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The Role of KDM3C in Oral Inflammation

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The Role of KDM3C in Oral Inflammation

A thesis submitted in partial satisfaction of

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Science in Oral Biology

by

Abdullah Mohammed A. Alshaikh

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ABSTRACT OF THE THESIS

The Role of KDM3C in Oral Inflammation

by

Abdullah Mohammed A. Alshaikh

Master of Science in Oral Biology

University of California, Los Angeles, 2017

Professor Mo K. Kang, Chair

Apical periodontitis (AP) occurs as a result of bacterial infection of the root canal and subsequent inflammatory signaling at the periapex. The disease is exacerbated by oral bacterial byproducts, including lipopolysaccharide (LPS), that trigger inflammatory cytokine release and bone destruction. Our laboratory recently demonstrated that expression of KDM3C, a histone lysine demethylase, is induced by LPS exposure and that KDM3C regulates inflammatory cytokine expression. In the current study, we investigated the role of KDM3C in generation of AP in mice after pulp exposure. AP was induced in these mice by exposure of dental pulp of maxillary first molars of KDM3C knockout (KO) and wild-type (WT) mice, and apical lesion development was assessed by uCT analysis, Hematoxylin and Eosin (H&E) staining, and immunohistochemical (IHC) staining. Bone marrow-derived macrophages (BMDM) from WT and KO mice were

exposed to LPS to assess the role of KDM3C in the regulation of inflammatory signaling. AP formation occurred in mice 7 – 21 days post-pulp exposure, and the size of the AP lesion was notably increased in KDM3C KO mice when compared with the WT mice. H&E staining revealed strong infiltration of inflammatory cells to the periapex in KO mice, as well as enrichment of macrophages when stained with F4/80 marker. Expression of inflammatory cytokines, e.g., IL-6, was significantly reduced in BMDM cultured from KDM3C KO mice. These findings indicate that KDM3C plays a critical role in immune response to oral bacterial infection in the root canal, and that loss of KDM3C compromises the inflammatory response and exacerbates development of AP.

The Thesis of Abdullah Mohammed A. Alshaikh is approved.

Wei Chen

Reuben Han-Kyu Kim

Yousang Gwack

Mo K. Kang, Committee Chair

University of California, Los Angeles

2017

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1 Introduction

1.1 The basis behind apical periodontitis and periodontitis development

Inflammation is the body's response to microbes and can be classified, based on the duration of inflammation, as either acute or chronic inflammation. Oral microorganisms are either classified based on their gram staining as Gram positive or Gram negative, or based on their oxygen needs as aerobic/oxygen dependent or anaerobic/oxygen independent (Bartold & Van Dyke, 2013; Costerton et al., 2003; Yoshimura, Mori, Ohishi, Aki, & Hanada, 2003). Gram-positive and gram-negative bacteria have distinct morphology. Gram-positive bacteria possess a plasma membrane, whereas gram-negative bacteria possess both a plasma membrane and an additional cytoplasmic membrane (Yoshimura et al., 2003). The gram-negative bacteria's outer cytoplasmic membrane contains a unique endotoxin component that stimulates the inflammatory response. This endotoxin, known as lipopolysaccharide (LPS), contains a lipid A moiety, an O antigen polysaccharide, and a core that holds the polysaccharide (Yoshimura et al., 2003). The release of LPS by anaerobic bacteria, such as Porphyromonas gingivalis (*P. gingivalis*), has become of great interest in the study of the development of periodontitis and apical periodontitis, as it accounts for the stimulation of inflammatory cells and promotion of inflammation at the site of infection (Aleo, De Renzis, Farber, & Varboncoeur, 1974; Berglund, Rizzo, & Mergenhagen, 1969; Johnson, Chen, Dombrowski, & Nowotny, 1976; Schonfeld et al., 1982; Yamasaki et al., 1992). Inflammation is typically accompanied by clinical symptoms such as redness, pain, and swelling. Radiographic findings including horizontal bone loss around the teeth or periapical bone loss are also commonly found in periodontitis (PD) or apical periodontitis (AP), respectively (Gemmell, Yamazaki, & Seymour, 2002; Gutmann, Baumgartner, Gluskin, Hartwell, & Walton, 2009; Preshaw, 2008). Both oral diseases share some similarities in their symptoms and mode of bacterial infection, as well as the molecular mechanism by which the immune response is activated in order to eradicate the infection (Bando, Henderson, Meghji, Poole, & Harris, 1993; Gemmell et al., 2002; Preshaw, 2008; Rossol et al., 2011;

Schonfeld et al., 1982; Yoshimura, Hara, Kaneko, & Kato, 1997). Conventional treatment for periodontitis and apical periodontitis largely relies on mechanical and chemical removal of the infection rather than targeted pharmaceutical treatment aimed at inhibiting the molecular activation of inflammatory molecules. Although conventional methods of disease management are generally effective, the persistence of unresolved inflammation in some cases indicates the need for combination therapy consisting of both mechanical removal of bacteria as well as pharmacological control of the inflammatory factors (Eke et al., 2015; Huumonen, Suominen, & Vehkalahti, 2017). The need for effective combination therapy becomes even more imperative when considering the association between many chronic oral infections and systemic diseases (Scannapieco, 2004; Scannapieco & Genco, 1999). In fact, a correlation between development of periodontitis and several systemic diseases, such as rheumatoid arthritis, cardiac disease, and diabetes, has been well documented in clinical and scientific literature (Scannapieco, 2004; Scannapieco & Genco, 1999). This association between oral infectious disease and systemic diseases indicates that both oral bacteria has the ability to induce a strong inflammatory signals that can cause systemic circulation of inflammatory cytokines and destruction of other tissues(Friedewald et al., 2009; Offenbacher et al., 2009), and that oral bacteria have the ability to travel from the site of infection to the other sites in the body (Arimatsu et al., 2014). In fact, several studies have demonstrated the unique ability of *P. gingivalis* to evade inflammatory cells such as macrophages and dendritic cells and travel to the other parts of the body (Carrion et al., 2012; Wang et al., 2007).

1.2 Basic molecular mechanism of Oral inflammation:

Once oral bacteria colonize in the oral tissue, they begin secreting byproducts such as endotoxins or lipopolysaccharide (LPS) (Johnson et al., 1976; Schonfeld et al., 1982), and LPS in turn binds to Toll-Like Receptors (TLR) 2 and 4 on epithelial cells (Costalonga, Batas, & Reich, 2009; Kikkert, Laine, Aarden, & van Winkelhoff, 2007). Once the TLR is activated, epithelial cells will undergo initiation of transcription of

inflammatory cytokines including TNF-α, IFN-γ, and IL-6 that will act as a signal to white blood cells (leukocytes) to recruit them towards the site of infection (Costalonga et al., 2009; Mahanonda & Pichyangkul, 2007). However, a recent study demonstrated that *P. gingivalis* can bypass TLR4 activation through the production antagonistic A lipid (Slocum et al., 2014). Interestingly, the secretion of antagonistic A lipid and inhibition of TLR4 has been associated with enhanced bacterial survival and activation of non-canonical inflammsome pathway (Slocum et al., 2014). The first leukocytes that arrive at the site of infection are neutrophils, followed by macrophages and natural killer (NK) cells. These leukocytes, or phagocytes, engulf the bacteria by a process known as phagocytosis. This fast process limits the spread of bacteria to the other parts of the body and this fast immune response is known as innate immunity (Cutler & Jotwani, 2004; Ji et al., 2007). Once innate immunity is established, the body begins to send signals to generate the adaptive immunity lead by T-lymphocytes. In fact, the elimination of intracellular pathogens, such as bacteria and viruses, is lead and organized by T-lymphocytes (Gemmell, Yamazaki, & Seymour, 2007). Previously, it was known that T-helper (Th) cells could be polarized into two main types, Th-1 and Th-2 depending on the cytokine that the Th cells receive. Generally, IL-6 and IFN-γ can differentiate Th cells to Th-1 lineage, while IL-4 will differentiate the Th-cells towards Th-2 lineage (Bluestone, Mackay, O'Shea, & Stockinger, 2009; Murphy & Reiner, 2002). Recently, a new subset of T-cells, Th-17 and Treg, were identified that are regulated by IL-17 and FOXP3, respectively (Park et al., 2005; Voo et al., 2009). Although the immune response initiated by innate and adaptive immunity aids in the removal of pathogens, uncontrolled or hyper-activation of the immune system may also lead to tissue damage. Therefore, controlling and limiting the inflammatory response may be imperative in maintaining tissue integrity and preventing excessive, unintended tissue damage. Oral inflammation involves the response of immune cells against oral bacterium, including gram-negative *P. gingivalis* bacteria. A hallmark of infection with gram-negative bacteria is the induction of chronic inflammatory response (Gemmell et al., 2002). *P. gingivalis* induces a local

low chronic inflammatory response that results in oral inflammatory bone destruction, which manifests as periodontal or pulpal disease (Yamaguchi et al., 2008). The chronic response induced by the oral bacteria elevates pro-inflammatory cytokine levels, eventually leading to bone loss by activation and proliferation of differentiated osteoclast cells. In fact, osteoclast cell activation requires the binding of receptor activator of nuclear factor kappa-B ligand (RANKL), presented by the osteoblast, to receptor activator of nuclear factor kappa-B RANK receptor (Yamaguchi et al., 2008). This osteoclast activation is negatively regulated by osteoprotegerin (OPG), which binds to RANKL and prevents it from binding to RANK. Thus, bone homeostasis in the oral cavity is manly regulated by the RANK/RANKL/OPG system (Liu et al., 2003).

1.3 Epigenetic regulation of gene expression:

Epigenetic regulation occurs post-translationally and involves the activation or silencing of gene expression without changing the nucleotide sequence by histone or DNA modification. Histone proteins can undergo several distinct epigenetic modifications, including methylation, ubiquitination, and acetylation. These modifications are carried out by specific epigenetic enzymes, including lysine-specfic methyltransfrases (KMTs) and lysine-specific demethylases (KDMs), which modify distinct sites on histones H2A, H2B, H3 and H4. KMTs serve to add methyl groups on lysine residues of specific histones by binding to the promoter regions of the targeted genes, leading to compacted chromatin that will disrupt the biochemical events of the regulatory factors. On the other hand, KDM's function by removing methyl groups from histone proteins, allowing regulatory factors to bind to the promoter site of genes of interest (Jaenisch & Bird, 2003). Histone modification enzymes modify distinct histone residues and are thus site specific, as is their effect on gene expression. In general, histone methylation results in transcription repression, while histone acetylation generally results in transcriptional activation (Jaenisch & Bird, 2003). However, methylation of lysine 4 on histone 3 (H3K4) results in transcriptional activation (Barski et al., 2007). Hence, histone

regulation can have different effects on gene expression depending on the histone site being modified and are important for tightly regulating gene transcription. Epigenetic regulation of gene expression plays an important role in modulating the secretion of inflammatory cytokines and limits hyperactivation of the immune system.

1.4 The epigenetic mechanism of inflammation:

Recently, the role of epigenetic regulation of immune response has become of great interest. De santa et. al. (De Santa et al., 2007) demonstrated the first link between JMJD3, a histone demethylase specific for H3K27me3, and regulation of immune response in macrophages. JMJD3 was found to be highly expressed in murine macrophages upon LPS exposure. De Santa et. al. also demonstrated that JMJD3 induction during inflammation is dependent on NF-κB activity (De Santa et al., 2009), suggesting that JMJD3 affects inflammatory cytokine regulation post-transcriptionally. Further, JMJD3 has been found to play a key role in differentiation of naïve CD4+ T-cells, with JMJD3 knockout resulting in preferential differentiation to Th2 and Treg subtypes (Li et al., 2014). A recent study by Antignano et. al. demonstrated the role of G9a, a histone methyltransferase for H3K9, in limiting Th17 and Treg differentiation both in vitro and in vivo, with G9a induction leading to preferential differentiation to Th17 and Treg in T-cells (Antignano et al., 2014). Our laboratory has identified KDM3C, an H3K9me1/me2 demethylase, as key regulator of inflammatory cytokine transcription. Thus, understanding the epigenetic regulation of cytokine secretion, immune activation and T-cell differentiation may aid in the development of novel therapeutics aimed at improving treatment outcomes of periodontitis and apical periodontitis.

2 Material and Methods:

2.1 Animal model:

C57BL/6 mice (WT) were purchased from the Jackson Laboratory (Bar Harbor, ME) and

kept in a pathogen-free vivarium in the Division of Laboratory Animal Medicine, University of California Los Angeles, Los Angeles, CA. All experimental protocols were approved by institutional guidelines from the Chancellor's Animal Research Committee. KDM3C heterozygous (KDM3C-hetero) mice were obtained from RIKEN BRC and were bred to generate KDM3C knockout mice (KDM3C-KO). Genotyping of these mice were done using polymerase chain reaction (PCR). Reverse and forward primers for WT mice were: 5"-AGTCCCCGCACTCAGGAGGCTGCTG-3" and 2C-144790-R- 5"-ATATACACTATGATAC AGGAACAGC-3". Reverse and forward primers for KO mice were: 5"-CGCCTTCTT GACGAGTTCTTCTGAGGGG-3" and 5"-ATATACACTATGATACAGGAACAGC-3".

2.2 Apical periodontitis model:

Mice were anesthetized with 100 and 5 mg/kg ketamine and xylazine, respectively. Pulpal exposure was performed on first maxillary molars using a high-speed quarter round bur powered by a portable dental unit (Aseptico Inc, Woodinville, WA) under endodontic microscopic magnification (10x) (BM-LED Stereo Microscope; MEIJI Techno, Saitama, Japan). Following pulpal exposure, the cavity was left open without restoration. In WT mice, the left maxillary first molar was exposed, whereas the right maxillary first molar was not exposed and served as a control. The mice were sacrificed and maxilla were harvested at 1, 3, 7, 21, and 42 days post-pulp exposure (n = 3). In KDM3C-KO mice, both left and right maxillary first molars were exposed and mice were harvested 7 days after pulp exposure.

2.3 Ligature-induced periodontitis:

Mice were anesthetized as described previously. 4-0 silk suture materials were used to ligate around the left maxillary second molar. Ligature was not placed on the right maxillary first molar, which was used as a control. The mice were sacrificed and maxillas were collected for further radiographical and histological analysis (n=3). Using micro-

computed tomography (μCT) analysis, bone loss was quantified by measuring the distance between the cementoenamel junction (CEJ) and the alveolar ridge on the palatal and buccal roots of the second molars.

2.4 Tissue preparation:

Harvested maxillas were fixed with 4% formalin solution for 24 hours. These maxillas were then scanned using μCT to assess bone loss, and were then decalcified with 5% EDTA and 4% sucrose in phosphate-buffered saline (pH = 7.4) for 3 weeks at 4°C. Decalcification solution was changed daily for 3 weeks, and decalcified tissue was sent to the University of California Los Angeles Translational Procurement Core Laboratory for paraffin embedding.

2.5 Micro-computed tomography (μCT) analysis:

Maxillas were placed inside a customized cylindrical tube and underwent scanning at 60 kVp and 166 μA , using 1 mm aluminum filter and voxel size of 10 μm^3 in high resolution settings. 3-D Images were reconstructed using NRecon Reconstruction software (Bruker microCT, Kontich, Belgium), and 3-dimensional images were structured using CTVOx and CTvol software (Bruker microCT, Kontich, Belgium).

2.6 Histologic Sample Preparation:

Decalcified maxillas were dehydrated in ethanol and embedded in paraffin. Serial sections of 5 μm thickness of the embedded tissue were mounted and stained with hematoxylin and eosin (H&E) or Tartrate-resistant Acid Phosphatase Staining (TRAP). For TRAP staining, 3 slides were selected containing the region of interest per sample. Deparaffinization was accomplished by incubating the slide at 60°C and washing the slide in xylene and 70%, 80%, 95%, and 100% ethanol. Slides were subsequently stained for either H&E or TRAP. For H&E, the slide was rehydrated after staining and prior to mounting the slide with a cover slip, whereas TRAP stained slides were not rehydrated. Quantitative measurement for TRAP staining was done using ImageJ software version

1.48 (NIH) on digital pictures taken using an Olympus microscope (model DP72; Olympus) at 100x magnification by counting the number of stained osteoclasts. Immunohistochemical (IHC) staining for F4/8 was done by covering the slides with proteinase K (20 μg/ml in TE buffer, pH 8.0), incubating them at room temperature for 3 minutes, and washing them with Phosphate Buffer Saline (PBS) (Agnello et al., 2003). Slides were subsequently incubated with 30% hydrogen peroxide diluted in methanol for 30 minutes and washed with PBS. Slides were then incubated with primary antibody (1:100) overnight at 4°C. Slides were then washed with PBST 3 times for a duration of 5 minutes and incubated with secondary antibody (1:200, anti-rat) for 45 minutes at room temperature. Slides were washed again with PBST and incubated with HRP for 30 minutes and DAP solution for an additional 3 minutes. The slides were soaked in water to stop the reaction, countered stained with hematoxylin, rehydrated and mounted.

2.7 Real-time qRT-PCR:

Liver and tongue tissue were collected from KDM3C-WT and KDM3C-KO mice used in the apical periodontitis model. Tongue tissue was used to confirm KDM3C knockout in the KDM3C-KO mice and liver tissue was used to assess cytokine levels in both WT and KO mice following pulp exposure. Extraction of mRNA was achieved by homogenization of tissue with 1ml of Trizol using tissue homogenizer, followed by conventional mRNA extraction and cDNA synthesis methods described elsewhere (Kang et al., 2004).

Table 1: Mouse Primer sequence used for Real-time PCR

Genes	Forward (5'-3')	Reverse (5'-3')
MAPK14	GCA-TCG-TGT-GGC-AGT-TAA-GA	GTC-CTT-TTG-GCG-TGA-ATG-AT
PIK3R1	CTT-GTC-CGG-GAG-AGC-AGT-AAG	TTG-TTG-GCT-ACA-GTA-GGC
KDM3C	GAG-GAC-TTC-AAG-GCC	AAT-TAG-GTG-TCT-TCC
G9A	TGC-CTA-TGT-GGT-CAG-CTC-AG	GGT-TCT-TGC-AGC-TTC-TTC-AG
GAPDH	AGC-TTG-TCA-TCA-ACG-GGA-AG	TTT-GAT-GTT-AGT-GGG-GTC-TCG
IL-6	GCT-ACC-AAA-CTG-GAT-ATA-ATC	CCA-GGT-AGC-TAT-GGT-ACT-CCA
IL-10	TGG-CCC-AGA-AAT-CAA-GGA-GC	CAG-CAG-ACT-CAA-TAC-ACA-CT
IL-15	CAT-CCA-TCT-CGT-GCT-ACT-TGT	CAT-CTA-TCC-AGT-TGG-CCT-CTG
IL-17A	CAG-GGA-GAG-CTT-CAT-CTG-TGT	GCT-GAG-CTT-TGA-GGG-ATG-AT

3 Results:

3.1 Pulp exposure induces time-dependent increase in PARL size, inflammatory cell infiltration and osteoclast activity in mice:

In order to determine the role of epigenetic regulation of inflammatory response on periapical lesion development, we developed an apical periodontitis model in which pulp tissue of maxillary first molars were exposed to the oral environment. Exposure of pulp tissue resulted in development of periapical radiolucency (PARL) as well as periapical infiltration of inflammatory cells as observed by H&E staining (Figure 1A). In order to observe time dependent progression of apical periodontitis in mice, we sacrificed the mice at 1, 3, 7, 21, and 42 days post-pulp exposure. PARL development progressed in a time dependent manner, and although PARL was radiographically observed 7 days post-exposure, periapical infiltration of inflammatory cells and osteoclast activation was observed as early as 3 days post-exposure (Figure 1B). A time point of 7 days post-pulp exposure was selected for additional experiments comparing periapical lesion

development between KDM3C-WT and KDM3C-KO, as osteoclast activation and number was at its peak at this time point. These data indicate that pulp exposure and bacterial infiltration into the canal space activates osteoclast cells and stimulates periapical infiltration of inflammatory cells.

3.2 Generation of KDM3C knockout mice:

In order to assess the role of KDM3C in the development of apical periodontitis, we obtained and bred KDM3C-KO mice. Due to KDM3C's important role in spermatogenesis and infertility of KDM3C-KO mice (Kuroki et al., 2013), we bred KDM3C-heterozygous mice to generate homozygous KDM3C-KO mice, which were distinguished by genotyping (Figure 2A). In order to confirm knockout of KDM3C, KDM3C-WT, KDM3C-heterozygous, and KDM3C-KO mice were sacrificed and tongue tissue was collected, and KDM3C expression was found to be completely lost in KDM3C-KO mice (Figure 2B). This data confirmed that complete knockout of KDM3C had been achieved in KDM3C-KO mice.

3.3 KDM3C ameliorates bone loss in apical periodontitis model in mice:

We investigated the role of KDM3C in generation of AP in mice by exposing the dental pulp of maxillary first molars of KDM3C-KO and KDM3C-WT mice for 7 days to induce AP. Pulp exposure led to greater AP formation in KDM3C-KO mice when compared to KDM3C-WT mice (Figure 3A,B). H&E and TRAP staining also revealed strong infiltration of inflammatory cells to the periapex, enrichment of macrophages stained with F4/80 marker, and osteoclast activation in KDM3C-KO mice (Figure 3C). These findings indicate that KDM3C plays a critical role in immune response to oral bacterial infection in the root canal, and that loss of KDM3C alters this inflammatory response and exacerbates development of AP.

3.4 KDM3C knockout induces increased inflammatory signaling:

Having demonstrated that KDM3C-KO mice exhibit more bone loss, osteoclast activation and macrophage recruitment than KDM3C-WT mice, we were interested in assessing the level of inflammatory cytokine expression in these mice. We found that mRNA expression of inflammatory cytokines were higher in KDM3C-KO mice when compared to KDM3C-WT mice, including TNF-α and IL-17, which play an important role in the development of apical periodontitis (Figure 4). This data suggests that KDM3C-KO mice exhibit dysregulation of immune response that may account for the exacerbation of apical periodontitis seen in these mice.

3.5 KDM3C ameliorates bone loss in ligature-induced periodontitis model in vivo:

In order to observe the effect of KDM3C-KO on development of periodontitis, we ligated the left maxillary first molar of KDM3C-KO and KDM3C-WT mice for 3 weeks. The ligature remained intact during the 3-week period and signs of clinical inflammation, such as edema and redness, were visibly distinguished from the non-ligatured side (Figure 5A). 3D-reconstration was also performed to demonstrate the extent of bone loss between the two sides (ligature vs. non-ligature) (Figure 5A). Placement of ligature led to periodontitis development and bone loss in both groups, however the amount of bone loss was notably increased in KDM3C-KO mice when compared with the KDM3C-WT mice (Figure 5B). Ligature-induced bone loss was measured from the CEJ of the second molar (from the mesiobuccal, distobuccal, and palatal roots) to the alveolar ridge using μCT images of the maxilla (Figure 5C, D). These findings indicate that loss of KDM3C exacerbates development of periodontitis induced by ligature in mice.

3.6 Loss of KDM3C induces osteoclast activation leading to enhanced bone loss in ligature induced periodontitis in-vivo:

We next sought to assess whether histological sections of ligature-induced periodontitis samples demonstrate a phenotype comparable to that found in our apical periodontitis

model. H&E staining revealed strong infiltration of inflammatory cells to the periapex of KDM3C-KO mice, as well as enrichment of osteoclast cells (Figure 6A). Similarly, quantitative measurement of the osteoclast cells demonstrated that KDM3C-KO mice exhibited significantly more TRAP staining when compared to KDM3C-WT mice (Figure 6B). These findings indicate that loss of KDM3C promotes enhanced infiltration of inflammatory cells and enrichment of osteoclast cells in the periapex during the development of periodontitis induced by ligature in mice.

4 Discussion:

Although several studies have investigated the molecular mechanisms underlying periodontitis and apical periodontitis (Bartold & Van Dyke, 2013; Preshaw, 2008), the role of epigenetic regulation in the development and progression of these oral inflammatory diseases is not well understood. In this study, we utilized periodontitis and apical periodontitis mice models in conjunction with KDM3C-KO mice in order to investigate the role of KDM3C in these inflammatory processes. We first established the apical periodontitis model and performed pulp exposure experiments using maxillary first molars in a time dependent manner. In our model, we were able to demonstrate that periapical lesion development and size of periapical radiolucency depends on the duration post-exposure of the pulpal cavity to oral microorganisms (Figure 1B), which is consistent with previous literature demonstrating the ability of pulp exposure to induce PARL development (Gutmann et al., 2009).

Our laboratory previously identified KDM3C as an important epigenetic regulator of inflammatory response which functions to regulate inflammatory cytokine expression by demethylating mono- and di-methylated histone 3 and lysine 9 (H3K9me1/2). Other studies have demonstrated the importance of inflammatory cytokine secretion or inhibition on PARL size (Bando et al., 1993). In this study, we were interested to the see the role of epigenetic regulation of inflammatory response by KDM3C on the development of periodontitis and apical periodontitis. Interestingly, we found that

KDM3C-KO resulted in significant increase in bone loss, osteoclast activation, and induction of inflammatory cytokines TNF-α and IL-17. We also found that that KDM3C-KO resulted in decreased expression of IL-6, demonstrating the anti-inflammatory nature of IL-6 in the context of apical periodontitis, as prevoiusly reported (Bando et al., 1993). Periodontitis is another oral inflammatory disease that is characterized by inflammation of soft tissue and loss of bone that surrounds and supports the tooth. Similar to apical periodontitis, oral bacteria *P. gingivalis* is also known to cause development of periodontitis(Hajishengallis, 2015). We were interested in determining whether exacerbation of periodontitis as observed in KDM3C-KO mice mimics that found in our apical periodontitis model. We utilized a ligature-induced periodontitis model and found that KDM3C-KO mice demonstrate significant bone loss as well as an increase in osteoclast activation similar to what was observed in the apical periodontitis model. However, it remains unclear whether KDM3C affects osteoblast or osteoclast cells directly, as it was previously reported that osteoblasts carrying RANKL are required to bind to the RANK receptor present on osteoclasts (Liu et al., 2003). Further study is necessary to fully understand the role of KDM3C on osteoclast and osteoblast cell activation. Although the findings of this study highlight the importance of KDM3C in the regulation of inflammatory response in the context of periodontitis and apical periodontitis, additional experiments are necessary to fully understand the molecular mechanism by which KDM3C induces the bone loss found in these oral inflammatory processes.

Collectively, in this study we were able to demonstrate that KDM3C regulates the inflammatory response in periodontitis and apical periodontitis. We also demonstrated that KDM3C may limit the over-activation of osteoclast cells; however, whether KDM3C directly affects osteoblast or osteoclast cells still requires further investigation. Although this study focused primarily on the phenotype of KDM3C knockout in oral inflammatory diseases, future studies will investigate the detailed inflammatory signaling pathway regulation by KDM3C in the context of periodontitis and apical periodontitis. It

will also be important to determine whether KDM3C overexpression can serve to abrogate inflammatory lesion development found in periodontitis and apical periodontitis as a possible treatment modality for these disease processes.

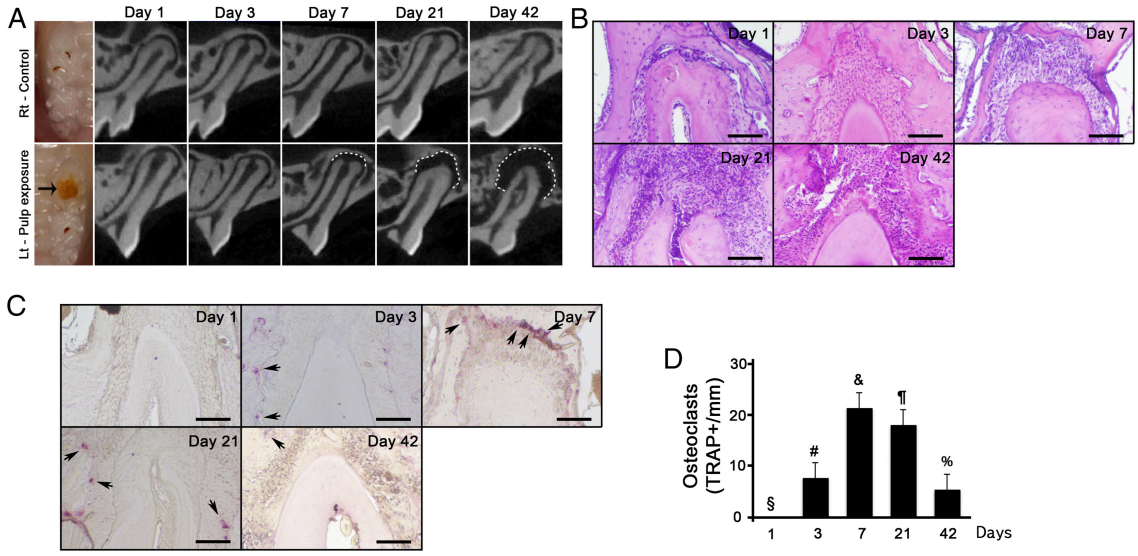

Figure 1. Pulp exposure induces time-dependent increase in PARL size, inflammatory cell infiltration and osteoclast activity in mice.

Pulp exposure was performed on maxillary left first molars, with the right side remaining intact. Mice were sacrificed after 1, 3, 7, 21, and 48 days. (**A**) Micro-Ct analysis of the mesio-buccul root (**B**) H&E staining for the apex of the disto-buccal roots and (**C**) TRAP staining for the apex was performed. (**D**) TRAP+ osteoclasts around the apex of the root ($P < .05$) were quantified. The distinct symbols represent significant difference by analysis of variance and the Tukey post hoc test ($P < .05$).

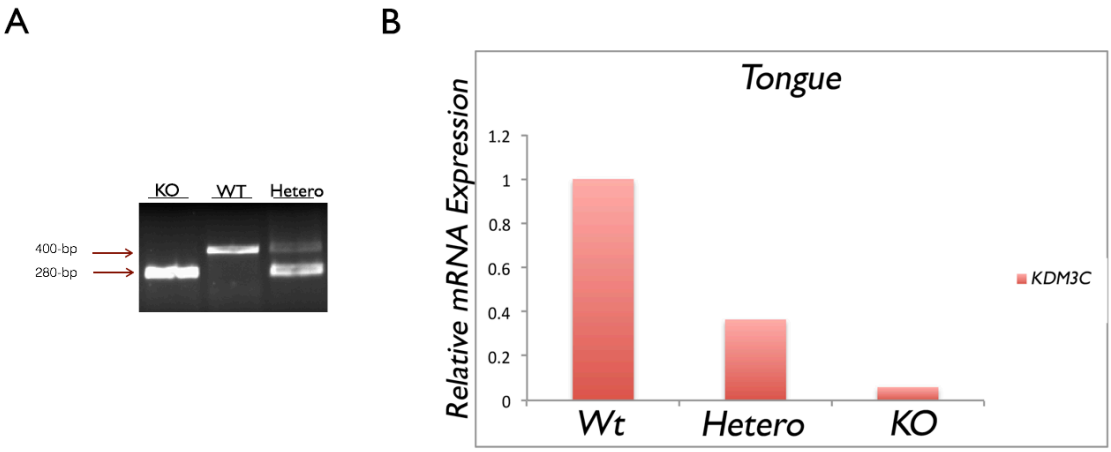

Figure 2. Generation of KDM3C knockout mice.

KDM3C knockout (KO) mice were established by crossing KDM3C heterozygous male mice and with heterozygous female mice. (**A**) Wild-type and mutant alleles of KDM3C were genotyped by semi-quantitative PCR. (**B**) KDM3C knockout was confirmed by assessing KDM3C mRNA levels in tongue tissue of WT and KDM3C KO mice tongue by qPCR.

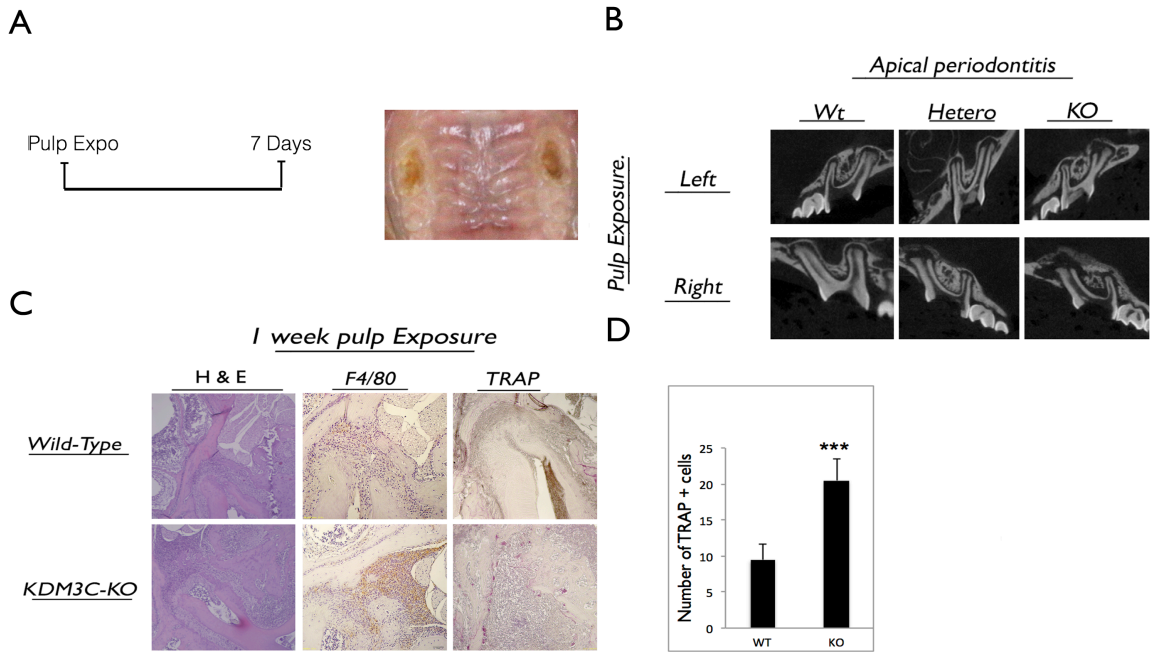

Figure 3. KDM3C ameliorates bone loss in apical periodontitis model in vivo.

(A) Schematic showing the duration of the pulp exposure and harvested maxilla harboring bilaterally pulp exposed first molars. (B) Micro-CT analysis of the maxillary first molar in KDM3C-WT, Hetero, KO was performed, followed by (C) histological analysis of the apex by H&E, F4/80, and TRAP staining. (D) TRAP+ osteoclasts around the apex of the root ($P < .05$) were quantified. Statistical difference was measured by t-test ($P < .05$).

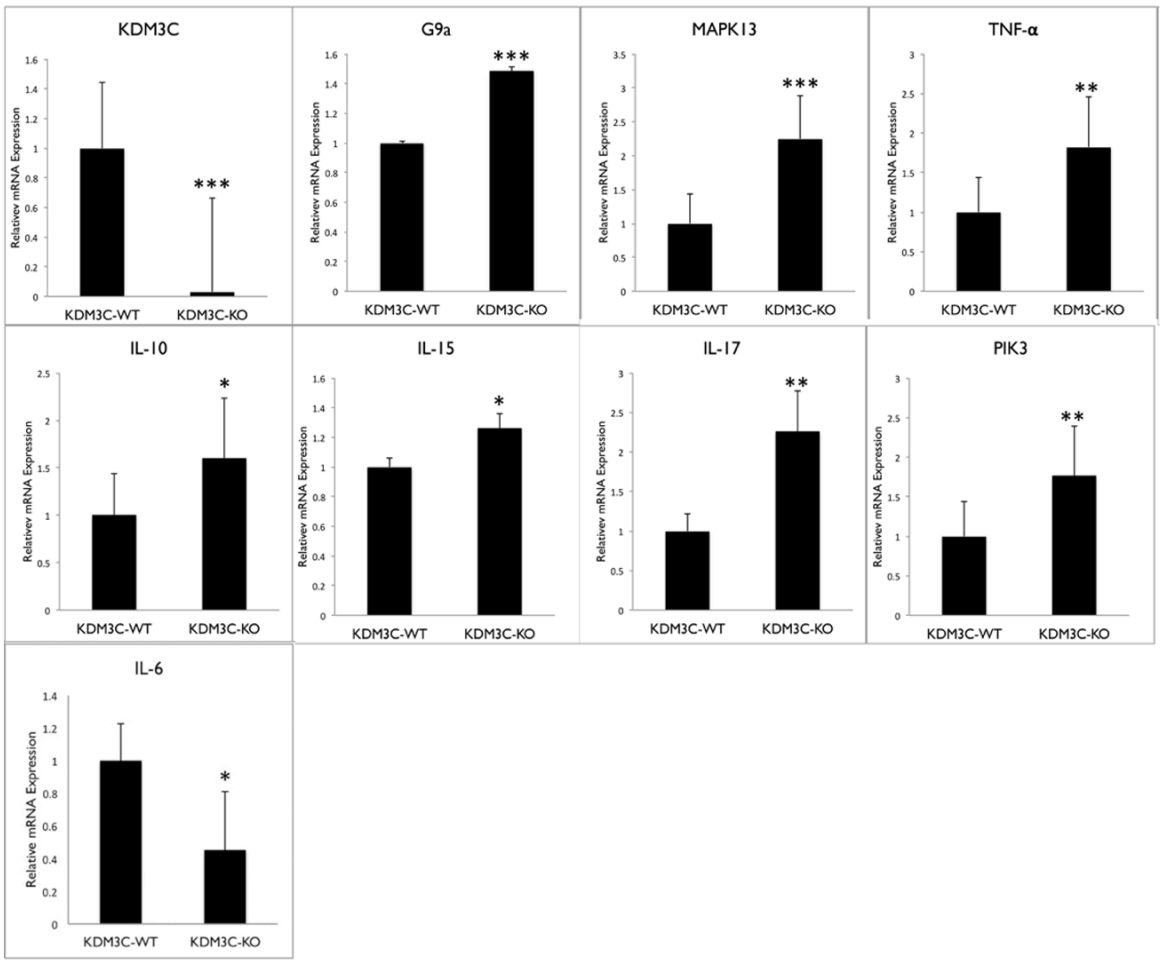

Figure 4. KDM3C knockout induces increased inflammatory signaling.

mRNA expression of KDM3C, G9a, MAPK13, TNF-a, IL-10, IL-15, IL-17, PIK3, and IL-6 were assessed using RT-PCR. Statistical difference was measured by t-test ($P < .05$).

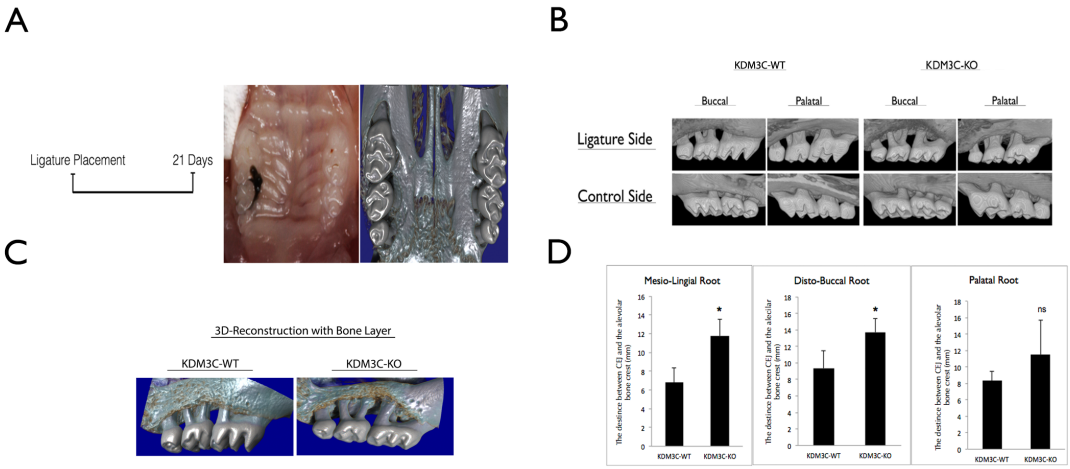

Figure 5. KDM3C ameliorates bone loss in ligature-induced periodontitis model in vivo.

Ligature was placed around the maxillary left second molar for 21 days. (**A**) Harvested maxilla possessing ligatured molar was visualized by 2D micro-CT analysis, and (**B**) micro-CT analysis of the buccal and palatal sides was also performed. (C) 3D reconstruction was performed with bone layer, and (D) the distance between CEJ and alveolar bone crest was measured. Statistical difference was measured by t-test ($P < .05$).

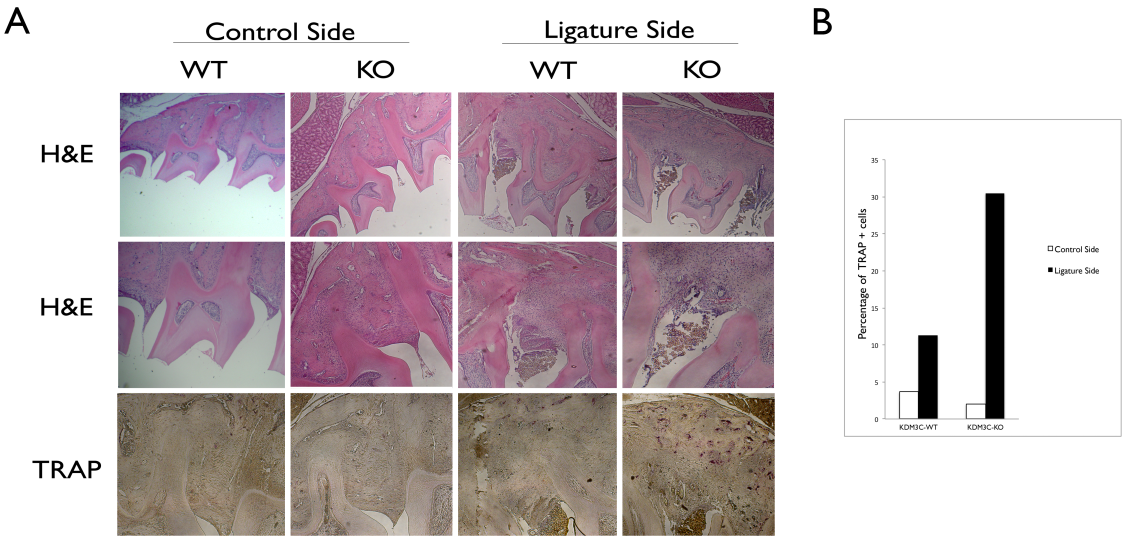

Figure 6. Loss of KDM3C induces osteoclast activation leading to more bone loss in ligature induced periodontitis in-vivo.

(**A**) Maxillas containing ligature-placed second molars were histologically assessed by H&E and TRAP staining. (**B**) TRAP+ osteoclasts around the second molar ($P < .05$) were quantified. Statistical difference was measured by t-test ($P < .05$)

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