitans-induced periodontal disease resulted in an increase in the expression of sEH which was abrogated when the mice were treated with TPPU. This is the first report demonstrating expression of sEH in
periodontal tissues and thus the importance of EET/sEH in the homeostasis of the periodontium.

The bacterial biofilm initiates periodontal disease, but destruction of the tissue in the periodontal lesions may occur because of the destructive processes mediated by the host’s immune response. Mannose binding lectin (MBL) was upregulated in mice treated with TPPU. This molecule stimulates the classical complement pathway as an opsonin and plays a role in the defense against invading microorganisms in periodontitis. Thus, MBL can recognize carbohydrate structures on the surface of a variety of microorganisms. In accordance with our findings in gingival tissue, the levels of MBL in plasma have been reported to increase during infections and inflammatory processes. The concentrations of MBL in plasma were not significantly different in moderate and severe periodontitis compared with controls, and MBL deficiency was not related to susceptibility for periodontitis. In our disease model, the immune response was provoked by inoculating A. actinomycetemcomitans; this may explain the dramatically increased expression of MBL.

Significant attention has been given to pattern recognition receptors similar to the toll receptors. TLRs 1 and 9 were significantly downregulated in the TPPU-treated mice. TLRs in periodontal disease play a key role in triggering the inflammatory response because they can mediate the release of inflammatory cytokines. Recently, TLR-2 was shown to form heterodimers with TLR-1 and TLR-6 that recognize triacylated lipopeptides from gram-negative bacteria. Interestingly, TLR-2/1 ligand induces osteoclastic bone resorption in mouse culture cells.

Human type I IFNs are produced and released by host cells in response to the presence of several pathogens. In inflammation, IFNs possess both pro- and anti-inflammatory functions, depending on the context of the particular pathology. Recently, an inhibitory role for type I IFNs in blocking IL-1β production has been reported. In this study, the IFN-α/p1 proteins are up-regulated, suggesting the importance of TPPU in controlling the inflammatory process in gingival tissue.

T helper 1 (Th1) cells secrete IL-2 and IFN-γ and there is strong evidence that the release of IFN-γ results in increased loss of alveolar bone during periodontal infections. Besides, high levels of IFN-γ in patients with periodontitis are related to the severity of the disease. Thus, the decreased expression of IFN-γ by treatment with TPPU may explain the decrease of bone resorption. In addition, TPPU also reduced the expression of receptors CD4 and CD8, and consequently lymphocytes.

The chemokine receptor, CCR, is expressed in pathological situations and is responsible for the development and function of the immune system during the inflammatory process. The administration of TPPU decreases the expression of CCR6, CCR4, and CCR8. Studies show that CCR6 is a specific marker for Th17 cells and regulatory T cells (Tregs). Furthermore, we demonstrated that treatment with TPPU also modulated the CXCR3 receptor. This receptor is responsible for guiding cells (mainly CD8+ T cells) out of the lymphoid compartment toward the sites of inflammation.

CCR4 and CCR8 are expressed predominantly in Th2 cells and Tregs. These two chemokines provide guidance to the T cells that express CCR4 so that they can reach the inflamed mucosa. CCL17 promotes vascular recognition and emigration of T cells that express CCR4 on the endothelial surface, while the CCL22 migration leader promotes expression of these same cells in mucosal tissue. CCR8 participates in induction and amplification of the inflammatory responses to pathogens or allergens recruiting effector memory T cells. Moreover, CCR8 participates in the recruitment of Tregs, contributing to the balance between effector and regulatory cells, and thus the outcome of the inflammatory response. Natural Tregs are CD4+ and CD25-FOXP3-expressing T cells that specifically regulate the activation, proliferation, and effector functions of activated conventional T cells and Tregs found in periodontal disease sites. Our results demonstrated that mice treated with TPPU statistically decreased the expression of CCR4 and CCR8 and, with borderline significance, FOXP3. It is important to note that the Treg influx was decreased in the gingiva of the treated mice. Previous results corroborate that immunomodulatory drugs, such as 15d-PGJ2, drive a shift to a rapid resolution of inflammation, which causes the natural decrease of Treg cells at the inflammatory site.

The interleukins IL-4, IL-5, and IL-13 are expressed by the gene GATA3 which is the only gene recognized by transcription factor expression in Th2 cells. The subtype, RAG1, is a gene involved in genetic recombination, in a process that leads to the organization of antigen receptor genes. This gene, along with Gata3, was suppressed by TPPU and demonstrates the importance of this pathway when you take into account that the adaptive immune system is characterized by the ability to form antigen-specific receptors. In the absence of GATA3, the differentiation of CD4+ is deficient before the CD4/CD8 interaction because thymocytes with deficiency of GATA3 and restricted to the major histocompatibility class II are redirected to the lineage of CD8+.

CD40 and CD40L were also downregulated in the mice treated with TPPU. CD40L is expressed on the surface of CD4+ cells and binds to CD40 on B cells to provide B-cell activation and differentiation and the production of antibodies against pathogens. The activated T and B lymphocytes in gingival tissue of patients with periodontitis express RANKL, as well as threonine synthase-like 2 (SOFAT), both major contributors to the activation of osteoclast cells. Besides, CD40 binding stimulates the production of cytokines by B cells, including IL-2, IL-6, IL-10, and TNF-α, some of which are essential in the development of periodontal disease.

In our study it was demonstrated that a classical standard Th2 cytokine, IL-5, was also decreased in the group of animals treated with TPPU. This cytokine, along with IL-6 and TNF-α, can promote survival of plasma cells in the presence of inflammation. The effects of IL-5 include the maintenance of survival and functions of B cells and eosinophils, and a lack of functional genes or receptors to IL-5 induce deficiencies in the development of these cells.

Interleukin-13, which is up-regulated in TPPU-treated mice, is another potent modulator of human monocyte/macrophage and B-cell function. Monocyte/macrophage major histocompatibility complex class II and several integrin molecules are up-regulated by
IL-13; however, the production of IL-1α, IL-1β, IL-6, IL-8, and TNF-α are inhibited by IL-13, suggesting an anti-inflammatory role.

The plasma protein serum amyloid P-component (SAP) reduces neutrophil adhesion, inhibits the differentiation of monocytes into fibroblast-like cells, and promotes phagocytosis of cell debris by macrophages. Together, these effects of SAP reduce important aspects of inflammation and fibrosis which help to explain the decreased bone resorption. sEH inhibitors have been shown, in multiple studies, to reduce inflammation and fibrosis.

In addition, it was observed that decrease of STAT4, a transcription factor for IL-12, IL-23, and IFN-1, leads to differentiation of Th1 and Th17 cells, as well as to the activation of monocytes, and stimulates the production of IFN-γ. By leading to the production of IFN-γ and IL-17, this transcription factor directs T cells to differentiate into Th1 cell lines. STAT4 in other cellular subtypes can also contribute to the progression of diseases as a result of the increase in inflammatory cytokines, prevention of apoptosis, presentation of antigens, or antibody production. The immunomodulation of this STAT is an important clue of the effect of TPPU.

Another aspect considered in our study was the decreased expression of the Ly22 and Mx1 genes in the mice treated with TPPU. The unbalanced patterns of Ly22 lead to increased bacterial load and consequently to persistent inflammation. Although not very clear in the literature, the Mx1 gene is related to inflammatory disease mediated by T cells.

Taken together, our results demonstrate that inhibition of sEH by TPPU may contribute to control of the inflammatory process by modulating cell activation and influx as well as the production of specific cytokines, suggesting that inhibition of sEH could be a therapeutic approach for periodontal disease treatment and bone loss and other inflammatory conditions.

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REFERENCES