Dual Role of 5-Lipoxygenase in Osteoclastogenesis in Bacterial-induced Apical Periodontitis

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Abstract

Introduction: The aim of this study was to evaluate the role of 5-lipoxigenase (5-LO) in the signaling for osteoclast formation and bone resorption in apical periodontitis (AP) after root canal contamination with oral bacteria. **Methods:** AP was experimentally induced in C57BL/6 mice because of contamination of the root canals left open to the oral environment. MK886 was used as a systemic inhibitor of 5-LO (5 mg/kg, daily). After 7, 14, 21, and 28 days, the animals were euthanized, and tissues were removed for gene evaluation by quantitative reverse transcriptase polymerase chain reaction, histologic analysis, and tartrate-resistant acid phosphatase staining. **Results:** Root canal contamination induced the expression of messenger RNA for 5-LO and leukotriene B4 receptors BLT1 and BLT2. The administration of the 5-LO inhibitor reduced early receptor activator of nuclear factor kappa-B and receptor activator of nuclear factor kappa-B ligand synthesis but augmented late receptor activator of nuclear factor kappa-B ligand and osteoprotegerin expression during the course of AP development. Interestingly, long-term inhibition of 5-LO resulted in increased bone resorption and induced tartrate-resistant acid phosphatase–positive osteoclast formation. The divergent findings related to 5-LO inhibition in osteoclastogenesis signaling, osteoclast formation, and bone resorption were accompanied by differently regulated inflammatory gene expression. *Il1b*, *Il11*, *Ccl3*, *Ccl7*, and *Spp* were down-regulated by the 5-LO inhibitor in early AP, but later on *Il11*, *Ccl3*, *Cxcl9*, *Cxcl15*, and *Spp* were up-regulated. **Conclusions:** 5-LO presented a dual role in osteoclastogenesis during the course of AP development. Early on, osteoclastogenesis signaling was down-regulated by the inhibition of 5-LO, but long-term inhibition failed to prevent synthesis of catabolic mediators that resulted in increased bone loss. *(J Endod 2016;42:447–454)*

Key Words

5-lipoxygenase, apical periodontitis, bone resorption, cytokines, osteoclast, osteoclastogenesis

Apical periodontitis (AP) represents a localized immune response against the microorganisms inside the dental root canal (1, 2) and is characterized by the presence of a mixed inflammatory infiltrate composed of lymphocytes, neutrophils, macrophages, and plasma cells depending on the stage of the disease (2, 3). An uncontrolled inflammatory response results in periapical bone resorption (2), albeit the mechanisms involved in the recruitment of hematopoietic lineage cells to differentiate into osteoclasts during development of the disease are not fully understood (4, 5).

Autocrine and paracrine signaling of cytokines and chemokines are important for osteoclast maturation and activity (6, 7). Receptor activator of nuclear factor kappa-B ligand (RANKL) is a soluble mediator; is a member of the tumor necrosis factor superfamily; is synthesized by osteoblasts, T lymphocytes, and endothelial cells; and binds to receptor activator of nuclear factor kappa-B in osteoclastic cells to promote tartrate-resistant acid phosphatase (TRAP) enzyme expression and bone resorption (4, 8). Osteoclastogenic signaling mediated by RANKL is blocked by the soluble decoy receptor osteoprotegerin (OPG) produced by osteoblasts under anabolic stimuli (8).

In an AP scenario, several inflammatory mediators are produced locally to orchestrate an immune response. Eicosanoids are among those molecules and represent a class of lipid mediators synthesized from arachidonic acid through the action of cyclooxygenases or lipoxygenases to generate prostaglandins and thromboxanes or leukotrienes and lipoxins, respectively (9). Lipoxygenase metabolites have been found in rat inflamed dental pulp and human AP (10–13), but only leukotriene B4 (LTB_4) is positively correlated to polymorphonuclear cell recruitment and pain (10, 11). LTB_4 binds G-coupled receptors (leukotriene B4 receptor 1 [BLT1] and leukotriene B4 receptor 2 [BLT2]), resulting in an increase in intracellular calcium and a reduction of cyclic adenosine monophosphate (cAMP) to mediate kinase activation, genic transcription, and, ultimately, cell recruitment (14, 15). Despite the fact that lipid mediators are produced in response to an infection in AP, the role of the 5-lipoxygenase pathway in disease severity and associated bone loss is not known.

Therefore, the aim of this study was to evaluate the role of 5-lipoxygenase in the signaling for osteoclast formation and bone resorption in AP after root canal contamination with oral bacteria.

Materials and Methods

Animals

C57BL/6 6-week-old male mice (*Mus musculus*, $n = 96$) were used for experimentation after institutional review board approval (#12.1.62.53.0). Animals were

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anesthetized intramuscularly with ketamine hydrochloride (150 mg/kg [Ketamine 10%; National Pharmaceutical Chemistry Union Agener S/A, Embu-Guaçu, Brazil]) and xylazine (7.5 mg/kg [Dopaser, Labs Calier S/A, Barcelona, Spain]).

Operative Procedures

Animals were placed on a surgical table with a device for mandibular retraction. The upper and lower first molars of each animal were used; the right side was used for root canal contamination, and the left side was used as the control.

Occlusal root canal accesses were gained with 1011 spherical diamond burs (KG Sorensen IND. com Ltda, Barueri, SP, Brazil), root canals were located with a #06 K-file (Les Fils d' Auguste Maillefer S/A, Ballaigues, Switzerland), and the radicular pulp tissue was removed. Then, the root canals were left open to the oral environment for 7, 14, 21, and 28 days (n = 6 teeth per group per period). Experiments were repeated twice.

5-Lipoxygenase Pharmacologic Inhibition

MK886, a 5-lipoxygenase activator protein inhibitor (Merck Frosst Inc, Kirkland, QC, Canada), was dissolved in alcohol (100 μL), diluted in distilled water (400 μL), and administered by gavage (0.5 mL, 5 mg/kg body weight) 1 hour before root canal contamination and daily throughout the experimental period. Control animals received the vehicle only.

Total RNA Extraction

Animals were euthanized by intramuscular anesthetic overdose; then, tissues containing bone and tooth from the periapical area were dissected, collected, and snap frozen. RNA was extracted from a pool of 3 teeth using the RNeasy Mini kit (RNeasy Mini; Qiagen Inc, Valencia, CA) and samples treated with DNAse I (RNase-Free DNase Set, Qiagen Inc) according to manufacturer protocol. RNA integrity was analyzed using 1% agarose electrophoresis, and quantity was estimated using the NanoDrop 1000 (Thermo Fisher Scientific Inc, Wilmington, DE) at 260-ηm wavelength.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Complimentary DNA (cDNA) was synthesized from 700 ng total RNA using random primers (High Quality cDNA Reverse Transcriptase Kits; Applied Biosystems, Foster City, CA). Aliquots of 2 μL of the total cDNA were amplified by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) using primers for *Alox5* (Mm01182747), *Ltb4r1* (Mm02619879), *Ltb4r2* (Mm01321172), *Tnfrsf11a* (Mm00437135), *Tnfsf11* (Mm00441906), and *Tnfrsf11b* (Mm01205928) (TaqMan Gene Expression Assay, Applied Biosystems) in an Eppendorf Mastercycler ep Realplex (Eppendorf AG, Hamburg, Germany). *Gapdh* (Mm99999915) was used as reference gene. qRT-PCR reactions were performed in duplicate, and amplification was done under the following conditions: denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative quantification was performed using the ΔΔCt method.

Morphometric Analysis of AP Size under Light Microscopy

Longitudinal tooth cuts were obtained, and analysis was performed in hematoxylin-eosin–stained sections using the microscope at 10× magnification in the bright field. In each specimen, the size of the periapical lesion was delineated in the section representing the most central part of the lesion, and the area was determined in μm^2 using Leica Application Suite V3.8 software and the Leica DM 5000B microscope (Leica Microsystems, Wetzlar, Germany). Delineation was performed excluding the intact tooth and bone structures (periodontal ligament, cementum, and alveolar bone).

TRAP Histoenzymology

Analysis of TRAP activity was performed as an indicator of active osteoclasts. The sections were deparaffinized in xylol, ethanol, and ethanol/acetone and left to dry at room temperature. Next, a solution was prepared with 10 mL acetic acid buffer, 0.1 mL N-N-dimethylformamide, 5 mg Fast Red Violet LB Salt (Sigma-Aldrich, St Louis, MO), and 1 mg Naphthol AS-BI phosphoric acid (Sigma-Aldrich) and then pipetted onto the sections that were maintained in a dark chamber at 37°C for 30 minutes. After incubation, the sections were counterstained with hematoxylin for 1 minute and examined with the microscope in the bright field. Quantitative analysis of the number of osteoclasts was determined by counting the number of multinucleate TRAP-positive cells in the resorption lacunae in direct contact with the alveolar bone around the periapical lesion. The results were expressed as the number of cells per lesion.

qRT-PCR Array

Global evaluation of genes involved in the inflammatory response was performed using a commercially available qRT-PCR array (Mouse inflammatory cytokines and receptors RT^2 Profiler PCR Array, PAMM-011A; Qiagen Inc). Total RNA was amplified starting with 300 ng using a commercially available kit (PreAMP cDNA synthesis, Qiagen Inc). *Gusb*, *Hprt*, *Hsp90ab1*, *Actb*, and *Gapdh* were used as reference genes. qRT-PCR reactions were performed in duplicate in an Eppendorf Mastercycler ep Realplex (Eppendorf AG) using SYBR Green (Qiagen Inc). Amplification was done under the following conditions: denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The dissociation curve was performed to determine the specificity of the primers considering the melting temperature of the amplicon under the following conditions: increasing the temperature to 95°C for 15 seconds followed by decreasing the temperature to 60°C for 15 seconds, gradually increasing the temperature to 95°C for 20 minutes, and maintaining the temperature at 95°C for 15 minutes. Relative quantification was performed using the ΔΔCt method.

Statistical Analysis

Data were analyzed using 1-way analysis of variance followed by the Dunnett test for comparison of gene expression over time in teeth with and without AP or using 2-way analysis of variance followed by the Bonferroni test for comparison of the effects of 5-lipoxygenase inhibition on AP development over time (α = 0.05).

Results

Root Canal Contamination Induces 5-LO, BLT1, and BLT2 Gene Expression and Administration of 5-LO Inhibitor Reduced Early RANK and RANKL Synthesis but Augmented Late RANKL and OPG Expression in AP

Root canal contamination induced 5-lipoxygenase (*Alox5*), LTB_4 receptor 1 (*Ltb4r1*), and LTB_4 receptor 2 (*Ltb4r2*) gene expression compared with healthy teeth at 14 days after AP induction. At 7, 21, and 28 days, *Alox5* and *Ltb4r2* were not modulated by root canal bacterial contamination, whereas *Ltb4r1* expression was inhibited compared with healthy teeth (Fig. 1*A–C*).

Because 5-lipoxygenase and LTB_4 receptors were induced by root canal contamination, we sought to investigate the functional role of 5-

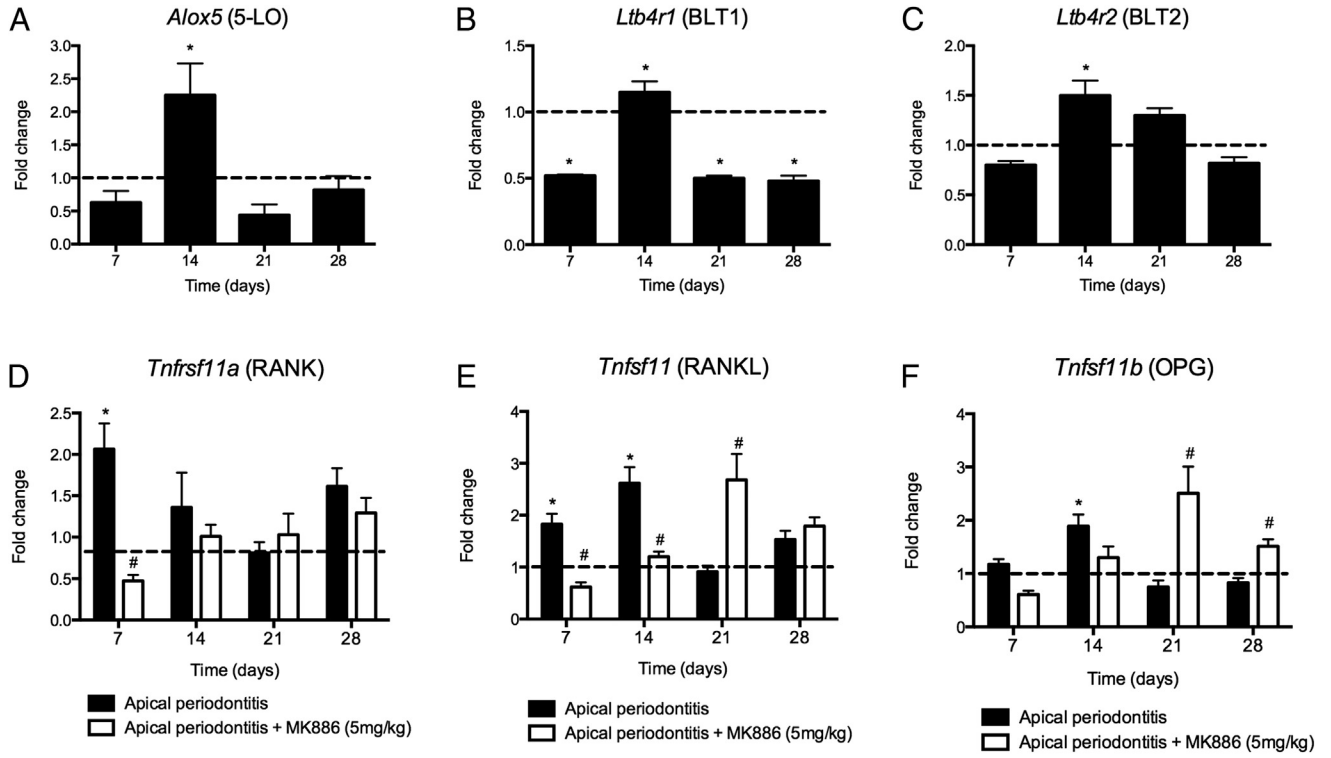

Figure 1. (*A–C*) Expression of mRNA for 5-lipoxygenase (5-LO, *Alox5*), leukotriene B_4 receptor 1 (BLT1, *Ltb4r1*), and leukotriene B_4 receptor 2 (BLT2, *Ltb4r2*) after root canal contamination with oral bacteria for 7, 14, 21, and 28 days. (*D–F*) The effects of 5-LO inhibition on osteoclastogenesis signaling (RANK, *Tnfrsf11a*; RANKL, *Tnfsf11*; OPG, *Tnfsf11b*) were investigated using a pharmacologic inhibitor of the 5-LO activating protein (MK886, 5 mg/kg). The *dashed line* indicates gene expression in teeth without AP. Data were normalized using *Gapdh* as the reference gene. The graphs depict the mean and standard error mean. $^{*}P < .05$ compared with teeth without AP. $^{\#}P < .05$ compared with teeth with AP without MK886 administration.

lipoxygenase in AP. We observed that 5-lipoxygenase is involved in the signaling for osteclastogenesis because inhibition of 5-lipoxygenase activity reduced the expression of *Tnfsf11* (RANKL) and *Tnfrsf11a* (RANK), a signaling that prevents osteoclastogenesis. Interestingly, messenger RNA (mRNA) for RANKL was augmented later on, at 21 days, accompanied by higher OPG expression (Fig. 1*D–F*). The ratio of *Tnfsf11* (RANKL)/*Tnfsf11b* (OPG) was higher from 7 to 28 days during AP development, and upon MK886 it remained similar to healthy teeth (Supplemental Figure S1 is available online at www.jendodon.com).

Inhibition of 5-LO Results in Larger AP and Increased TRAP-positive Osteoclast Formation

To clarify the effects of osteclastogenesis signaling modulated upon 5-lipoxygenase inhibition in AP, we examined the course of AP development and osteoclast formation. At 7 days, an increase in periodontal ligament thickness was observed, and then at 14 days inflammatory cells were recruited to the periapical area. At 21 days, bone resorption was initiated and became more evident at 28 days. The inhibition of 5-lipoxygenase did not impact bone resorption up to 21 days; however, an increase in bone loss was detected at 28 days compared with animals with AP that did not receive MK886 (Fig. 2*A–J*).

Because osteoclasts are responsible for bone resorption, we performed TRAP histoenzymology to detect these cells within AP. We observed a time-dependent increase in TRAP-positive cells during AP development initiated at 14 days after root canal contamination and remaining higher than teeth without AP after that. 5-LO inhibition for 21 and 28 days induced a higher number of TRAP-positive osteoclast formations compared with teeth with AP without MK886 administration (Fig. 3*A–H*). Taken together, our findings show that 5-LO is involved in canonic RANK-RANKL-OPG osteoclastogenesis signaling, but 5-LO inhibition did not prevent osteoclast formation and periapical bone resorption.

5-LO Inhibition Differently Regulates Inflammatory Gene Expression in Early versus Late AP Development

To further explore the divergent findings related to 5-LO inhibition in canonic osteoclastogenesis signaling, osteoclast formation, and bone resorption, we took advantage of a broad analysis of genes involved in the inflammatory response to investigate other molecules that would be involved in bone metabolism.

We observed that several mRNAs for cytokines and chemokines were up-regulated in AP including interleukin (IL)-1β (*Il1b*), IL-11 (*Il11*), CCL3 (*Ccl3*), CCL7 (*Ccl7*), and CXCL9 (*Cxcl9*). *Spp*, a mediator of osteoclast function that encodes osteopontin (OPN), was also induced. Upon administration of MK886, a dual response was observed in early versus late AP development. Early on, MK886 inhibited mRNA for IL-1β (*Il1b*), IL-11 (*Il11*), CCL3 (*Ccl3*), CCL7 (*Ccl7*), and OPN (*Spp*). However, after 21 and 28 days, MK-886 administration stimulated IL-11 (*Il11*), CCL3 (*Ccl3*), CXCL9 (*Cxcl9*), CXCL15 (*Cxcl15*), and OPN (*Spp*) (Fig. 4*A–G*). Detrimental regulation of cytokines, chemokines, and osteopontin upon 5-LO inhibition in early versus late AP development corroborate with divergent RANKL and OPG osteoclastogenic signaling, suggesting possible mechanisms involved in dual role of 5-LO in apical periodontitis.

Discussion

Here we show that 5-LO presents a role in osteoclastogenesis signaling against bacteria from dental root canal during bone loss in AP development. Interestingly, 5-LO and its metabolites seem to induce

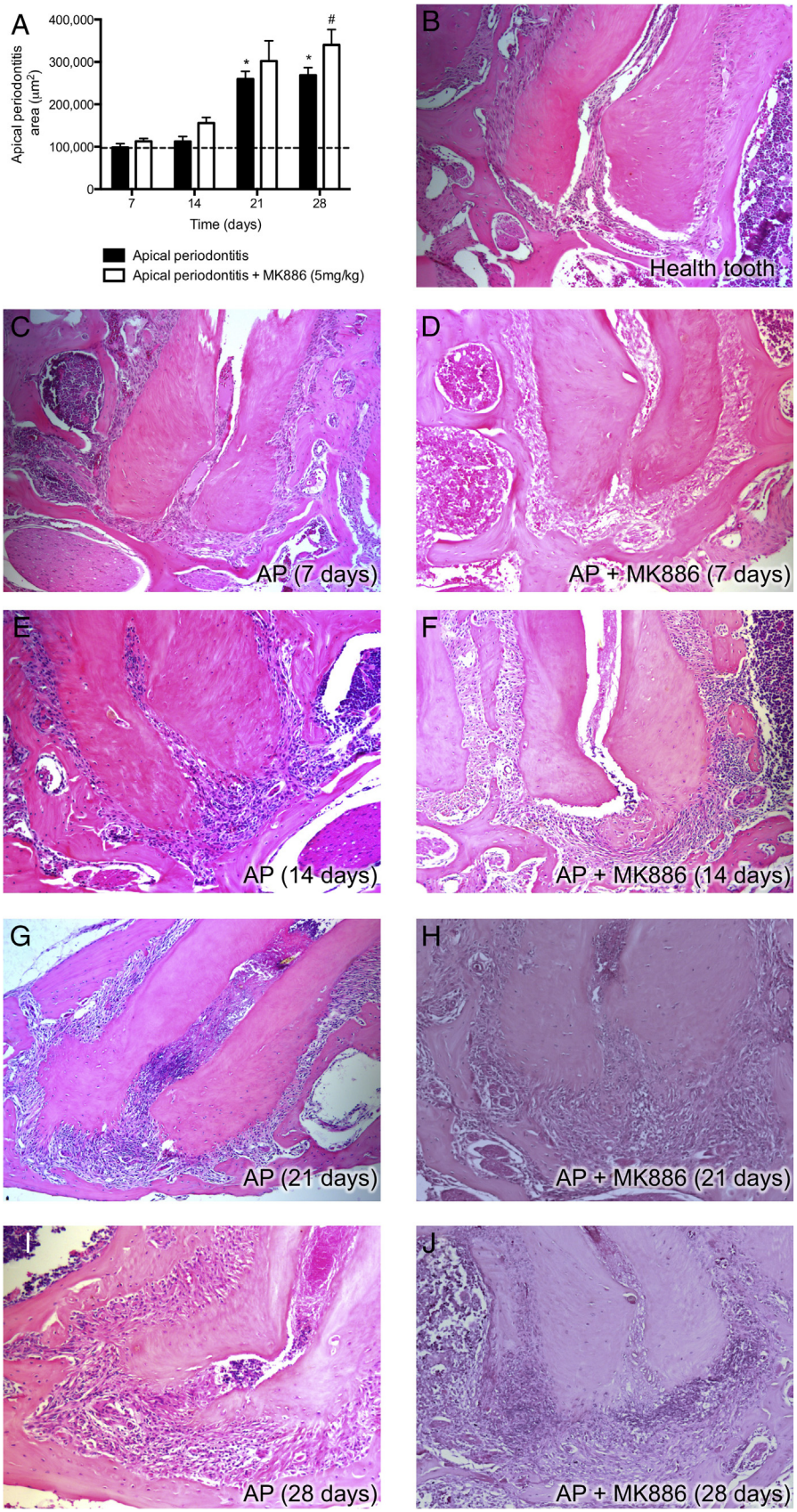

Figure 2. AP measured in histologic sections after root canal contamination with oral bacteria for 7, 14, 21, and 28 days with or without the 5-LO inhibitor (MK886, 5 mg/kg). The graph depicts mean and standard deviation, and the *dashed line* represents the periodontal ligament area in teeth without AP. $^{*}P < .05$ compared with teeth without AP. $^{\#}P < .05$ compared with teeth with apical periodontitis without MK886 administration (*A*). Representative photomicrographs from (*B*) healthy teeth, teeth with apical periodontitis at (*C*) 7, (*E*) 14, (*G*) 21, and (*I*) 28 days or teeth with AP and MK886 administration for (*D*) 7, (*F*) 14, (*H*) 21, and (*J*) 28 days are presented. (*B–J*) Hematoxylin-eosin, magnification 10×.

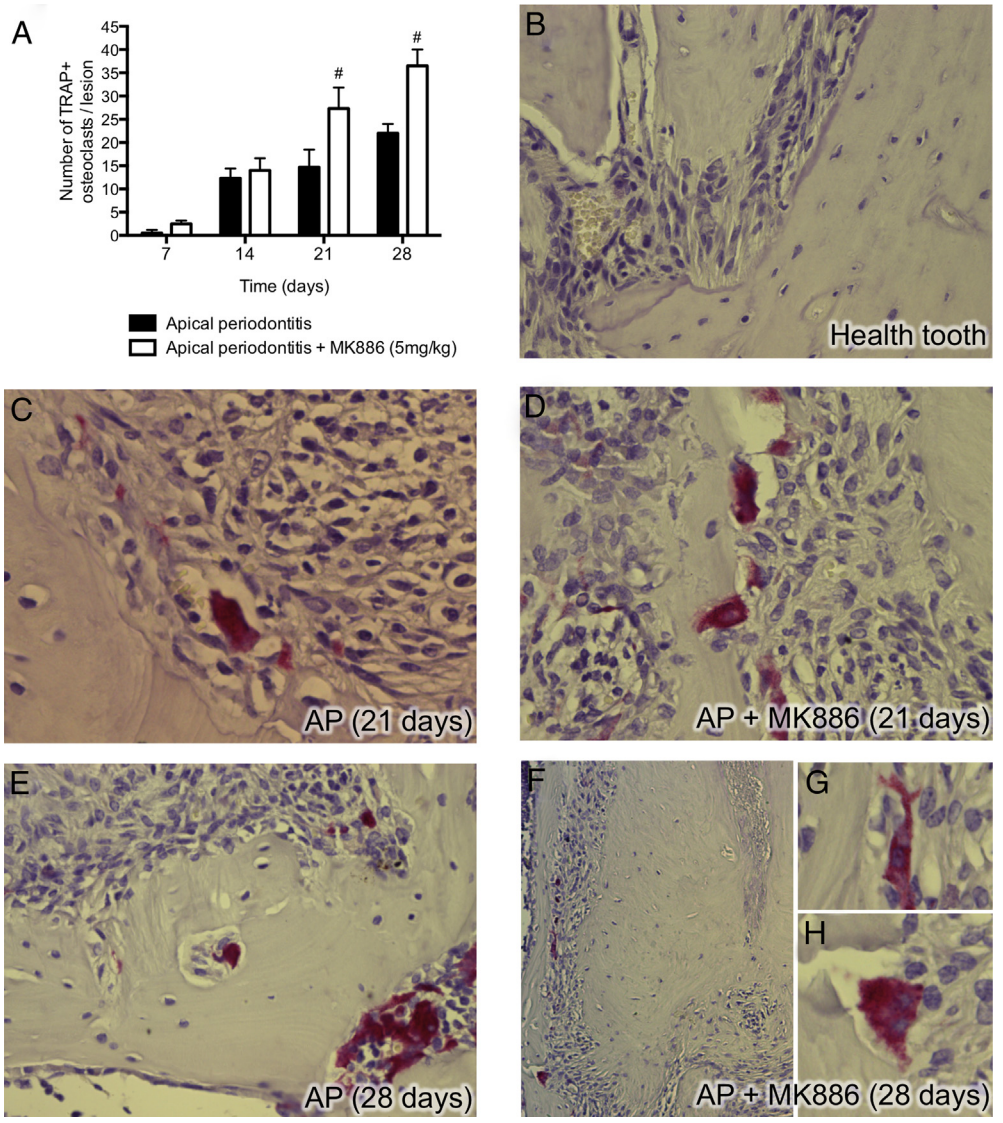

Figure 3. TRAP-positive osteoclasts were counted in histologic sections after root canal contamination with oral bacteria for 7, 14, 21, and 28 days with or without the 5-LO inhibitor (MK886, 5 mg/kg). The graph depicts the mean and standard deviation. $^{\#}P < .05$ compared with teeth with AP without MK886 administration (*A*). Representative photomicrographs from (*B*) healthy teeth, teeth with AP at (*C*) 21 and (*E*) 28 days or teeth with AP and MK886 administration for (*D*) 21 and (*F–H*) 28 days are presented. (*B*, *E*, and *F*) Magnification 20×, (*C* and *D*) 40×, and (*G* and *H*) 63×.

osteoclastogenesis signaling in early AP; however, later on, its inhibition augmented late synthesis of RANKL and other cytokines and chemokines that culminate with enhanced bone loss.

5-LO enzyme has been imunohistochemically shown in fibroblasts, osteoclasts, and endothelial cells during callus formation after fracture healing in mice (16). Treatment with pharmacologic inhibitors of 5-LO accelerates femoral fracture healing and enhanced bone regeneration (17, 18). Those findings might be explained by the fact that exogenous or endogenous LTB_4 inhibits osteoblast cell proliferation (19) and bone formation *in vitro* (20) and stimulates osteoclasts to resorb calcified matrices *in vitro* (21, 22) and murine calvaria *in vivo* (23).

The mechanism involved in osteoclast formation induced by RANKL via 5-LO has been shown (24). The BLT1 receptor but not BLT2 presents a distinctive role in osteoclastic activity during bone loss after ovariectomy or lipopolysaccharide intraperitoneal injection (25), suggesting 5-LO as a potential therapeutic strategy for treating bone diseases. Our results after 7 days of inhibition of 5-LO corroborate with that, but after long-term inhibition an exacerbation of catabolic effects on bone was observed. One possible explanation for that is the time-dependent invasion of bacteria from the oral cavity through the root canal system that might shift the course of the inflammatory response because of the paradoxic relationship of infection, inflammation, and bone remodeling (26). Increased expression of mRNA for BLT2 was observed at 14 days upon root canal contamination, whereas mRNA for BLT1 was inhibited at 21 and 28 days, indicating that downstream signaling would be different in our model compared with previous studies. Indeed, BLT1 and BLT2 receptors present nonredundant roles in inflammatory arthritis progression (27).

The ratio of Tnfsf11 (RANKL)/Tnfsf11b (OPG) was higher from 7 to 28 days during AP development, and MK886 administration changed this pattern to a physiological expression but it did not prevent bone loss. Therefore, we also investigated other genes involved in the inflammatory response to shed light on detrimental findings related to 5-LO inhibition in osteoclastogenesis signaling and bone resorption. We observed that mRNAs for IL-1β, IL-11, CCL3, and

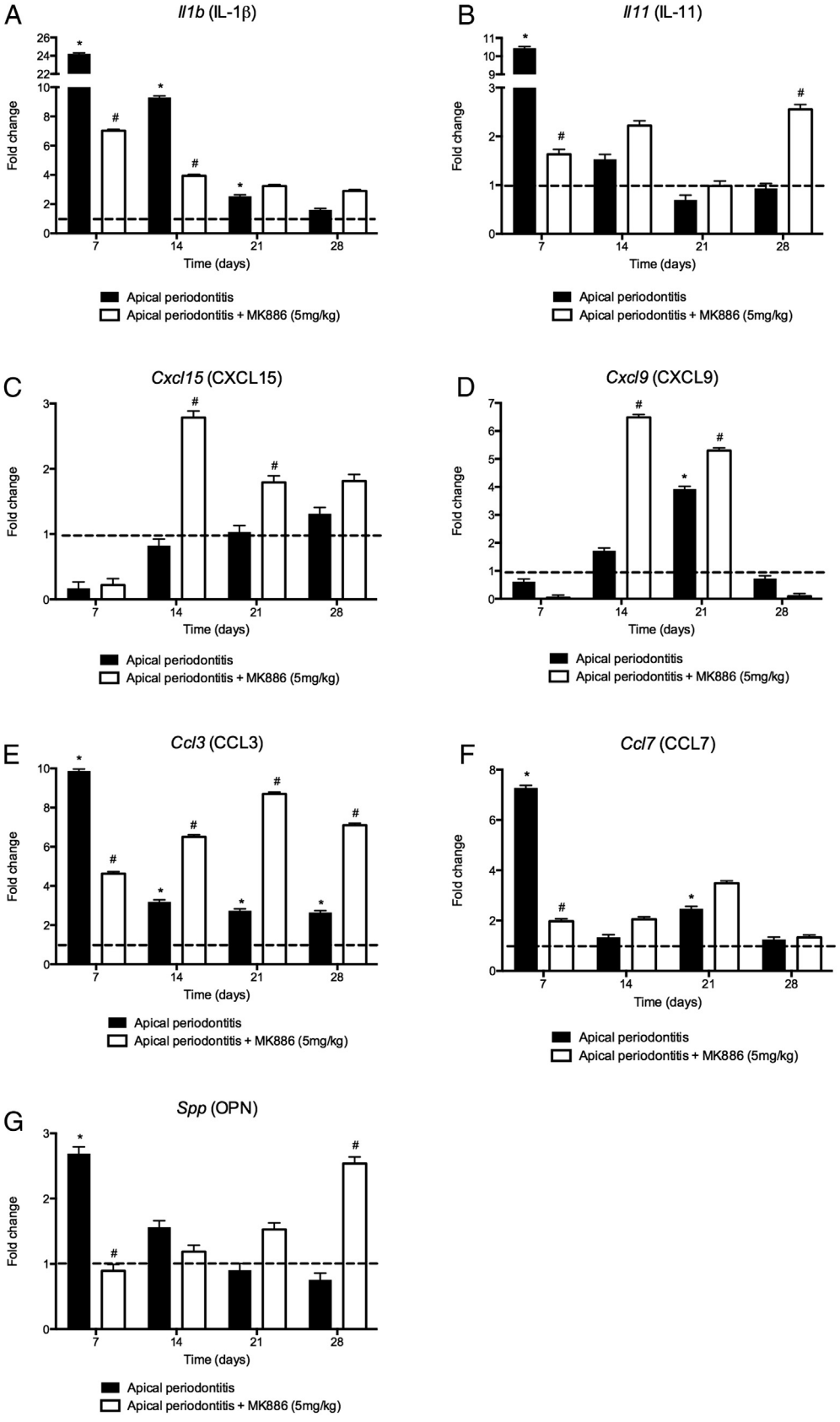

Figure 4. The expression of mRNA for IL-1β (*Il1b*), IL-11 (*Il11*), chemokine (CXC motif) ligand 15 (CXCL15, *Cxcl15*), chemokine (CXC motif) ligand 9 (CXCL9, *Cxcl9*), chemokine (CC motif) ligand 3 (CCL3, *Ccl3*), chemokine (CC motif) ligand 7 (CCL7, *Ccl7*) and osteopontin (OPN, *Spp*) after root canal contamination with oral bacteria for 7, 14, 21, and 28 days. (*A–G*) The effects of 5-LO inhibition were investigated using a pharmacologic inhibitor of 5-LO activating protein (MK886, 5 mg/kg). The dashed line indicates gene expression in teeth without AP. Data were normalized using *Gusb*, *Hprt*, *Hsp90ab1*, *Actb*, and *Gapdh* as reference genes. The graphs depict the mean and standard error of the mean. $^{*}P < .05$ compared with teeth without AP. $^{\#}P < .05$ compared with teeth with AP without MK886 administration.

CCL7 were inhibited under MK886 administration for 7 days, and those cytokines are involved in osteoclast formation (6, 28, 29). Late induction of mRNA for IL-11 by MK886 observed here might explain the increased osteoclast formation at 21 and 28 days, probably because of the maintenance and development of osteoclast progenitor cells by this cytokine (28). *Ccl3*, a chemokine known for stimulating chemotaxis of osteoclast precursors and RANKL-mediated differentiation (6), was also up-regulated upon 5-LO inhibition from 14 to 28 days. *Cxcl9* and *Cxcl15*, induced by MK886 administration, promote osteoclast adhesion, migration, and differentiation under the influence of several cytokines such as IL-6, tumor necrosis factor alpha, and IL-1α (30–32). Of note, it has been shown that osteopontin (Spp) is important to mediate bone resorption and is involved in the regulation of immune responses such as Th1/Th2 balance, leukocyte recruitment, and dendritic cell function (33, 34). Here, we observed that MK886 inhibited Spp expression at 7 days and stimulated expression at 28 days, which correlates with higher osteoclast formation observed in histologic sections. In bone tissue, the role of osteopontin has been detrimental because it has largely been thought of as the protein that promotes the bridge between osteoclast and the bone matrix (35) but might also prevent a protective effect on endodontic polymicrobial infection, affecting phagocyte recruitment and persistence at the sites of infection (36). Two important limitations of our investigation is that first, we cannot point out the reason for this change in the effects mediated by MK886 if we consider early versus late disease development, and second, the mediators presented here correlate with the phenomena of osteoclast formation and bone resorption, but a cause-effect relationship has not been shown. Therefore, we recommend that further long-term investigation should be conducted on the effects of the 5-LO inhibitor on the dynamic interplay between bone and immune system before using this medication for treatment purposes.

The main role of LTB_4 in the inflammatory response is the recruitment of inflammatory cells, directly or through induced secretion of mediators at the inflammatory milieu to promote the host defense against pathogens (37). Another explanation for the divergent effects observed here is the fact that LTB_4 modulates phagocytosis and killing of microorganisms (15, 37), and its long-term inhibition might have impaired infection control resulting in bone loss. Because we have shown that the early inhibition of 5-LO impacts osteoclastogenic signaling, further studies should be conducted to investigate if clinical removal of microorganisms in combination or associated with antimicrobial agents would prevent bone loss in AP.

Another limitation of this study was the use of histologic investigation instead of micro–computed tomographic imaging to measure the area of AP. Previous investigations have shown the superiority of micro–computed tomographic imaging over conventional histology regarding the possibility of analyzing several planes (axial, coronal, or sagittal) and obtaining volumetric measurements (38, 39). Nevertheless, our findings show that 5-lipoxygenase presents a dual role in osteoclastogenesis during the course of AP development. Early on, osteoclastogenesis signaling is down-regulated by the inhibition of 5-LO, but long-term inhibition fails to prevent the synthesis of catabolic mediators that results in increased bone loss.

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The authors deny any conflicts of interest related to this study.

Supplementary Material

Supplementary material associated with this article can be found in the online version at www.jendodon.com (http://dx.doi.org/10.1016/j.joen.2015.12.003).

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