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Effects of 5-lipoxygenase gene disruption on inflammation, osteoclastogenesis and bone resorption in polymicrobial apical periodontitis

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ABSTRACT

Objectives: To investigate the regulation of inflammatory and osteoclastogenic signaling by 5-lipoxygenase (5-LO) in apical periodontitis induced by oral contamination of dental root canals in mice.
Design: Apical periodontitis was induced in 5-lipoxygenase enzyme knockout (129-Alox5^{tm1Fun}) and 129 wild-type mice (n = 96) by exposure of the dental root canal to the oral cavity. After 7, 14, 21, and 28 days, the animals were euthanized and the tissues removed (n = 12 teeth per period) for histopathological and histometric analyses (hematoxylin and eosin [HE]), evaluation of osteoclastogenic activity (tartrate-resistant acid phosphatase enzyme [TRAP]), and determination of inflammatory and osteoclastogenic signaling (qRT-PCR).
Results: Oral contamination of dental root canals induced recruitment of neutrophils and osteoclasts to the periodontal ligament, resulting in bone resorption. Absence of 5-LO did not impair neutrophil recruitment while osteoclastic formation was increased. Nonetheless, early bone resorption progressed similarly to lesions in wild-type animals. Interestingly, in the absence of 5-LO, the synthesis of mRNAs for cytokines, chemokines, and their receptors was significantly reduced while that of regulators of osteoclastogenesis (RANK, RANKL, and OPG) was increased in comparison with the corresponding levels in wild-type animals.
Conclusions: The 5-LO pathway plays a role in the stimulation of inflammatory mediator synthesis and inhibition of osteoclastogenesis in apical periodontitis in mice. However, the paradoxical inflammatory-osteoclastogenic signaling did not impair inflammatory cell recruitment and bone resorption during early development of the disease.

1. Introduction

The progression of tissue necrosis toward the apical region, concomitant with contamination of dental root canals, leads to the recruitment of inflammatory cells to periapical tissues (Martón & Kiss, 2014). In human teeth with apical periodontitis, microorganisms that invade dental root canals diffuse widely through the canal system and reach the cementum reabsorption areas, either planktonic or extensive microbial biofilms (Leonardo, Rossi, Silva, Ito, & Bonifácio, 2002; Rocha et al., 2008). The contamination of the root canals initially generates an inflammatory response, and if not controlled, it results in the degradation of the components of the extracellular matrix and resorption of mineralized bone tissue (Martón & Kiss, 2014).

The recruitment of inflammatory cells to the apical and periapical regions is caused by the local release of chemotactic factors (chemokines) and cytokines such as interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α) (Silva, Garlet, Fukada, Silva, & Cunha, 2007; Wang & Stashenko, 1991, 1993). In the presence of periapical inflammatory infiltrates, common response patterns can be generated, such as those mediated by T helper 1 (Th1) and T helper 2 (Th2) cells. The former is characterized by the production of interleukin-2 (IL-2), interleukin-12 (IL-12), and interferon gamma (INF-γ), while the latter is characterized by the synthesis of interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-10 (IL-10), and interleukin-13 (Araujo-Pires et al., 2014; Brito et al., 2012; Carvalho Fraga et al., 2013; De Rossi, Rocha, & Rossi, 2008; Teixeira-Salum et al., 2010; Yamasaki et al., 2006). Several

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microorganisms, including bacteria, induce the recruitment of Th1 cells, which produce IFN-γ, the cytokine responsible for activation of macrophages to perform phagocytosis and microbial killing (Bambirra et al., 2015; Campos et al., 2013).

Among the local inflammatory mediators, leukotrienes are widely recognized for their biological functions, including efficient chemotactic activity, polymorphonuclear cell degranulation, induction of phagocytosis as well as stimulation of leukocyte adhesion in the endothelial vessel wall for transmigration of the inflammatory cell infiltrate (Boudreau et al., 2011; Leite et al., 2009; Le Bel, Brunet, & Gosselin, 2014; Medeiros et al., 2008; Peters-Golden & Henderson, 2007; Pidgeon et al., 2007; Rådmark & Samuelsson, 2009, 2010; Secatto et al., 2014; Singh, Gupta, Dastidar, & Ray, 2010; Thomas & Puleo, 2011; Wymann & Schneiter, 2008; Zoccal et al., 2016). Leukotrienes are synthesized by the activation of the 5-lipoxygenase (LO) enzyme by the 5-LO activating protein (FLAP). Initially, the substrate derived from the cell membrane phospholipids is converted to 5-hydroperoxyalkidonate (5-HPETE) and subsequently to leukotriene A_4 (LTA_4). LTA_4 in turn can be converted to leukotriene B4 (LTB_4) by the action of the enzyme LTA_4-hydrolase or converted to cysteinyl leukotriene C4 (LTC_4) after addition of glutadione to LTA_4 by the enzyme LTC_4-synthase. LTB_4 and LTC_4 are secreted and the latter can be converted to LTD_4 and LTE_4 by sequential hydrolysis of amino acids (Murphy & Gijón, 2007; Rådmark, Werz, Steinhilber, & Samuelsson, 2015). Leukotrienes act in autocrine signaling either by inducing production of cytokines or in neighboring cells in paracrine signaling in order to regulate immune response. Upon binding to a subfamily of G protein-coupled receptors, these mediators activate cascades of kinases, leading to diverse cellular events ranging from cellular mobility to gene transcription. (Kanaoka & Boyce, 2004; Tager & Luster, 2003; Zoccal et al., 2014, 2015).

We had demonstrated that the inhibition of 5-LO by a drug (MK-886) has a dual role in osteoclastogenic signaling in apical periodontitis, inhibiting the formation of osteoclasts in early periods, but stimulating them later on (Paula-Silva, Petean, da Silva, & Faccioli, 2016). Then, the effect of 5-LO in periapical bone loss was further investigated using 5-LO-deficient animals infected with *Fusobacterium nucleatum* (Wu, Sun, Yang, Liu, & Wang, 2018). The periapical lesion area, measured at 21 days following infection with *F. nucleatum,* was larger in 5-LO-deficient mice than in the wild-type littermates, indicating exacerbated bone loss. However, the effect of 5-LO impairment on the kinetics of periapical inflammation and bone resorption deserves investigation in 5-LO-deficient mice by using oral contamination of root canals as an infection model. Therefore, the purpose of this study was to investigate the role of the 5-LO pathway in the regulation of inflammatory mediators, osteoclastogenic signaling, and periapical bone resorption induced by oral contamination of the root canals in 5-LO knockout and wild-type mice.

2. Materials and methods

2.1. Animals

The protocols used in the present study were approved by the Ethics Committee on Animal Use, from the Ribeirão Preto Campus, University of São Paulo (USP) (process# 12.1.60.53.8). We used 48 knockout mice for the 5-LO enzyme (129-Alox5^{tm1Fun}; 129-Alox5$^{-/-}$; The Jackson Laboratory, Bar Harbor, ME, USA) and 48 129 wild-type 6–8-week-old littermates. For the operative procedures, the animals were anesthetized with ketamine hydrochloride (150 mg/kg, ketamine 10 %, Agener União Química Farmacêutica Nacional S/A, Embu-Guaçu, SP) and xylazine (7.5 mg/kg, Dopaser, Laboratorios Calier S/A, Barcelona, Spain).

2.2. Induction of apical periodontitis

Apical periodontitis was induced in mice placed on a surgical table with a device for mandibular retraction that allowed immobilization of the animals and maintenance of the open mouth for access to the molars. Coronary opening was performed by the occlusal face in the lower and upper right first molars using a 1011spherical diamond tip (KG Sorensen Ind. Com. Ltda., Barueri, SP). Next, the root canals were located through a type K file number 06 (Les Fils d'Auguste Maillefer S / A, Switzerland) and the root pulp was removed. The root canals were exposed to the buccal environment, as previously described (Paula-Silva et al., 2016). Healthy molar teeth on the left side from the same mice, without pulp exposure, were used as controls. Mice were euthanized at 7, 14, 21, and 28 days after root canal contamination (n = 12 teeth per period).

2.3. Histological processing

The lower molars were used for histological processing. The jaws were dissected and removed with surgical scissors. The blocks containing tooth and bone were fixed in 10 % buffered formalin for 24 h at room temperature and demineralized in 10 % EDTA (Merck S.A. Chemical Industries, Rio de Janeiro, RJ) for approximately 21 days. After demineralization, the pieces were submitted to routine histological processing, washed in running water for 24 h, dehydrated in increasing concentrations of alcohol, diaphanized in xylol, and embedded in paraffin. The blocks were sectioned longitudinally, in a bucco-lingual direction, to obtain cuts with a thickness of 5 μm. Sections were stained by hematoxylin and eosin (HE) for histopathological and histometric evaluation and for tartrate-resistant acid phosphatase (TRAP) enzyme for histoenzymological analysis.

2.4. Histopathological and histometric evaluation

Histological sections stained by HE were evaluated using conventional light microscopy (Zeiss Axio Imager, Carl Zeiss AG Light Microscopy, Göttingen, Germany), and apical periodontitis was measured according to previously described parameters (Paula-Silva, D'Silva, Silva, & Kapila, 2009, 2016). Polymorphonuclear leukocytes were identified by their morphological characteristics, *i.e.*, darkly stained cells with multi-lobed and horseshoe-shaped nuclei. Cells were counted under conventional light microscopy at a fixed distance from the apical foramina using HE-stained sections. For morphometric analysis of the apical periodontitis size, videomicroscopy was used with the Zeiss AxioVision Software (Carl Zeiss AG Light Microscopy), in conjunction with the AxioCam MRc5 (Carl Zeiss AG Light Microscopy) microscope and camcorder, at a magnification of 10 × . For each specimen, the apical periodontitis area was delineated and measured in m^2 on sections representing the largest diameter of the lesion. The delimitation of the lesion excluded intact structures (periodontal ligament, cementum and alveolar bone), and included areas of resorption and inflammatory infiltrate. The groups were compared by means of the two-way ANOVA test followed by the Sidak or Dunnett post-test (α = 0.05).

2.5. Determination of the presence and activity of osteoclasts by histoenzymology analyses for TRAP

The deparaffinized tissue sections were incubated in a solution containing 8 mg of naphthol AS−MX di-sodium phosphate (Sigma-Aldrich) in 500 μL of NN-dimethylformamide followed by the addition of 50 mL of a 0.2 mol buffer solution/L sodium acetate (pH 5.0) containing 70 mg of Fast Red ITR (Sigma-Aldrich). Subsequently the sodium tartrate substrate dihydrate (50 mmol/L) was added to the solution and incubated at 37 °C for 12 h. Subsequently, the blades were washed in distilled water and stained with hematoxylin. As a control, blades were incubated with a medium without substrate. Quantitative analysis of the number of osteoclasts positive for the TRAP enzyme was performed taking into consideration the total number of osteoclasts per

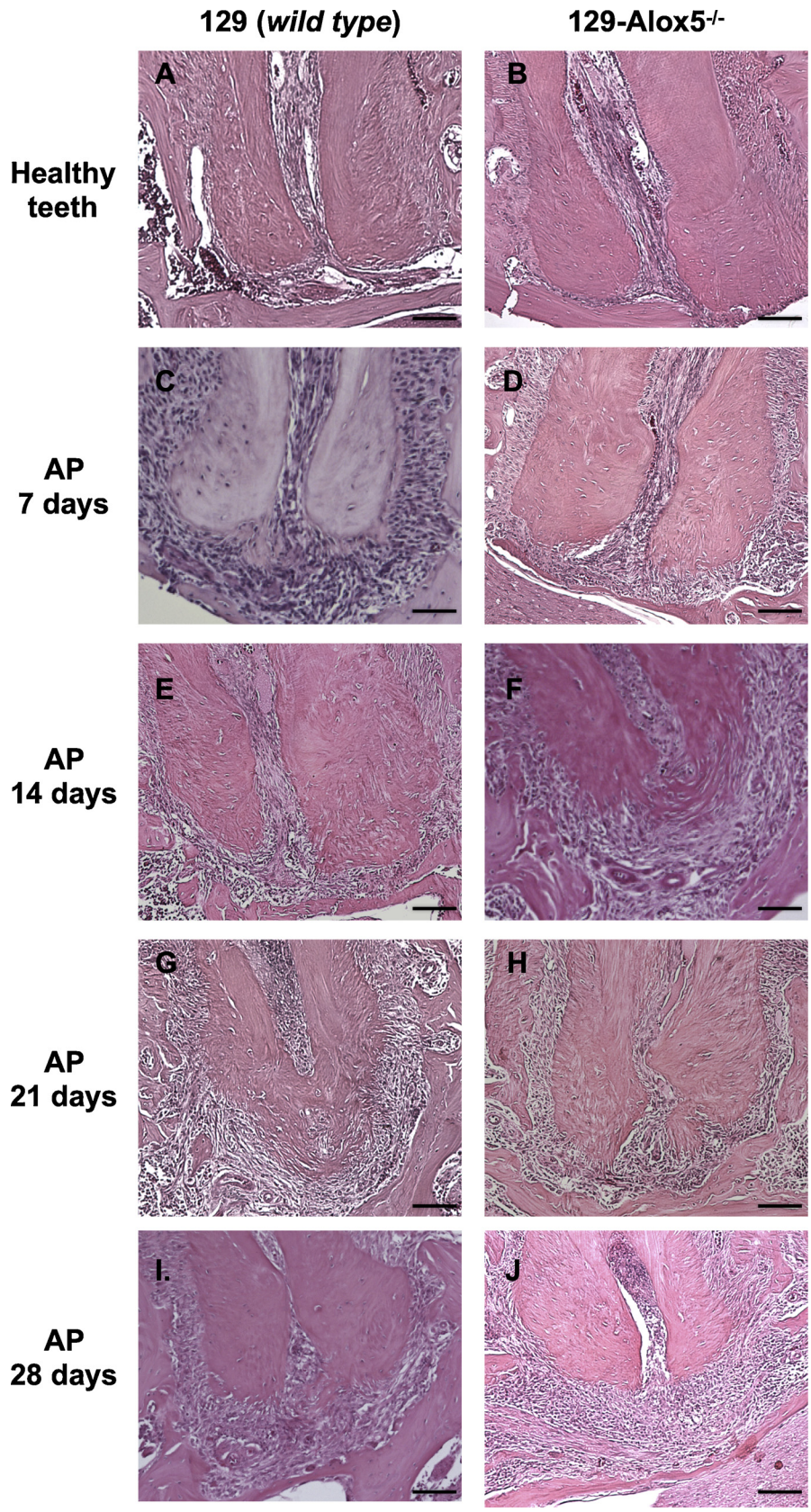

Fig. 1. Photomicrographs representative of the periapical region of wild-type (129; A, C, E, G, I) and 5-LO knockout mice (129-Alox5$^{-/-}$; B, D, F, H, J) after contamination of the root canals by microorganisms from the oral cavity at 7 (C, D), 14 (E, F), 21 (G, H), and 28 (I, J) days of exposure. Healthy teeth (A, B). HE, original magnification 10× (scale bar = 100 μm).

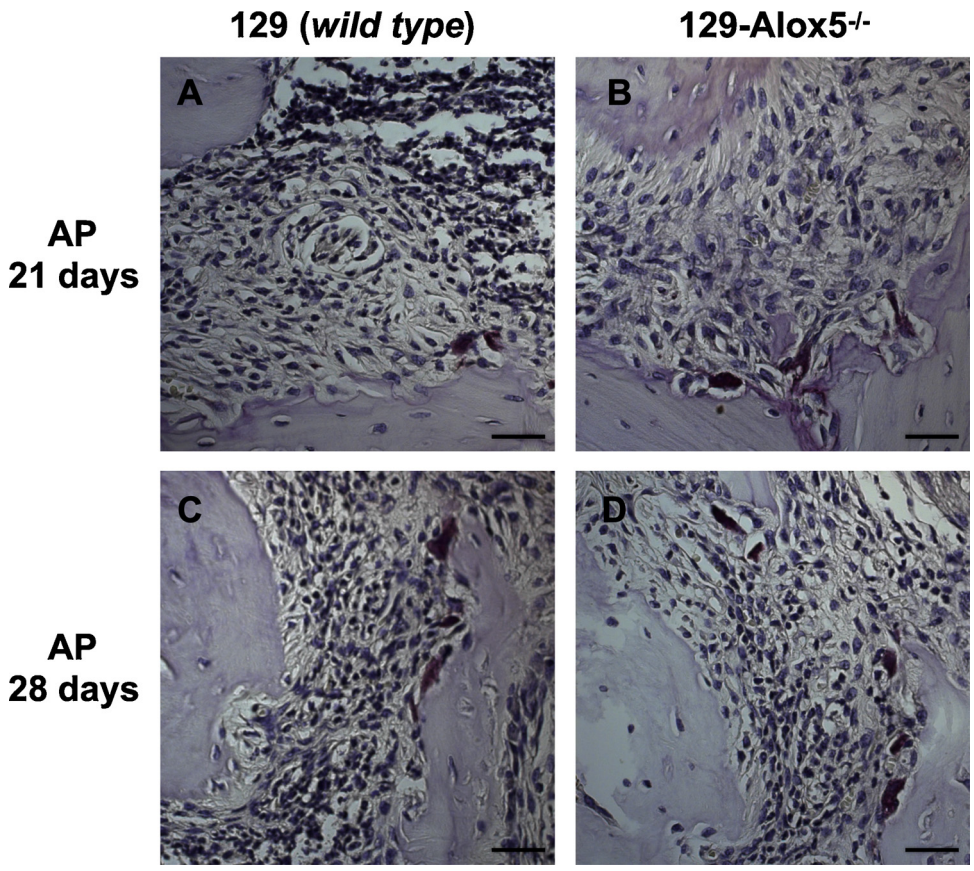

Fig. 2. Photomicrographs representative of histoenzymological findings for the tartrate-resistant acid phosphatase enzyme (TRAP) in the periapical region of wild-type (129; A, C) and 5-LO knockout mice (129-Alox5$^{-/-}$; B, D) at 21 (A, B) and 28 (C, D) days of exposure. Original magnification 40× (scale bar = 20 μm).

apical periodontitis lesion. The groups were compared by means of the two-way ANOVA test followed by the Sidak or Dunnett post-test (α = 0.05).

2.6. Determination of inflammatory activity by evaluation of gene expression using qRT-PCR arrays

For extraction of total ribonucleic acid (RNA), a pool containing 3 upper molar teeth with a mean total weight of 30 mg was used with the RNeasy Mini kit, which employs a guanidine thiocyanate extraction protocol (RNeasy® Mini, Qiagen Inc., Valencia, USA). The quality of total RNA was assessed by 1 % agarose gel electrophoresis (Sigma-Aldrich Corp.) containing ethidium bromide (Sigma-Aldrich Corp.) using 1x concentrated TBE (Tris-Borate-EDTA) buffer. The purity and nucleic acid mass were analyzed using spectrophotometry in NanoDrop 1000 (Thermo Fisher Scientific Inc., Wilmington, USA) at wavelengths of 230, 260, and 280 nm. The cDNA was synthesized by reverse transcription of 2 μg of total RNA using the First Strand RT2 kit (Qiagen Inc.) and stored at −20 °C until use. Overall analysis of gene expression was performed by means of RT-PCR arrays (*Inflammatory Cytokines* PAMM-011Z, Qiagen Inc.) containing 18 target sequences (chemokines *Ccl1*, *Ccl3*, *Ccl4*, *Ccl5*, *Ccl6*, *Ccl8*, *Cxcl5*, *Cxcl9*, *Cxcl10*, *Cxcl11*, *Cxcl13*, *Cxcl15*, and *C3xcl1* a and cytokines *Il1a, IL1B, IL10, IL13,* and *IL20*). *Gusb*, *Hprt*, *Hsp90ab1*, *Actb*, and *Gapdh* genes were used as reference. Controls for detecting mouse genomic DNA contamination (MGDC), controls for the efficiency of the reverse transcription reaction (RTC), and the positive controls (PPC) consisting of a passive artificial DNA sequence to be detected during the reaction were used. qRT-PCR reactions were performed in duplicate in an Eppendorf Mastercycler® ep Realplex (Eppendorf AG) using SybrGreen. Amplification was performed under the following conditions: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The dissociation curve was evaluated to determine the specificity of the primers, considering the melting temperature of the amplicon under the following conditions: temperature increased to 95 °C for 15 s, followed by a decrease to 60 °C for 15 s, with a gradual increase to 95 °C for 20 min and maintained at 95 °C for 15 min. Relative quantification was performed using the ΔΔCt Method. The groups were compared to each other using the two-way ANOVA test followed by the Sidak post-test or Dunnett's post-test (α = 0.05).

2.7. Evaluation of the gene expression of enzymes and receptors of the metabolism of the 5-LO pathway and of osteoclastogenesis mediators through real-time polymerase chain reaction (qRT-PCR)

Complimentary DNA (cDNA) was synthesized from 1 μg of total RNA using random primers (High Quality cDNA Reverse Transcriptase Kits, Applied Biosystems, Foster City, CA). Next, 2-μL aliquots of total cDNA were amplified by qRT-PCR using primers for *Alox5* (Mm01182747), *Alox5ap* (Mm00802100), *Ltb4r1* (Mm02619879), *Ltb4r2* (Mm01321172), *Ppara* (Mm00440939), *Ppard* (Mm00803184), *Pparg* (Mm01184322), *Tnfrsf11a* (Mm00437135), *Tnfsf11* (Mm00441906), and *Tnfrsf11b* (Mm01205928) (TaqMan® Gene Expression Assay, Applied Biosystems). The gene for the enzyme glyceraldehyde-3-phosphate dehydrogenase (Gapdh; Mm99999915) was used as a reference. Amplification was performed under the following conditions: activation of AmpliTaq Gold Enzyme polymerase at 95 °C for 2 min, followed by 40 cycles of 95 °C for 1 s for DNA denaturation and 60 °C for 20 s for primer annealing and polymerization. Relative quantification was performed using the ΔΔCt Method. The groups were compared by means of the two-way ANOVA followed by the Sidak or Dunnett post-test (α = 0.05) or one-way ANOVA followed by Dunnett's

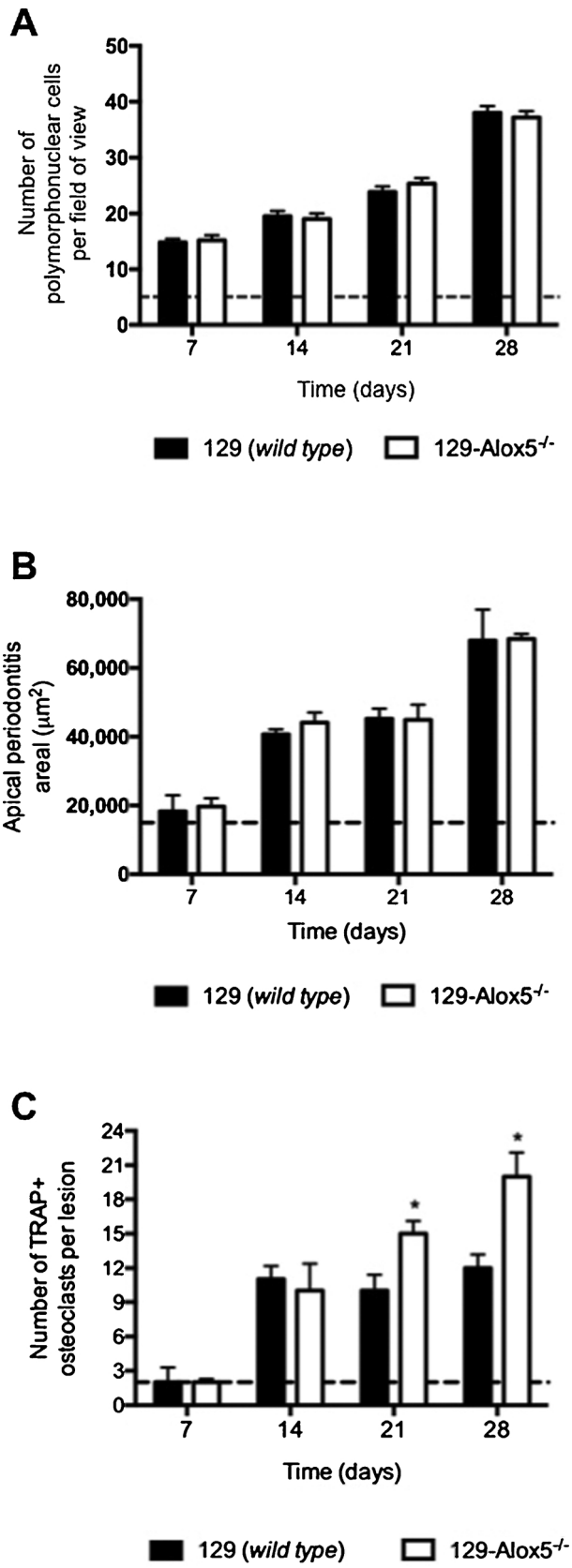

Fig. 3. Number of polymorphonuclear cells (A), measurement of the area of apical periodontitis or periodontal ligament in μm^2 (B), and number of osteoclasts positive for the tartrate-resistant acid phosphatase ($TRAP^+$) enzyme (C). Measurements were performed at 7, 14, 21, and 28 days after contamination of the root canals of wild-type and 5-lipoxygenase (129-Alox5$^{-/-}$) knockout mice. *$p < 0.05$ compared to the measurement of the periodontal ligament of healthy teeth (dashed line) or the number of $TRAP^+$ osteoclasts in teeth without periapical periodontitis (dashed line) and #$p < 0.05$ compared to wild-type mice.

post-test ($\alpha = 0.05$).

3. Results

3.1. Contamination of root canals induced osteoclast formation and expansion of apical periodontitis in both 5-LO and wild-type animals

Contamination of dental root canals led to the development of apical periodontitis in wild-type animals (129) and 5-LO-deficient mice (129-Alox5$^{-/-}$), with no histopathological difference between them. This lesion was characterized at 7 and 14 days by thickening of the apical periodontal ligament and recruitment of neutrophils, culminating in intense infiltration of inflammatory cells, loss of tissue continuity, and periapical bone resorption at 21 and 28 days (Figs. 1 and 2).

Polymorphonuclear cells were found during apical periodontitis development and their number increased with time ($p < 0.05$). Deficiency of 5-LO did not impair polymorphonuclear cell recruitment ($p > 0.05$) (Fig. 3A).

Histometric assessment of the apical periodontitis revealed an increase in the periapical space due to bone resorption in teeth that underwent oral contamination at 14, 21, and 28 days after exposure of the root canals, unlike the healthy teeth with intact periodontal ligament and alveolar bone ($p < 0.05$), but there were no differences in development kinetics when 5-LO-deficient animals were compared with wild-type animals ($p > 0.05$; Fig. 3B).

5-LO-deficient mice showed higher number of osteoclasts than wild-type animals at 21 and 28 days of development of apical periodontitis ($p < 0.05$; Figs. 2A–D and 3 C).

Together, these results show that 5-LO deficiency did not change the recruitment of polymorphonuclear cells to the periapical area following root canal contamination, but osteoclastogenesis was enhanced. However, it did not impact apical periodontitis bone loss, which was similar in 5-LO-deficient and wild-type mice.

3.2. The 5-LO pathway prevents expression of mediators of osteoclastogenesis

In order to further study the role of the 5-LO pathway in osteoclastogenesis and bone resorption induced by periapical inflammation, we investigated the synthesis of osteoclastogenic mediators (RANK, RANKL, and OPG) after contamination of the root canals by exposure to the oral cavity. The expression of mRNAs for RANK (*Tnfrsr11a*), RANKL (*Tnfsf11*), and OPG (*Tnfsf11b*) in wild-type animals was increased at 7 and 14 days during development of the apical periodontitis. Interestingly, the expression of the mRNA for RANKL, a mediator of osteoclast formation and bone resorption, in 129-Alox 5$^{-/-}$ animals was more intense than that in wild-type mice at 7 and 21 days of root canal exposure ($p < 0.05$). Gene expression of OPG, on the other hand, was not increased in 129-Alox5$^{-/-}$ animals compared to that in wild-type mice ($p > 0.05$). Since OPG acts as an inhibitory competitor, blocking the RANK-RANKL interaction and therefore inhibiting osteoclastic differentiation, we compared the ratio of RANK to OPG gene expression. This comparison showed that osteoclastogenic signaling in wild-type animals is pro-resorptive at 7 and 28 days of root canal contamination ($p < 0.05$) and that in 5-LO-deficient animals is pro-

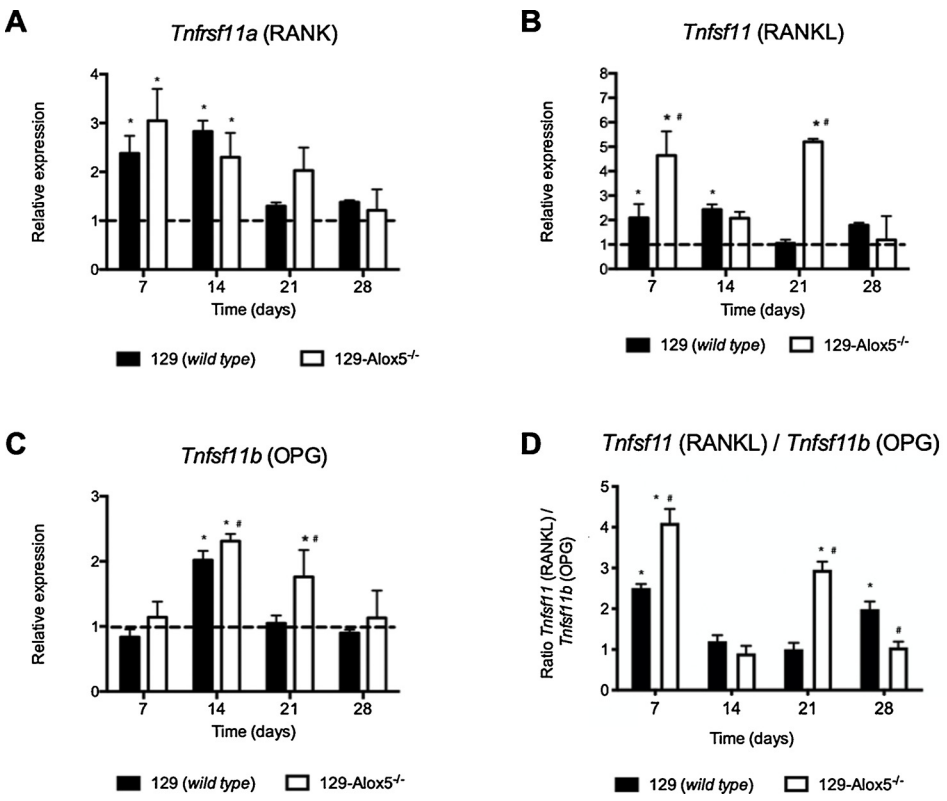

Fig. 4. mRNA for RANK (*Tnfrsf11a*) (A), RANKL (*Tnfsf11a*) (B), OPG (*Tnfsf11b*) (C), and RANKL/OPG ratio (D) evaluated at 7, 14, 21, and 28 days after root canal contamination of wild-type (129) and 5-lipoxygenase knockout (129-Alox5$^{-/-}$) mice. *$p < 0.05$ compared to the basal production of target genes in teeth without periapical periodontitis (dashed line) and #$p < 0.05$ compared to wild-type animals.

resorptive at 7 and 21 days. Interestingly, this ratio is higher in 5-LO-deficient animals at 7 and 21 days ($p < 0.05$), but lower at 28 days ($p < 0.05$) in comparison with the corresponding ratios in wild-type animals (Fig. 4).

3.3. Absence of 5-LO impairs synthesis of inflammatory mediators induced in wild-type animals following oral contamination of root canals

In wild-type mice, at 7 days after oral contamination, we observed induction of *Ccl1, Ccl3, Ccl4, Ccl8, Cxcl10, Cx3cl1, Il1a, Il1b, Il10, Il13, Il20* expression ($p < 0.05$) in comparison with healthy teeth not subjected to pulp exposure. At 14 days, we observed induction of *Ccl3, Ccl8, Cxcl10, Cxcl15, Cxcl15, Cx3cl1, Il1b, Il10, Il13, and Il20* ($p < 0.05$). At 21 days, we observed induction of *Ccl1, Ccl3, Ccl8, Cxcl9, Cxcl10, Cxcl11, Cxcl11, Cxcl13, Il1b, Il13,* and *Il20* ($p < 0.05$), while at 28 days, there was induction of *Ccl1, Ccl3, Ccl5, Ccl8, Cx3cl1, Il1b, Il10, Il13,* and *Il20* ($p < 0.05$).

In 5-LO-deficient animals (129-Alox5$^{-/-}$), 7 days after root canal contamination, the genes induced in the periapical region were *Ccl3, Ccl5, Ccl6, Cxcl9, Cxcl11, Il1a, Il10* ($p < 0.05$). On the other hand, the *Ccl1, Ccl4, Ccl8, Il1b, Il13, Il20* genes were inhibited ($p < 0.05$). At 14 days, the genes stimulated by apical periodontitis in knockout mice were *Ccl3* and *Cxcl11* ($p < 0.05$) and the genes inhibited were *Ccl8, Cxcl15, Cx3cl1, Il1b, Il13,* and *Il20* ($p < 0.05$). At 21 days, the genes stimulated by apical periodontitis in knockout mice, compared to wild-type animals, were *Ccl5, Il1a, Il1b,* and *Il10* ($p < 0.05$). The genes inhibited were *Ccl1, Ccl3, Ccl8, Cxcl9, Cxcl10, Cclcl13, Cx3cl1, Il13,* and *Il20* ($p < 0.05$). At 28 days, no gene were stimulated in apical periodontitis in knockout mice, compared to wild-type animals ($p > 0.05$). On the other hand, the genes inhibited were *Ccl1, Ccl4, Ccl5, Ccl8, Il1b, Il13,* and *Il20* (Figs. 5–7).

4. Discussion

Our results showed that the 5-LO enzyme plays an important role in the synthesis of inflammatory and osteoclastogenic mediators during microbial invasion of the root canals and the development of the experimental apical periodontitis. Interestingly, although this role played by 5-LO in osteoclastogenesis *in vivo* was protective, both in signaling and in the formation of TRAP^{+} osteoclasts, it was not sufficient to impair periapical bone catabolism.

Previously, 5-LO deficiency has been shown to aggravate *F. nucleatum*-induced apical periodontitis in an experimental murine model (Wu et al., 2018). Interestingly, we found that 5-LO modulates bone loss differently in polymicrobial apical periodontitis induced by root canal contamination from microorganisms arising from the oral cavity. This could be due to the pattern of the inflammatory response generated during microbial invasion of root canals. Indeed, we found that, in the absence of 5-LO, there was lower production of several inflammatory mediators and receptors in the periapical region.

In apical periodontitis in rats, the expression of the proinflammatory cytokines IL-1 and TNF-α increases concomitantly to RANKL expression, suggesting a synergistic effect of RANKL and pro-inflammatory cytokines for the progression of periapical periodontitis (Kawashima et al., 2007). In the periapical milieu, the synthesis of OPG and IL-10, which are RANKL-negative regulators, is rapidly induced as a probable host defense mechanism to prevent exacerbated bone loss (Kawashima et al., 2007). In the present study, this positive correlation was observed between mRNA synthesis of IL-10 and RANKL in wild-type animals and, interestingly, it increased in 5-LO-deficient animals, especially at 7 and 21 days after root canal contamination. IL-10 may therefore be a key mediator in the control of periapical resorption, since even with a greater number of osteoclasts, the extent of the lesion in 5-LO-deficient animals was not greater than that in wild-type animals. These interpretations are supported by previous studies demonstrating that IL-10 is an important endogenous suppressor of periapical bone resorption *in vivo* induced by root canal contamination (De Rossi et al., 2008; Sasaki et al., 2000). Other interleukins such as IL-1α and IL-1β and IL-1 receptors of type 1 and 2 showed an increase in the levels of their mRNAs in 5-LO-deficient animals during periapical periodontitis development, corroborating previous findings showing that these cytokines are important regulators of osteoclastogenesis and bone metabolism (Lee

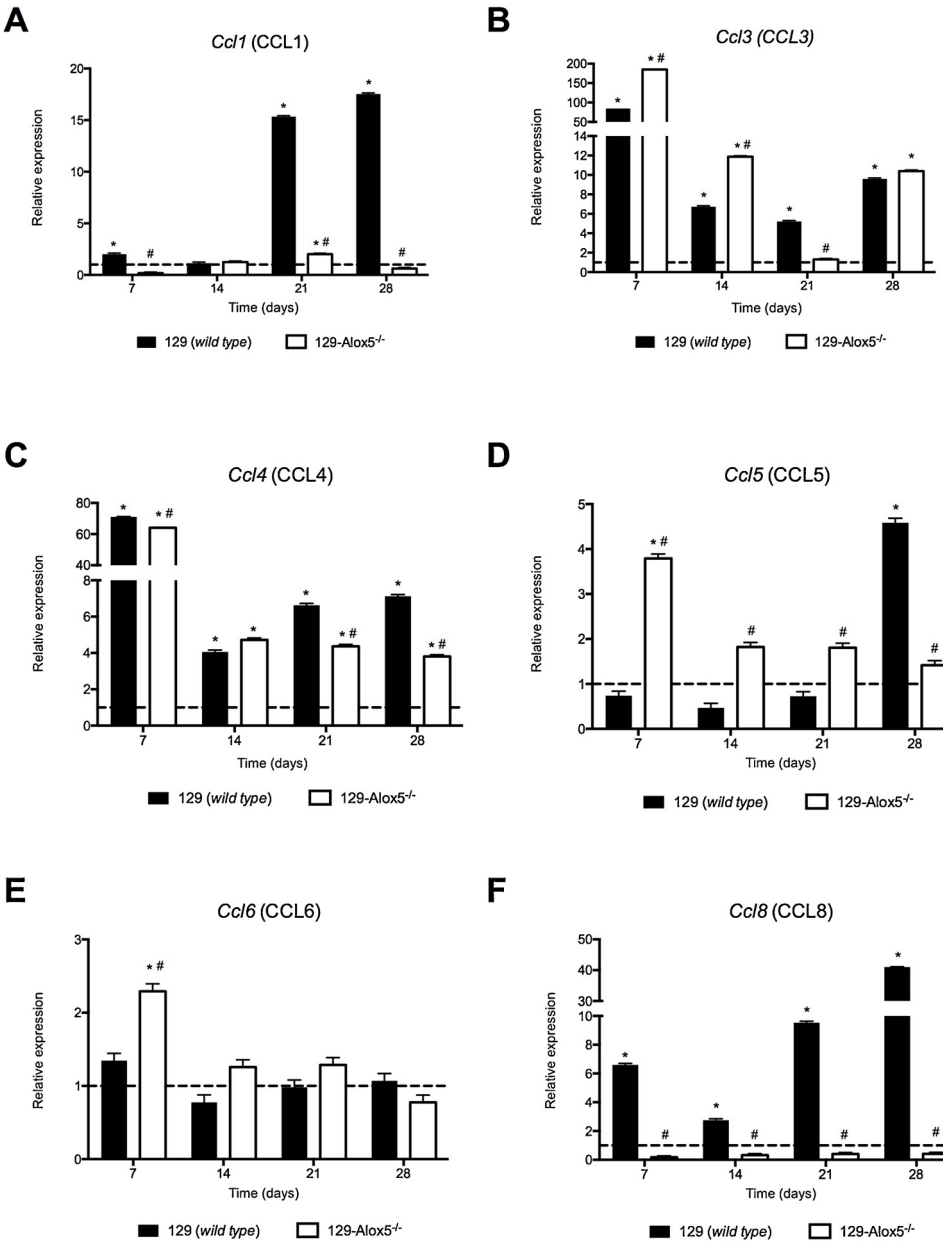

Fig. 5. (A) mRNA expression for CCL1 (*Ccl1*) (A), CCL3 (*Ccl3*) (B), CCL4 (*Ccl4*) (C), CCL5 (*Ccl5*) (D), CCL6 (*Ccl6*) (E), and CCL8 (*Ccl8*) (F) at 7, 14, 21, and 28 days after dental root canal contamination of wild-type (129) and 5-lipoxygenase (129-Alox5$^{-/-}$) knockout mice. *$p < 0.05$ compared to the basal expression of target genes in teeth without apical periodontitis (dashed line) and #$p < 0.05$ compared to wild-type mice.

et al., 2014).

Chemokines are also important regulators of bone tissue metabolism, both in physiological and disease situations. These mediators can act independently of RANKL, facilitating recruitment and direction of precursors of osteoblasts and osteoclasts to specific sites (Bendre et al., 2003; Silva et al., 2007; Wright et al., 2005). Similar to interleukins, the chemokine *Ccl3*, which is important for chemotaxis of osteoclast precursors and for the differentiation mediated by the RANKL soluble factor (Yu, Huang, Collin-Osdoby, & Osdoby, 2004), and the chemokine *Cxcl9*, which promotes the adhesion, migration, and differentiation of osteoclasts under the influence of cytokines (Kwak et al., 2005), were also increased in 5-LO-deficient animals, especially in early periods of recruitment of inflammatory cells and the immune system, which may have resulted in increased formation of osteoclasts in late periods.

The CCR1 receptor, expressed on the surface of osteoclast precursors, binds to chemokines CCL3, CCL5, CCL7, CCL9, CCL9, and CCL23, stimulating the targeting of cells to specific sites for fusion and bone resorption (Yang et al., 2006; Yu et al., 2004). Although gene expression of *Ccr1* was poorly modulated in both 5-LO and wild-type animals, mRNA expression of *Ccl3, Ccl5*, and *Ccl9* was greatly increased in 5-LO-deficient animals.

Another interesting result was the correlation between the low expression of the mRNA for *Infg* (INF-γ) and chemokine *Cxcl10* in 5-LO-deficient animals and the highest number of TRAP^{+} osteoclasts. During the immunoinflammatory response, the CXCR3 receptor and its CXCL10 ligand lead to the recruitment of Th1 cells, which produce *Infg* (INF-γ) and activate macrophages by the classical pathway (Garlet, Martins, Ferreira, Milanezi, & Silva, 2003; Kabashima, Yoneda, Nagata, Hirofuji, & Maeda, 2002). However, INF-γ is an inhibitor of osteoclastogenesis (Nakashima & Takayanagi, 2008). Thus, the negative modulation of the CXCL10-INF-γ axis in 5-LO-deficient animals may have contributed to the higher number of TRAP $^{+}$ osteoclasts in the periapical region.

The presence and intracellular location of the 5-LO enzyme was previously demonstrated by immunohistochemistry in fibroblasts,

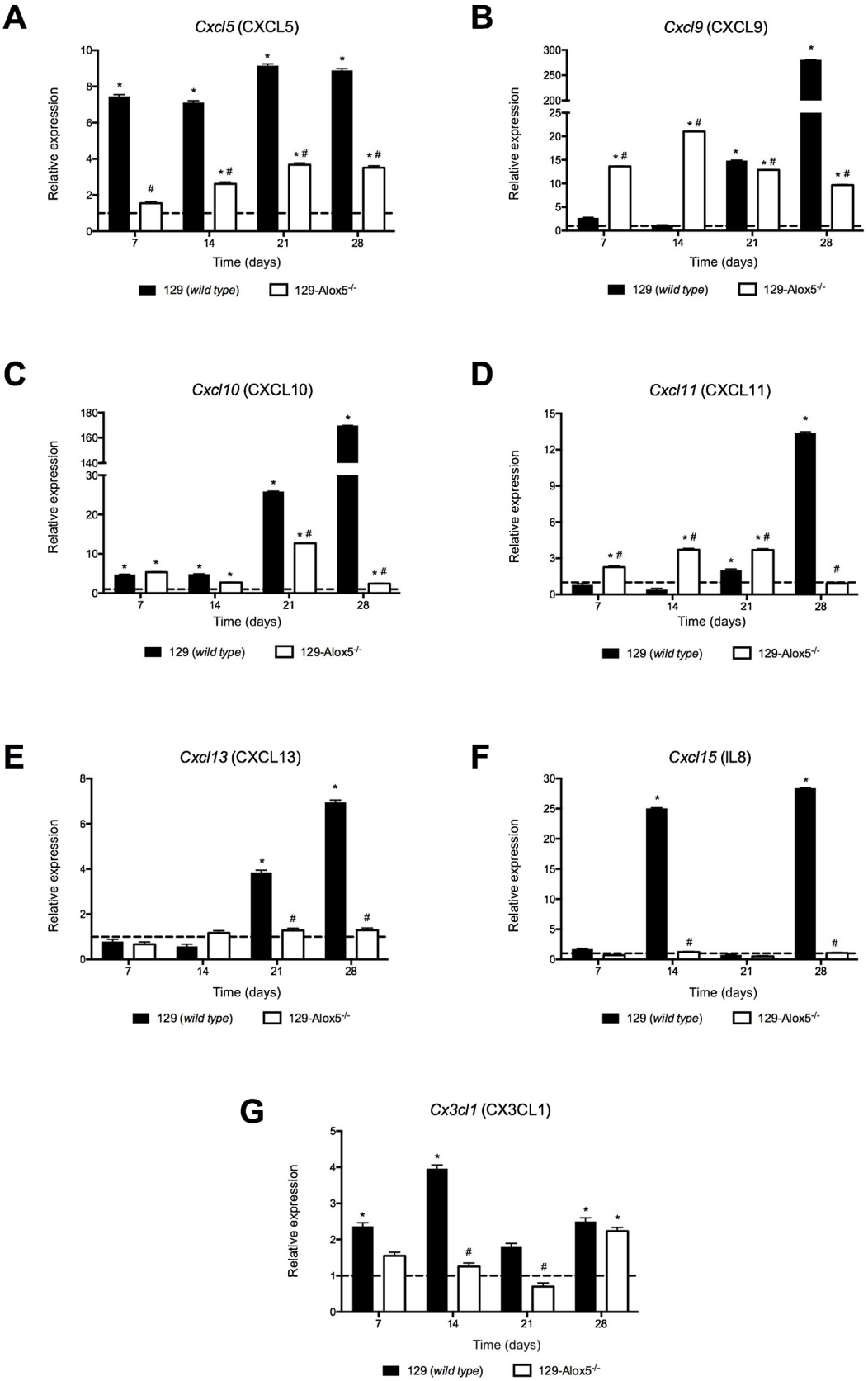

Fig. 6. (A) mRNA expression for CXCL5 (*Cxcl5*) (A), CXCL9 (*Cxcl9*) (B), CXCL10 (*Cxcl10*) (C), CXCL11 (*Cxcl11*) (D), CXCL13 (*Cxcl13*) (E), CXCL15 (*Cxcl15*) (F), and CX3CL1 (*Cx3cl1*) at 7, 14, 21, and 28 days after dental root canal contamination of wild-type (129) and 5-lipoxygenase (129-Alox5$^{-/-}$) knockout mice. *$p < 0.05$ compared to the basal expression of target genes in teeth without apical periodontitis (dashed line) and #$p < 0.05$ compared to wild-type mice.

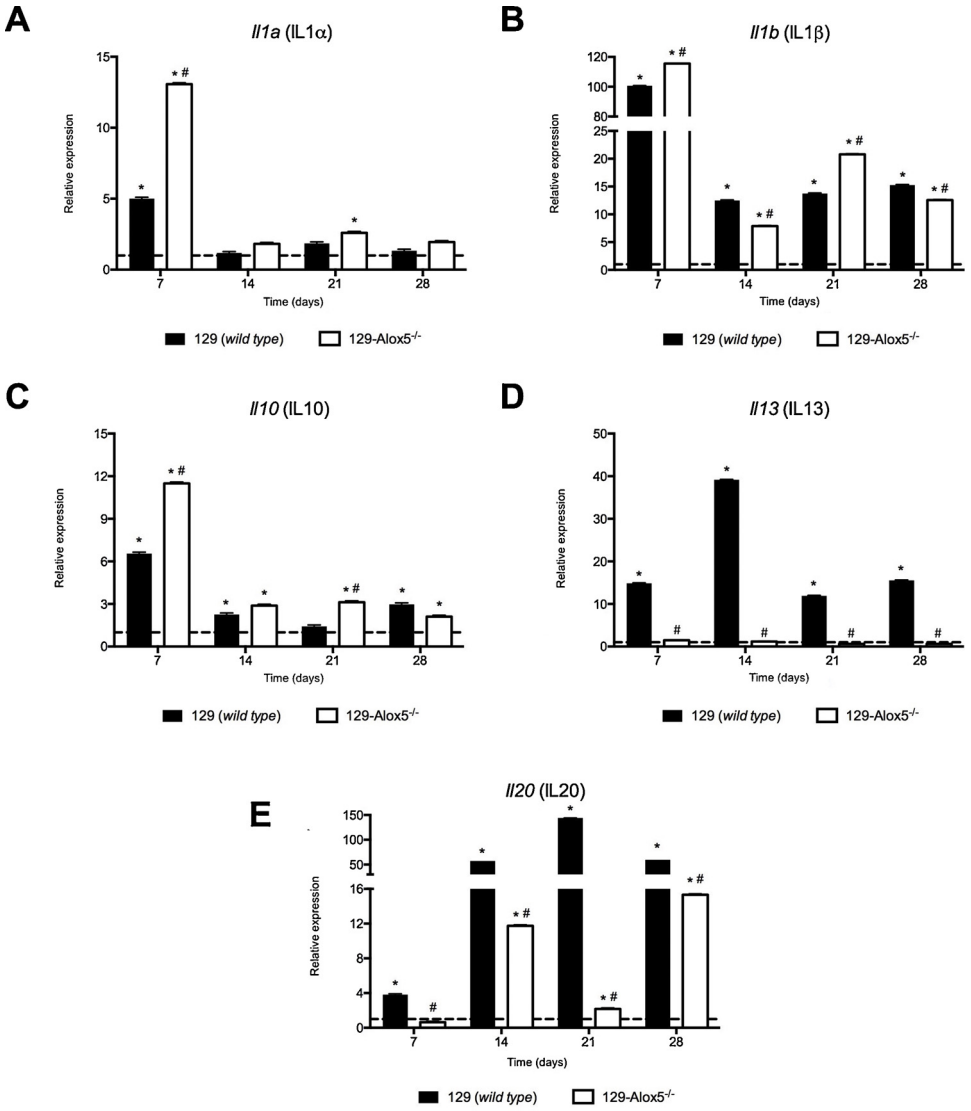

Fig. 7. (A) mRNA expression of IL1α (*Il1a*) (A), IL1β (*Il1b*) (B), IL10 (*Il10*) (C), IL13 (*Il13*) (D), and IL20 (*Il20*) (E) at 7, 14, 21, and 28 days after dental root canal contamination of wild-type (129) and 5-lipoxygenase (129-Alox5$^{-/-}$) knockout mice. *$p < 0.05$ compared to the basal expression of target genes in teeth without apical periodontitis (dashed line)and #$p < 0.05$ compared to wild-type mice.

osteoclasts, and endothelial cells during bone callus formation after long bone fracture in mice (Lin & O'Connor, 2014). Treatment with pharmacological inhibitors of the 5-LO pathway accelerated the repair of femoral fractures, characterized by improved bone regeneration (Cottrell & O'Connor, 2009; Wixted et al., 2009). These results were explained by the fact that both endogenous and exogenous LTB_4 inhibit the proliferation of osteoblasts (Ren & Dziak, 1991) and the formation of mineralized tissue *in vitro* (Traianedes, Dallas, Garrett, Mundy, & Bonewald, 1998) and stimulate osteoclasts to reabsorb calcified matrices *in vitro* (Gallwitz et al., 1993; Jiang, Lv, Lin, Jiang, & Chen, 2005) and murine calvaria *in vivo* (Garcia et al., 1996). Induced tooth movement in animals in the absence of the 5-LO enzyme has been recently shown to result in reduced bone resorption, recruitment and formation of $TRAP^+$ osteoclasts, and expression of *Tnfa*, *Il10*, and *Runx2*. Treatment with LTB_4 and LTD_4, in the presence of RANKL induced *in vitro* differentiation of osteoclasts and the production of TNF-α (Moura et al., 2014). These results are also divergent from ours, since we observed that the 5-LO pathway plays a protective role in the formation of osteoclasts *in vivo*, although this does not result in reduced bone loss. Considering that these divergent results were observed in different bone resorption models, *i.e.*, force application, mechanical fracture, LPS intraperitoneal injection, ovariectomy, or microbial contamination of the root canals, shifting this pathway with the aim of controlling bone catabolism under physiological or pathological conditions deserves future investigation. Moreover, 5-LO-deficient animals present, as a phenotypic characteristic, a bone tissue with a higher mineral density (Mehrabian et al., 2005) and bone catabolism in these animals may differ from that in wild-type animals not only by the different molecules involved in the regulation of bone resorption but also by the microhardness of the tissue. In fact, when we used the biochemical inhibitor of the 5-LO pathway (MK-886) to block the synthesis of mediators in this pathway during apical periodontitis development, we observed that at 21 and 28 days, the animals receiving the drug showed increased apical periodontitis size compared to untreated animals (Paula-Silva et al., 2016).

It is widely accepted that osteoclastogenesis and bone loss are regulated by the balance in the expression of RANKL and OPG, the canonical pathway of osteoclastogenesis. However, in the present study, signaling and osteoclastogenesis were more intense in 5-LO-deficient mice, which was not accompanied by a greater extent of bone loss. Thus, the presence of other cross-regulatory mechanisms that must be finely orchestrated for the balance of the immune response should

not be discarded.

5. Conclusions

The 5-LO pathway plays a role in the stimulation of inflammatory mediator synthesis and inhibition of osteoclastogenesis in polymicrobial-induced apical periodontitis in mice. However, the paradoxical inflammatory-osteoclastogenic signaling did not impair bone resorption during early development of the disease.

Authors contribution

Francisco Wanderley Garcia Paula-Silva. - Designed the experiments, Performed the experiments, Wrote the manuscript, Revised the final version of the manuscript.

Maya Fernanda Manfrin Arnez - Performed the experiments, Wrote the manuscript, Revised the final version of the manuscript.

Igor Bassi Ferreira Petean – Performed the experiments, Wrote the manuscript, Revised the final version of the manuscript.

Luciano Aparecido de Almeida Junior - Performed the experiments, Critical revision, Revised the final version of the manuscript.

Raquel Assed Bezerra da Silva - Designed the experiments, Critical revision, Revised the final version of the manuscript.

Léa Assed Bezerra da Silva - Designed the experiments, Critical revision, Revised the final version of the manuscript.

Lúcia Helena Faccioli - Designed the experiments, Critical revision, Revised the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.archoralbio.2020.104670.

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