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The Role of KDM3C in Oral and Systemic Inflammation During Aging

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The Role of KDM3C in Oral and Systemic Inflammation During Aging

A dissertation submitted in partial satisfaction of the

requirement for the degree Doctor of Philosophy in

Oral Biology

by

Abdullah Mohammed A. Alshaikh

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ABSTRACT OF THE DISSERTATION

The Role of KDM3C in Oral and Systemic Inflammation During Aging

by

Abdullah Mohammed A. Alshaikh

Doctor of Philosophy in Oral Biology

University of California, Los Angeles, 2022

Professor Mo K Kang, Chair

Aging is associated with a decline in innate and adaptive immune functions resulting in a weakened immune response against infection. Clinically, this decline in immune function leads to age-associated pathologies, including atherosclerosis, type II diabetes, dementia, obesity, and cancer systemically and prevalence of periapical bone loss the prevalence and severity of periodontitis orally. Previously, our lab identified an epigenetic marker KDM3C as a novel regulator of inflammatory signaling. In addition, we demonstrated that KDM3C regulate inflammatory signaling against oral bacterial infection through suppression of NK-κB signaling in macrophage (MΦ). In addition, our preliminary results showed KDM3C expression was inhibited in the aged mice compared to a younger

cohort; thus, we hypothesize KDM3C is a key regulator for macrophage polarization during aging. In this project, we found that 1) old (18-20mo) mice had increase in apical bone loss and alveolar bone loss compared to young (2mo) mice following pulp exposure model (PE) and Ligature induced periodontitis model (LIP) respectively; 2) *Kdm3c*$^{-/-}$ mice revealed increased periapical bone loss compared to the *Kdm3c*$^{+/+}$ mice upon PE. Similarly, *Kdm3c*$^{-/-}$ mice developed increased alveolar bone loss with ligature-induced periodontitis (LIP). 3) KDM3C deletion in mice resulted in increase in inflammatory cytokines levels in liver and spleen. Additionally, we characterize the KDM3C role in macrophage polarization. Here we found that, 1) Anti-inflammatory MΦ (M2) markers were significantly decreased in bone-marrow derived macrophages (BMDM) and in gingival tissue. 2) Histological staining of CD206 expression, a surface marker for M2, showed significant decrease in the positive staining of CD206 in *Kdm3c*$^{-/-}$ following LIP.

The thesis of Abdullah Mohammed A. Alshaikh is approved.

Flavia Queiroz de Mo Pirih

Renate Lux

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Dedication

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Biographic sketch

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INTRODUCTION

1.1 The process of aging and the evolution of the immune system.

The human life cycle involves multiple stages starting from infancy to elderly. During each stage, the human being undergoes several physiological and biological changes that affect different parts of the individual body system, including the immune system [1]. In the early life of an individual the immune system is exposed to many environmental conditions including, viruses, pathogens, and fungi that shapes the immune system, as the human ages, the immune system will gradually develop and mature[1]. At a certain age, which could be different from an induvial to other, the immune system will start to decline leading to several aging associated diseases [1].

Aging refers to the process of getting old. Aging research start to attract many researchers in the last decade due to the increase in the number of aged individuals, known as 65 and older. In fact, it's been predicted that by 2050, 25% of the United States population will be considered aged [2] Aging is a slow process occurs over lifetime of an induvial and is associated with changes in several physiological and biological process that evolves to adapt to certain environment[1, 3]. One of the physiological systems that shows marked changes during aging is the immune system [1, 3]. The immune system undergoes significant changes in aged individuals that involves both arms, the innate and the adaptive systems [3-5]. The impact of aging on the immune system is known as "immunosenescence" [5, 6]. Immunosenscenece refers to the decline in the immune response in aging [5, 6]. Immunosenscenece has been associated with many age related diseases, including autoimmune disease, cancer, dementia and periodontitis [6]. Another aging phenotype that is related to the immune dysfunction is inflammaging [4, 5]. Inflammaging refer to the increase of inflammatory cytokines in aging and it was coined by an Italian research Claudio Franceschi[7]. Inflammaging is associated with an increase in the proinflammaotry status including (pro-

inflammatory cytokines and chemokines) in aged individuals in the absences of disease "Sterile inflammation" [4, 5, 7]. Further studies aimed to establish distinct definitions for inflammging and Immunosenscenece, some scholars reported that inflammaging is more related to the innate immune system [8], whereas Immunosenscenece refers to the decline in the adaptive part of the immune system [6]. Collectively, its important to note that aging affects both the innate and the adaptive immunity.

1.2 The immune system.

The immune system composed of several cells that originate from the hematopoietic stem cell [9, 10]. The hematopoietic stem cell differentiates into two main cells that give the rise to the immune cells that regulate the immune system. These two cells are known as myeloid and lymphoid progenitor cells [9, 10]. The myeloid progenitor cells differentiae to cells that play an important role in the innate immune cells including, myeloblast, mast cells, and erythrocyte (red blood cells) [11]. Myoblast produce granulated cells such as basophil, neutrophil, eosinophil and monocytes [11]. Furthermore, monocytes give the rise to macrophage and dendric cells both of which are initiator of the inflammatory process [9, 11]. On the other hand, lymphoid progenitor cells produce lymphoid cells including B-lymphocyte and T-lymphocyte [9, 11]. Both of which play a key role in the adaptive immune system.

The immune system reacts against pathogen through a process known as inflammation [9, 11]. Inflammation refers to the mechanism by which the immune system defends the body from harmful agents/pathogens such as bacteria [9, 11]. Upon the exposure of the body to bacteria and bacteria byproducts, the body start to send signals to activate the immune system [12, 13]. The first immune cells to respond are the innate cells such as macrophages and dendric cells, this action

takes place in the first few hours of exposure to pathogens [12, 13]. Later on, the immune system start to recruit the adaptive immune cells to the site of inflammation, these includes T-cells and B-cells [12, 13]. Once the infection is eliminated, the body will activate a series of signals to start the healing process [12, 13]. This process involves multiple cells and signals that is strictly controlled by specific enzymes and regulators [12, 13]. A defect in the immune reaction, may lead to an overreaction in the inflammatory process (chronic inflammation) which will lead to tissue damage. On the other hand, an underreaction may lead to systemic dissemination of the infection. Thus, its critical to regulate the inflammatory response to an infection as well as to regulate the transition from the innate immunity to the adaptive immunity to achieve pathogens elimination with minimum tissue damage[9, 12, 13].

One of the key regulators in the inflammatory process is macrophage[9, 14]. Macrophage take part of the innate response as well as presenting the antigen and activating the adaptive immune response[9, 14]. In addition, macrophage are known as an important regulator for inflammation due to their role in the initiation and resolving the inflammatory process.[9, 14]

1.3 The changes in the innate immune cells during aging.

The innate immune cells is composed of multiple cells that play an important role in defending the body from harmful pathogens [9]. The immune cells are capable of recognizing and reacting to pathogens through receptors that can identify the molecular patterns of pathogens known as pattern-recognition receptors (PRRs) [5]. There are three different categorizes of PRRS, each one of them have a unique role to initiate some sort of inflammation. The first categories of PRR is toll-like receptors (TLRs) [4, 5, 15]. TLRs, are proteins that are expressed by innate immune cells such as macrophage and they have the capacity to recognize pathogens byproducts such as

lipopolysaccharide (LPS)[15]. Activation of TLRs by LPS leads to an intracellular signal that eventually activates the nuclear factor-keppa beta (NF-Kβ). NF-Kβ activation results in the production of various cytokines and chemokines including IL-6 and TNF-a [4, 5]. The second category of PRR involve receptors that activate inflammasome known as the NOD-like receptors [16]. Inflammasome activation results in the production and secretion of IL-1, IL18, and IL-33 [16]. The last category of PRR include Rog-like receptors (RLRS) which recognize viruses leading to the production of interferons [16].

Aging-associated immune changes such as inflammaging affect many innate immune cells [5]. These changes can be summarized into a couple of phenotypes. 1- Overstimulation of the immune system [17]. 2- Underfunction or immune paralysis [5]. Regulating gene expression and inflammatory cytokines to over/under produce inflammatory factors could be epigenetic modifications[18]. Epigeneitc regulation play a role in both innate and adaptive immune cells[18]. Recent studies have focused on the impact of epigenetic regulation in macrophages[18, 19], Macrophages are considered keystone of inflammation and inflammaging due to their ability to modify their phenotype to produce pro and anti-inflammatory factors including cytokines and chemokines[14].

1.4 Macrophage polarization.

Macrophage are part of the myeloid immune cells distinguished by phagocytic activity to remove hazardous agents[20]. In addition, macrophages have the capacity to promote and resolve inflammation as well as tissue healing[20]. Macrophages are capable to change their phenotype based on the signals they receive from the inflammatory site e.g. presence of LPS and/or IFN-γ produces pro-inflammatory macrophages (M1). On the other hand, the presence of IL-10 and IL-

13 promotes anti-inflammatory macrophages (M2) [14, 21]. Macrophage can switch from one phenotype to another depending on their signal [14, 21]. Previously, it was hypothesized that native macrophage (M0) can polarize to either of M1 or M2. However, recent studies started to demonstrate that macrophage can repolarize, meaning that an M1 can change to an M2 based on the inflammation status[22]. Additionally, recent studies identified subtypes of polarized macrophage including M2a, M2b, M2c, and M2d[23].

1.5 The basis behind apical periodontitis (AP) and marginal periodontitis (PD) 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, gram positive or gram negative, or based on their oxygen needs , aerobic/oxygen dependent or anaerobic/oxygen independent [24-26]. 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 [26]. The gram-negative bacteria's outer cytoplasmic membrane contains a unique endotoxin component that stimulates the inflammatory response, known as LPS, contains a lipid A moiety, an O antigen polysaccharide, as well as a core that holds the polysaccharide [26]. 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 [27-31]. 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 PD or AP, respectively [32, 33] . 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 [30, 32-36]. 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 [37, 38]. The need for effective combination therapy becomes even more imperative when considering the association between many chronic oral infections and systemic diseases [39, 40]. 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 [39, 40]. 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[41, 42], and that oral bacteria have the ability to travel from the site of infection to the other sites in the body [43]. 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 [44-46].

1.6 Basic molecular mechanism of Oral inflammation.

Once oral bacteria colonize in the oral tissue, they begin secreting byproducts such as endotoxins or lipopolysaccharide (LPS) [29, 30], and LPS in turn binds to Toll-Like Receptors (TLR) 2 and 4 on epithelial cells [47, 48]. 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 [48, 49]. However, a recent study demonstrated that *P. gingivalis* can bypass TLR4 activation through the production antagonistic A lipid [50]. 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 [50]. 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 [51, 52]. 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 [53]. 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 [54, 55]. Recently, a new subset of T-cells, Th-17 and Treg, were identified that are regulated by IL-17 and FOXP3, respectively [56, 57]. 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 [32]. *P. gingivalis* induces a local low chronic inflammatory response that results in oral inflammatory bone destruction, which manifests as periodontal or pulpal disease [58]. 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 [58]. 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 [59].

1.7 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 [60]. Epigenetic regulation involves micro-RNA (miRNA), DNA methylation (DNMTs), and histone modification [60]. Histone proteins can undergo several distinct epigenetic modifications, including methylation, ubiquitination, and acetylation[60]. These modifications are carried out by specific epigenetic enzymes, including lysine-specific 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[18, 60]. 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 [18, 60, 61]. Histone modification enzymes modify distinct histone residues and are thus site specific, as is their effect on gene expression [18]. In general, histone methylation results in transcription repression, while histone acetylation generally results in transcriptional activation [61]. However, methylation of lysine 4 on histone 3 (H3K4) results in transcriptional activation [62]. 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.

1.8 The epigenetic mechanism of inflammation:

Recently, the role of epigenetic regulation of immune response has become of great interest. De santa et. al. [63] 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 [64], 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 [65]. Another study by Satoh et. al. demonstrated the role of Jmjd3, a histone demethyltransferase for H3K27, in promoting M2 macrophages, with Jmjd3 inhibition leading to impairment differentiation BMDM[66]. Our laboratory has identified KDM3C, an H3K9me1/me2 demethylase, as key regulator of

inflammatory cytokine transcription[19]. Thus, understanding the epigenetic regulation of cytokine secretion, immune activation and macrophage polarization may aid in the development of novel therapeutics aimed at improving treatment outcomes of periodontitis and apical periodontitis.

MATERIALS AND METHODS

2.1 Mice

Heterozygous Kdm3C$^{+/-}$ mice were obtained from Riken (Tsukuba, Japan), courtesy of Dr. Makoto Tachibana (Tokushima University, Tokushima, Japan), and crossed to generate homozygous Kdm3C$^{-/-}$ mice. Kdm3C $^{+/+}$. Mutant alleles were identified by PCR according to the method published by Kuroki et al[67]

We used the WT primers and mutant primers (table 1), yielding products of 670 bp(WT) and 500 bp (KO). Aged male mice (18-20 month) C57BL/6 were obtained from the NIA colony at the Charles River Laboratory (Stone Ridge, NY). All mice were maintained on a 12-hour light/dark cycle with food and water available ad libitum.

To induce experimental apical periodontitis, mice were anesthetized with ketamine/xylazine (100 and 5 mg/kg body weight, respectively) by intraperitoneal injection. Pulp exposure was performed on the left maxillary first molar using a high-speed. round bur on a portable dental unit (Aseptico Inc, Woodinville, WA) under 10x magnification of an endodontic microscope (BM-LED Stereo Microscope; MEIJI Techno, Saitama, Japan). Exposed teeth were left open to the oral environment without any coverage. Mice were sacrificed, and maxillae were harvested at 21 days after pulp exposure.

To induce experimental periodontitis, mice were anesthetized by intraperitoneal injection of 100 mg/kg of ketamine hydrochloride and 5 mg/kg of xylazine, and a ligature was placed around the left maxillary second molar using 6-0 silk suture (Teleflex, Wayne, PA, USA) with a surgical knot in accordance with Abe et al [68]. The suture was gently placed to avoid any damage to periodontal tissue. After 21 days, the mice were euthanized, and maxillae were collected for further analysis.

To induce systemic inflammation, bacterial endotoxin Lipopolysaccharides from *Escherichia coli (Ec-LPS,* O111:B4 - Sigma-Aldrich) was given intraperitoneally (5mg/kg). mice were then anesthetized with isoflurane inhalation and organs were collected and immediately frozen until further needed for analysis. Non-injected mice were used as control. All animal studies were performed according to the guidelines of the University of California–Los Angeles (UCLA) Institutional Animal Care and Use Committee.

2.2 Micro Computed Tomography (µ-CT) analysis.

The harvested maxillae were fixed in 4% paraformaldehyde (Thermo Fisher Scientific) for 24 h and scanned for µ-CT using Scancom-CT 40 (Scanco Medical, Br ¨uttisellen, Switzerland) using 1-mm aluminum filter and a voxel size of 10 mm^3 in high-resolution settings at 60 kVp and 166 mA. The reconstruction was performed by NRecon Reconstruction software (Bruker, Billerica, MA, USA), and 3-dimensional images were reconstructed using DataViewer CTVOx and CTvol software (Bruker). Morphologic parameters of apical periodontitis in maxilla were assessed using the CTAn software (Bruker) as previously described by Bouxsein et al[69]. The extent of periapical radiolucent area was measured by calculating the difference between the bone volume and tissue volume from the first molar mid root to the apex of the radiolucent area. To measure the alveolar bone height changes in the periodontitis model, we calculated the distance from the cemento-enamel junction to the alveolar crest from the infected site and normalized with the control site. After the scanning, the tissues were decalcified in 5% EDTA and 4% sucrose in PBS (pH 7.4) for 3 weeks at 4°C and sectioned for histologic analyses.

2.3 Tissue processing and Histology analysis.

After completion of μ-scanning, maxillae were sent to the Translational Pathology Core Laboratory (TPCL) for paraffin embedding and sectioned to 5-μm thickness using microtome and slides were dewaxed before any histological staining. Hematoxylin-eosin (H&E) staining and tartrate-resistant acid phosphatase (TRAP) histochemical staining were performed as previously described [70]. Briefly, H&E staining was performed with sectioned tissues using Richard Allen signature series™ Hematoxylin 7211 solution and Eosin-Y solution (Thermo Fisher Scientific). To detect TRAP-positive cells, tissue sections were stained using the Acid Phosphate Leukocyte (TRAP) Kit (Millipore Sigma) following the manufacturer's instructions. The stained sections were washed with in deionized water, counterstained with Aniline blue or Hematoxylin, and mounted with Cytoseal™ (Thermo Fisher Scientific). Histomorphometry analysis of osteoclast was carried out using the TrapHisto software (University of Liverpool, Liverpool, UK) as previously described [71]. *In situ* expression of CD206 were determined in mouse oral mucosal tissue specimens by immunohistochemistry (IHC). Positive CD206 staining was quantified per each sample and scored using ImageJ software version 1.48 (National Institutes of Health, Bethesda, MD) on digital pictures taken using an Olympus microscope (model DP72; Olympus, Tokyo, Japan) at 100× magnification.

2.4 Cell culture.

Bone marrow cells were obtained by flushing the femurs and tibia of (6–8wk old) Kdm3c wildtype (WT) or KO C57BL/6J mice. BMDMs were cultured in a–minimum essential medium (a-mem) (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution and differentiated with 30 ng/ml macrophage colony-stimulating factor

(M-CSF) (R&D Systems, Minneapolis, MN, USA) for 7 days. To induce macrophage polarization, BMDM cultures from young, old, and young *Kdm3C* ko mice were fully differentiated for 7 days with M-CSF, medium was changed every 2 days and M-CSF is added every time the medium is changed. On day 7 (M0), the medium with M-CSF is removed and the cells are washed with sterile phosphate-buffered saline (PBS) and a fresh a–mem without M-CSF is added. To induce M1 macrophage BMDM cells were stimulated with *Pg* LPS (100 ng/ml; Invivogen) + IFNγ (20 ng/ml; R&D Systems) and to induce M2 cells were stimulated with IL-4 (10 ng/ml; Invitrogen) + IL-13 (10 ng/ml; Invitrogen). After 24 hour of stimulation M1/M2 polarized cells were harvested qRT-PCR analysis. Spleen cells (splenocyte) were cultured by homogenizing spleen from young (2-3 month) and old (18-20 month) mice. Following mouse euthanization, spleen was collected and placed in a petri dish containing PBS. Carefully the spleen was minced into small pieces using micro-scissors. The excised spleen pieces were placed on 70μm mesh cell strainer (Corning Falcon™) over 50 ml conical tube. Using the end of the syringe as a plunger, the spleen tissue was mased through the strainer. The cells were centrifuge for 5 minutes at 400g. supernatant was discarded, and the cell pellet was resuspended with red blood cell (RBC) lysis buffer and incubated on ice for 5 minutes. Next, the cells were suspended with PBS and centrifuge for 5 minutes at 400g. supernatant was discarded, and the cells were resuspended and cultured in Roswell Park Memorial Institute (RPMI)1640 Medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. To induce acute inflammatory signaling, M-CSF– differentiated BMDMs and splenocyte were challenged with *Pg*LPS from InvivoGen (SanDiego, CA,USA) and LPS from Escherichia coli (*Ec*LPS) (MilliporeSigma) respectively, for various time points from 0 to 24 h, and the cells were harvested for biochemical assays.

2.5 RNA extraction and quantitative real-time RT-PCR.

Total RNA was isolated from cells or tissues using the TRIzol reagent (Invitrogen™) and PureLink™ RNA mini kit (Invitrogen™) according to the manufacturer's instructions. DNA-free total RNA (1 µg) with Random Primer (Thermo Fisher Scientific) was used for reverse transcription by Super- Script II Reverse Transcriptase (Thermo Fisher Scientific). Real-time qPCR was performed using a QuantStudio 3 (Thermo Fisher Scientific) and PowerUp SybrGreen MasterMix (Thermo Fisher Scientific) to measure the expression of genes under the following conditions: 45 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 60 s. The comparative Ct (DDCt) method was used to determine the relative gene expression levels. For normalization of the cycling threshold values obtained with the experimental samples, 18s ribosomal RNA was amplified in the same condition. Primer sequences of the genes are detailed in the Table 1 . All experiments were independently repeated at least 3 times to ensure reproducibility.

Genes	Forward (5'-3')	Reverse (5'-3')
mKdm3C	CCTTGGTGATGCCATTGTTT	TCGGTCACCTGAACACAGC
mIl6	GCTACCAAACTGGATATAATC	CCAGGTAGCTATGGTACTCCA
mIl1b	CACAGCAGCACATCAACAAG	GTGCTCATGTCCTCATCCTG
mTnf	TCAGGTTGCCTCTGTCTCAG	GCTCTGTGAGGAAGGCTGTG
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Nos2	GCTACCAAACTGGATATAATC	CCAGGTAGCTATGGTACTCCA
mArg1	TGGCCCAGAAATCAAGGAGC	CAGCAGACTCAATACACACT

mRetnla	CATCCATCTCGT-GCACTTGT	CATCTATCCAGTTGGCCTCTG
mChil3	CAGGGAGAGCTTCATCTGTGT	GCTGAGCTTTGAGGGATGAT
mKdm3C WT	CTCTGTAGTCCCCGCACTCA	CTAGCATTGAATCACAGGGGCTG
mKdm3C KO	CCTTCTTGACGAGTTCTTCTGAGG	CTAGCATTGAATCACAGGGGCTG

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

2.6 Western blotting.

Cells were harvested in cold PBS by scraping and tissue were homogenized using a plunger. Both were lysed in the lysis buffer (20 mM Tris-HCl [pH 7.5], 150mM NaCl, 1% Triton X-100, 10% Glycerol, 1X Roche Complete Protease Inhibitor cocktail and 1X Roche PhosSTOP™ phosphatase inhibitor). Protein concentration was determined using the Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). The 4X sample buffer, containing 200 mM Tris-HCl (pH 6.8), 400 mM β-mercaptoethanol, 8 % SDS, 0.02 % Bromophenol Blue, and 40 % Glycerol, was added to same amount of proteins, and samples were boiled at 95 °C for 10 min. Protein samples were separated by SDS-PAGE, transferred to a PVDF membrane filter with 0.45 µm pore size (EMD Millipore, Burlington, MA, USA), and subjected to Western blot analysis. Chemiluminescence signal was detected using a ChemiDoc™ Touch Imaging system (Bio-Rad) with the Clarity™ or Clarity Max™ Western ECL Substrate (Bio-Rad). Following antibodies were used for Western blot analyses: mouse anti-P-Stat6 (#56554; Cell signaling technology, Danvers, MA, USA); mouse anti-Stat6 (#5397; Cell signaling technology); mouse anti-KDM3C (D356-3) from Medical & Biologic Laboratories (Nagoya, Japan); mouse anti-AMPK (#2532; Cell signaling technology); mouse anti-P-AMPK (#2535**;** Cell signaling technology) anti-GAPDH (sc-25778)

from Santa Cruz Biotechnology (Dallas, TX, USA); and anti-β-ACTIN (A5316; Millipore Sigma).

2.7 Flow cytometry

Following mouse euthanization with isoflurane, thymus, spleen and lymph nodes were isolated and collected in 15 ml conical tube with (RPMI)1640 Medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. Tissues were transferred to 70 μm cell strainer 50 mL tube and were homogenized using a plunger. When every single tissue is fully homogenized, the cells were centrifuged for 5 min at 400 x g. Supernatant was discarded and the cells were resuspended with the RPMI medium. Cell number for each tissue was counted using counting chamber. Staining of splenic, thymic and lymph nodes single-cell suspensions with fluorophore-conjugated anti-mouse antibodies according to manufacture instructions Anti-mouse CD4 APC-eFluor 780 (eBioscience); Anti-mouse CD8a PE-Cy7 (eBioscience); Anti-mouse CD62L eFluor 450 (eBioscience); Anti-mouse CD44 Alexa Fluor 700 (eBioscience).

2.8 ELISA for cytokines

The concentration of mouse IL-6, IL-1β, and TNF-α in supernatant from splenocyte and blood serum was measured with Ready-Set-Go ELISA kits (Thermo Fisher Scientific) following manufacturer's instruction. Each sample was tested in triplicates in every experiment, and values were calculated on the basis of a standard curve constructed for each assay.

2.9 Statistical analysis.

Statistical analyses were performed with an unpaired Student's t test for 2 groups using Prism 6 software (GraphPad Software, La Jolla, CA, USA). A value of P, 0.05 was considered as a statistically significant difference. The graphs represent mean values and include SD unless stated otherwise. All experiments were repeated 2 or 3 times. The sample size of the animals used in our experiments and of the public datasets are indicated in the respective figure legends.

RESULTS

3.1 Ligature induces time-dependent increase in periodontitis in mice.

To determine the role of epigenetic regulation of inflammatory response on oral diseases, we established a ligature-induced periodontitis (LIP) model in which we ligated maxillary second molars with a silk suture material and left the ligature intact for a specific time point. Ligature placement resulted in development of alveolar bone loss known as periodontitis. In order to observe time dependent progression of periodontitis in mice, we sacrificed the mice at 3, 7, 10, and 21, days post-ligature placement (Appendix Figure 1A). Periodontitis development progressed in a time dependent manner, and although periodontitis was radiographically observed 7 days post-ligature placement (Appendix Figure 1B, C) increase in inflammatory cytokines *IL-6* was observed as early as 3 days post- ligature (Appendix Figure 1D). Our observation from this time point experiment that acute inflammation occurred as early as 3 days which reflect the acute phase of the inflammatory process. In addition, we observed that *IL-6* started to go down following 3 days and reach the lowest expression by 10 days. At 21 days, we noticed *IL-6* started to increase again, possibly reflecting the chronic phase of the inflammatory reaction in our oral disease model. Moreover, we observed an increase in the expression of *KDM3C* at 21 days. Thus, a time point of 21 days post-ligature was selected for additional experiments comparing the periodontitis development between young and old mice as well as KDM3C-WT and KDM3C-KO mice. These data indicate that ligature placement and bacterial accumulation around the tooth and the gingival tissue induces inflammation to the alveolar mucosa.

3.2 Aging exacerbate bone loss in oral inflammatory diseases in mice.

Next, we investigated the role of aging in the development of Ligature induced periodontitis (LIP) and pulp exposure induced apical periodontitis (PE) in mice. Periodontitis was induced by placing a ligature around maxillary second molars in young (2 mo.) and old (18-20 mo.) mice. Similarly, apical periodontitis was induced by exposing the pulp tissue of maxillary first molars in young and old mice. After 21 days, μCT scanning revealed significantly increased bone loss in the LIP mice **(Figure 1A, B, and C),** and infiltration of inflammatory cells to the alveolar mucosa as observed by H&E staining on the histological specimens **(Figure 1A).** Additionally, we were interested to observe the effect of aging in the development of apical periodontitis. Following 21 days, μCT images revealed significant increase in the bone loss at the periapical area as well as periapical infiltration of inflammatory cells as observed by H&E staining (Figure 1D and E). These findings indicate that aging exacerbate the development and severity of oral inflammation in both apical periodontitis and alveolar periodontitis.

3.3 Aging enhances and exacerbate the inflammatory response to systemic exposure to bacterial endotoxin LPS.

In order to study the impact of aging in response to external toxins. Young and old mice were exposed to Ec-LPS via intraperitoneal administration. Following 5 days of Ec-LPS exposure mice were sacrificed, lung, liver, and spleen as well as blood serum were collected for further analysis. As expected, tissues from aged mice without Ec-LPS exposure revealed increase in tissue inflammation compared to tissues from young mice, this was evident in histological sections in liver, spleen and lung tissues (Figure 2A). Following Ec-LPS exposure, substantial increase in inflammation and infiltration of immune cells was evident in liver, spleen, and lung from aged

tissue. In addition, inflammatory cytokines levels in blood serum and splenocytes in aged mice were significantly increased after Ec-LPS exposure compared to young mice (Figure 2B and C). To further test the role of aging on the expression of inflammatory cytokines, we harvested bone marrow cells from young and aged mice and induced their differentiation to BMDMs using M-CSF. Fully differentiated BMDMs were then challenged with pg LPS, and the levels of TNF-a , IL-6 and Il-1b were analyzed. Real-time qPCR revealed that the expression of these cytokines was strongly induced by Pg-LPS in BMDMs of both young and old mice, but the level of induction was severe in BMDMs from the old mice compared with those of the young (Figure 2D).

3.4 KDM3C is negatively regulated during aging.

To investigate the role of epigenetics in inflammatory process during aging, we screened for differential mRNA expression for mouse KDMs in BMDM. We utilized splenocyte for the screening. KDMs levels were determined by qPCR from young and old mice splenocyte. Among 7 KDMs, aging led to down regulation of *Kdm3C* and *Kdm2A* as well as an up-regulation of *Kdm6B* (Figure 3A). To confirm the altered expression of *Kdm3C* in aging, real time qPCR was performed for *Kdm3C* in liver, spleen, skin, thymus, and tongue. We found a significant decrease in *Kdm3C* mRNA expression in spleen, thymus, and tongue. Furthermore, western blotting was performed to address the protein expression of Kdm3C in various tissue in aged mice (Figure 3B). We found that Kdm3C protein expression was lost in spleen and skin tissues from aged mice (Figure 3C). These data suggest that *Kdm3C* expression is negatively regulated during aging specifically in spleen, thymus and oral tissue (tongue).

3.5 Kdm3C deficiency leads to increased alveolar bone loss in oral inflammatory lesions.

We next determined the role of *Kdm3C* in modulating the inflammatory responses to oral bacterial infections. This was accomplished by pulpal exposure to induce apical periodontitis in maxillary first molars or by ligature placement on maxillary second molars to induce periodontitis in Kdm3C WT and KO mice. After 21 days of post-pulpal exposure, μ-CT scans revealed enlarged periapical lesions in the Kdm3C KO mice compared with those of the WT littermates (Fig. 4A). H&E and TRAP staining confirmed increased infiltration of inflammatory cells and an abundance of osteoclasts at the periapex of *Kdm3C* KO mice compared with the WT mice (Fig. 4B, C). Likewise, ligature-induced periodontitis led to increased alveolar bone loss and an abundance of osteoclasts in *Kdm3C* KO mice (Fig. 4D–F).

3.6 Loss of Kdm3C did not affect T-cell number, activation, and differentiation.

Since we found that *Kdm3C* expression was lost in spleen and thymus, we questioned whether *Kdm3C* has a role on adaptive immune cells, specifically T-cells. Thymus, spleen, and lymph nodes were collected from $Kdm3C^{+/+}$ and $Kdm3C^{-/-}$. Total T-cell number was calculated through flowcytometry including both CD4 and CD8 T-cells. We found no significant difference in the total cell number between $Kdm3C^{+/+}$ and $Kdm3C^{-/-}$ (Figure 5A). Next, we examined whether KDM3C effect T-cell activation, CD4 T-cells from $Kdm3C^{+/+}$ and $Kdm3C^{-/-}$ were gated for CD25 and CD69 (T-cell activation surface markers). We found that both groups had the same percentage of cells expressing CD25 and CD69 (Figure 5B). Lastly, we tested the number of effector memory T-cells. Its generally accepted that T-cell population shifts from naïve cells to memory cells during aging. Thus, we questioned whether T-cell from $Kdm3C^{-/-}$ mouse will have similar phenotype to that aged mouse. Flowcytometry was utilized to assess the expression CD62L and CD44 surface

markers in both $CD4^+$ and $CD8^+$ T-cells. Similar to previous results, there was no difference noted in the frequency of effector memory T-cells between *Kdm3C*$^{+/+}$ and *Kdm3C*$^{-/-}$ (Figure 5C). These data suggest that KDM3C does not affect T-cell maturation, differentiation, and activation.

3.7 Kdm3C deficiency alters macrophage polarization.

We previously identified KDM3C as negative regulator to pro-inflammatory cytokines in macrophage[19]. Next, we questioned whether KDM3C affect macrophage polarization. we assayed oral tissues generated from *Kdm3C*$^{+/+}$ and *Kdm3C*$^{-/-}$ with and without Ligature. IHC staining for CD206 was utilized as surface marker for M2 macrophages, we found a significant increase in the CD206 positive staining in oral tissue from *Kdm3C*$^{-/-}$ compared to *Kdm3C*$^{+/+}$ (Figure 6A). Furthermore, we assessed M2 markers including Rentla, Chil3, and Mrc1 in gingival tissue from *Kdm3C*$^{+/+}$ and *Kdm3C*$^{-/-}$ mice with and without ligature placement. We observed a significant decrease in Chil3 mRNA expression but not in Rentla and Mrc1 in *Kdm3C*$^{-/-}$ mice (Figure 6B). In addition, the same markers were analyzed in macrophages from bone marrow cells from the same groups. BMDM were fully differentiated to M2 macrophage, M2 markers were analyzed using RT-qPCR. Our results demonstrate a significant decrease in Rentla, Chil3, and Mrc1 in M2 macrophages from Kdm3C$^{-/-}$ compared to Kdm3C$^{+/+}$ (Figure 6C). These data suggest that KDM3C play a role in modulating macrophage polarization.

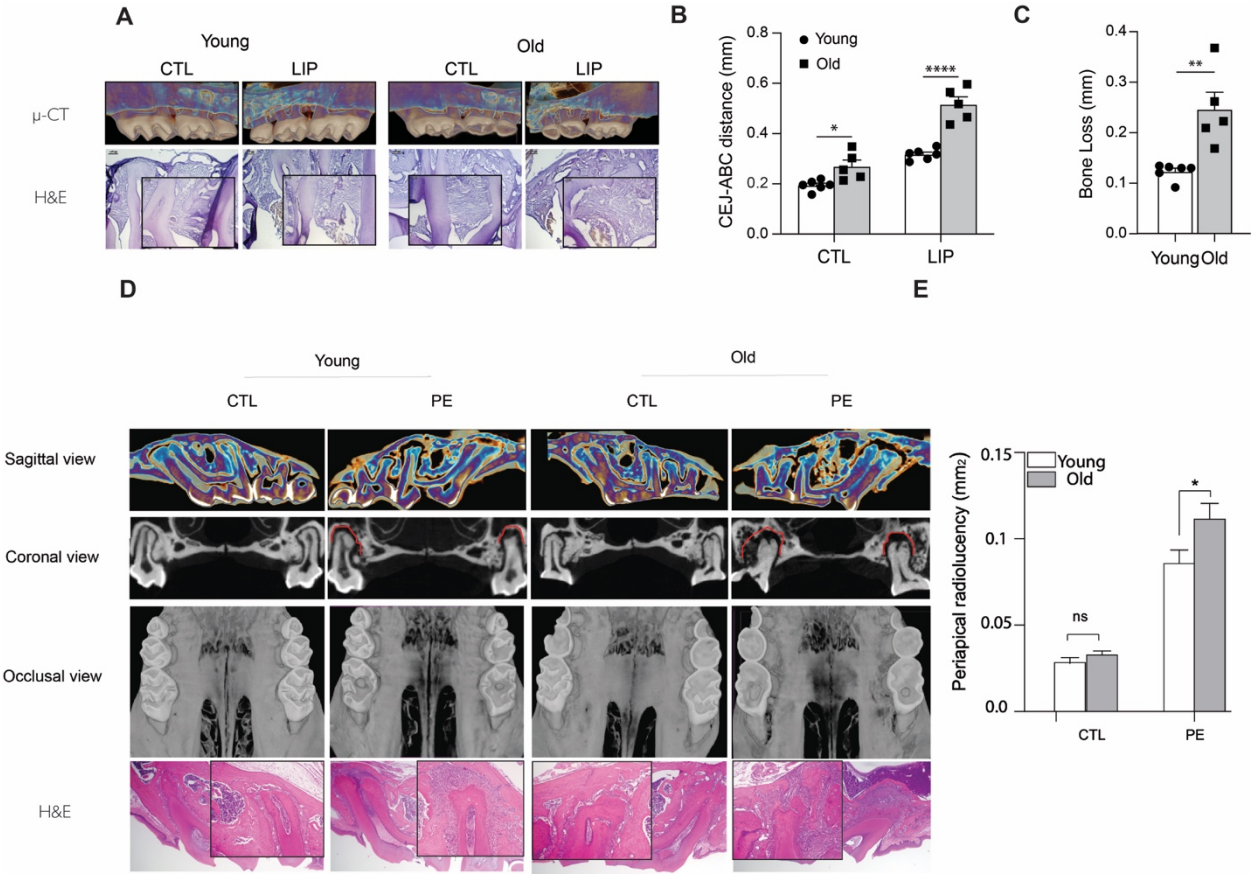


Figure 1. Aging increases inflammatory response against oral infection. (A) Experimental periodontitis was induced by ligature placement around maxillary second molar. After 21 days, μCT scan and H&E staining shows increased bone loss in aged mice. (B) Bone loss measured from cementoenamel junction (CEJ) to alveolar bone crest (ABC). (C) total bone loss was measured by subtracting the CTL (control) values from LIP values in both groups. (D). Experimental apical periodontitis was induced by exposing the pulp tissue in maxillary first molar. After 21 days, representative μCT and H&E images shows increased bone loss in aged mice. (E) increase in the bone loss was measured by quantifying the periapical radiolucency area around the first maxillary molar. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, $****P < 0.0001$. Results represent the means ± SEM performed in triplicate

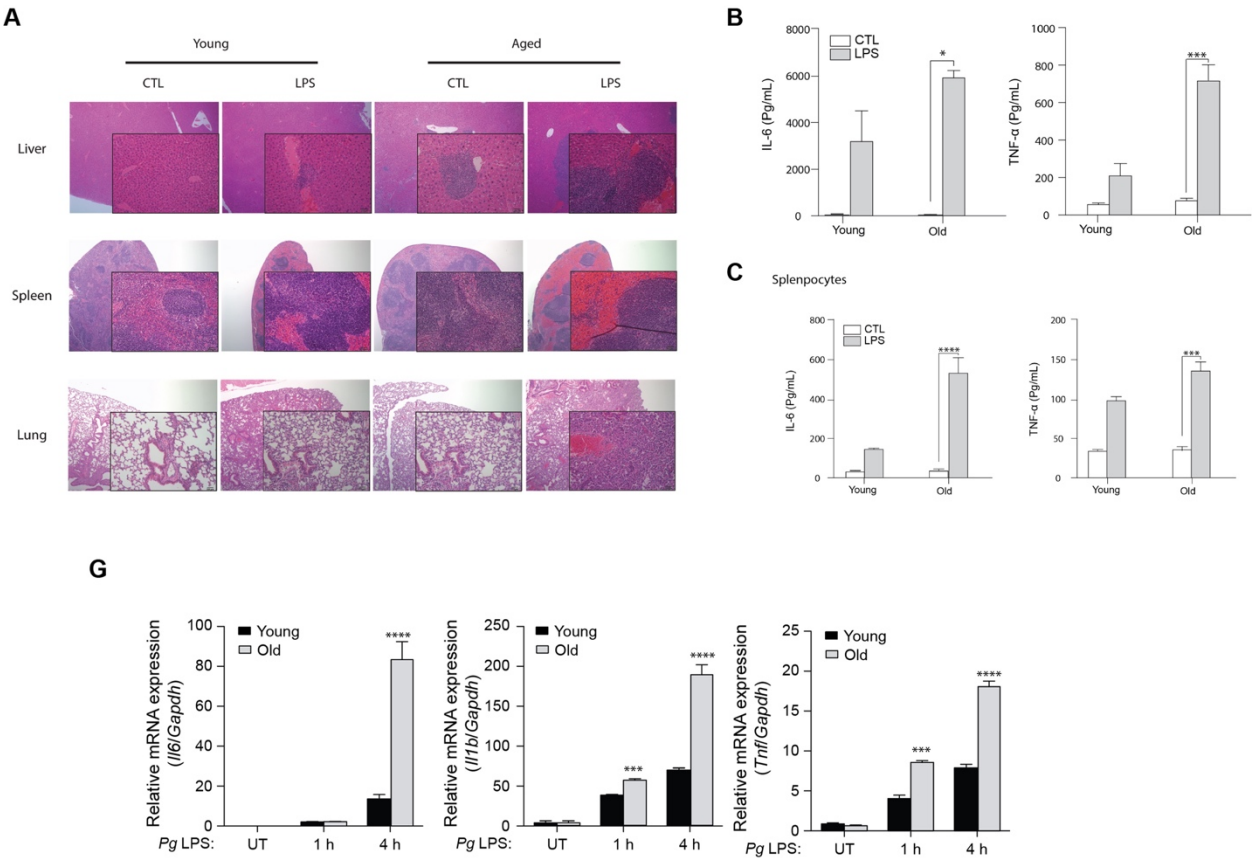


Figure 2. Aging exacerbates inflammatory response to LPS. (A) Young and old mice received an intraperitoneal injection with E-coli lipopolysaccharide (Ec-LPS). After 5 days, tissue were harvested and analyzed histologically. (A) Representative H&E stained images in Liver, spleen, and lung. (B) Secretions of cytokines TNF-a and IL-6 into blood serum were measured by ELISA. (C) Spleenocyte from untreated young and old mouse were challenged with Ec-LPS for 24 hours. Secretions of cytokines was measured in culture supernatant using ELISA. (D) BMDM from young and old were challenged with Ec-LPS, expression levels of il6, il1β and tnf were determined by qRT-PCR. Expressions values were normalized by 18S. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, $****P < 0.0001$. Results represent the means ± SEM performed in triplicate.

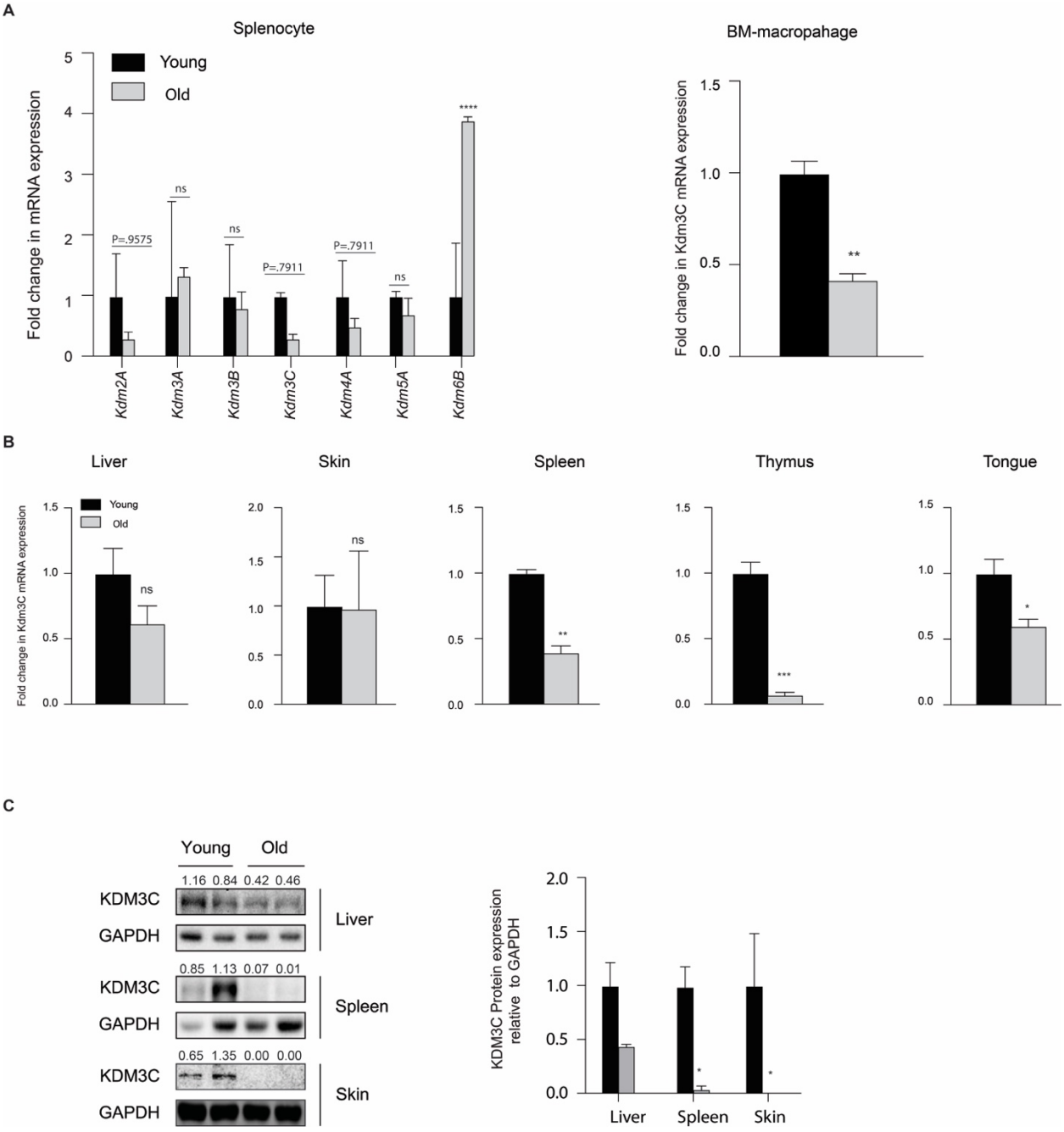


Figure 3. KDM3C expression is reduced in aging (A) mRNA expression profiling of KDMs in splenocyte was assessed by using real-time qPCR. Expressions values were normalized by 18S. (B) mRNA expression of Kdm3C in several tissues, including liver, skin, spleen, thymus and tongue, from young mice and old (n = 2). (C) KDM3C protein level was determined by western

blotting in liver, spleen and skin from young and old mice. GAPDH was used as loading control. Normalized densitometric values (from Image J) are shown above each band and quantified in the bar. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, ****$P < 0.0001$, ns >0.9999. Results represent the means ± SEM performed in triplicate

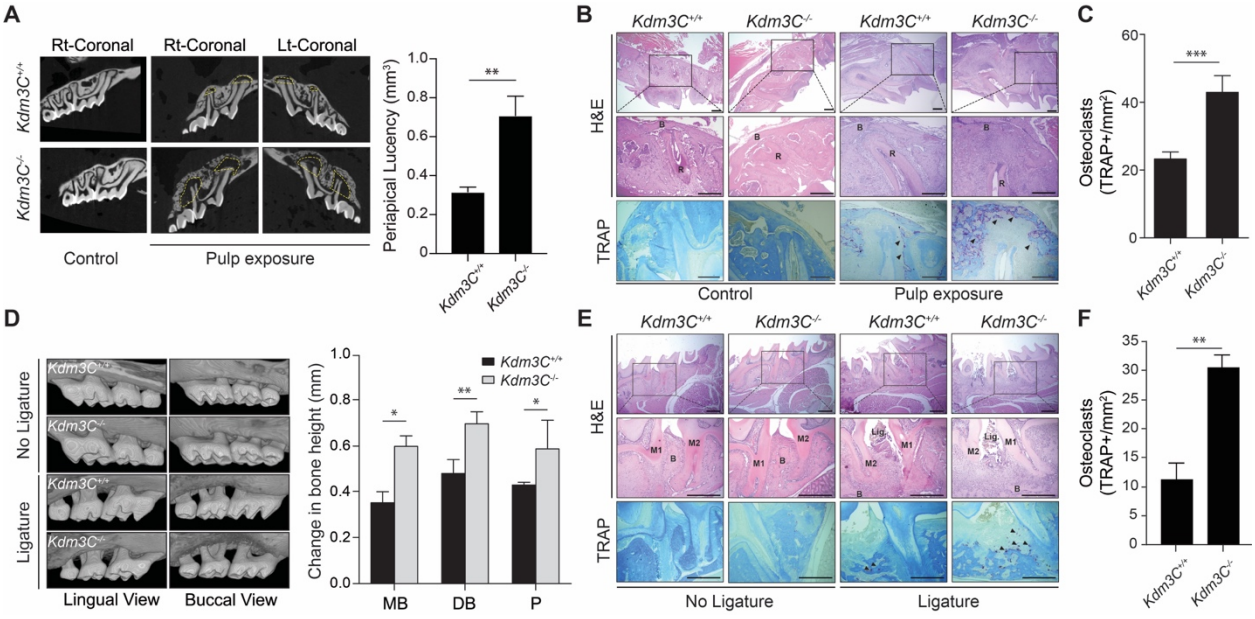


Figure 4. Loss of KDM3C exacerbates inflammatory response to oral infection.

Loss of KDM3C exacerbates inflammatory response to oral infection. *A*) Pulp exposure was done on both sides of the maxillary first molar from *Kdm3C* WT (*Kdm3C*+/+) and *Kdm3C* KO (*Kdm3C*–/–) mice. After 21 d, the maxilla was harvested for μ-CT analysis. Size of periapical lesions was measured by calculating the total loss of bone volume using μ-CT images. Data were expressed as means ± sem with 10 mice per group. *B*) Representative H&E- and TRAP-stained images. Arrows depict TRAP-stained areas. *C*) Quantification of TRAP$^+$ multinucleated osteoclasts number per square millimeter. *D*) Experimental periodontitis was induced by the ligature placement around the maxillary second molar for 21 d. μ-CT images of maxillary molars from *Kdm3C*$^{+/+}$ and *Kdm3C*$^{-/-}$mice were reconstructed. *E*) Representative H&E- and TRAP-stained photographs obtained. *F*) Quantification of TRAP$^+$ osteoclasts number per square millimeter (n = 3). Arrows depict TRAP-stained areas. B, bone; DB, distobuccal; Lig.; ligature; M1, first molar; M2, second molar; MB, mesiobuccal; P, palatal roots; R, root. Scale bars, 500 μm. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with *Kdm3C*$^{+/+}$

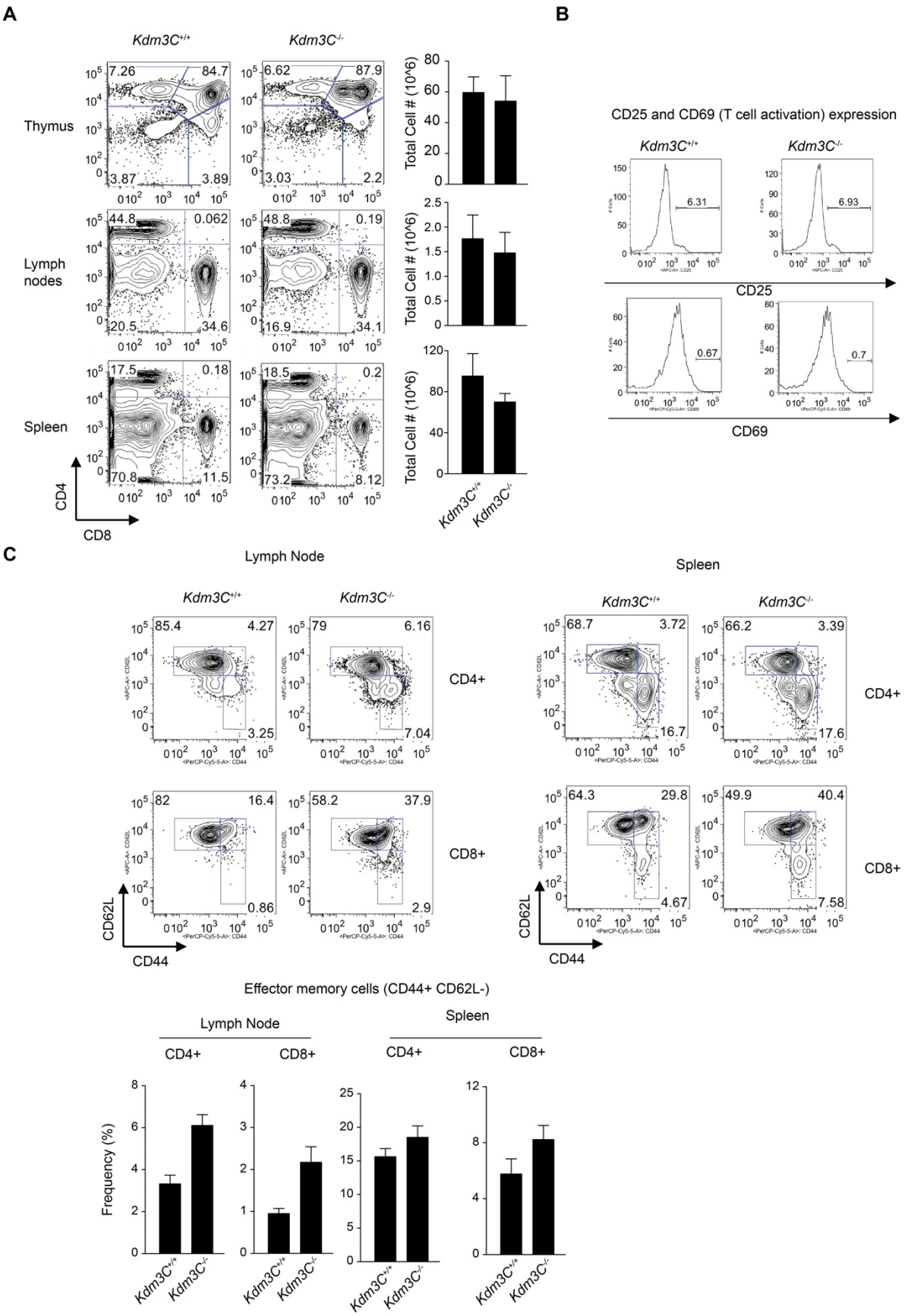

A
Kdm3C+/+
Kdm3C-/-
Thymus
Lymph nodes
Spleen
7.26
84.7
3.87
3.89
6.62
87.9
3.03
2.2
44.8
0.062
20.5
34.6
48.8
0.19
16.9
34.1
17.5
0.18
70.8
11.5
18.5
0.2
73.2
8.12
CD4
CD8
Total Cell # (10^6)
Kdm3C+/+
Kdm3C-/-
B
CD25 and CD69 (T cell activation) expression
Kdm3C+/+
Kdm3C-/-
6.31
6.93
CD25
0.67
0.7
CD69
C
Lymph Node
Spleen
Kdm3C+/+
Kdm3C-/-
85.4
4.27
3.25
79
6.16
7.04
68.7
3.72
16.7
66.2
3.39
17.6
CD4+
82
16.4
0.86
58.2
37.9
2.9
64.3
29.8
4.67
49.9
40.4
7.58
CD8+
CD62L
CD44
Effector memory cells (CD44+ CD62L-)
Lymph Node
Spleen
CD4+
CD8+
CD4+
CD8+
Frequency (%)
Kdm3C+/+
Kdm3C-/-

Figure 5. Loss of KDM3C has no impact on T-cell differentiation and activation.

Thymus, Lymph nodes and spleen were collected from *Kdm3C* WT (*Kdm3C*+/+) and *Kdm3C* KO (*Kdm3C*−/−) mice ($n = 3$). Tissue were homogenized and prepared for flowcytometry analysis (**A**) CD4+ and CD8+ cell counts calculated by flow cytometry (**B**) CD25 and CD69 cell counts calculated from lymph nodes. (C) CD44 and CD62 cell counts calculated in lymph nodes and spleen by flow cytometry.

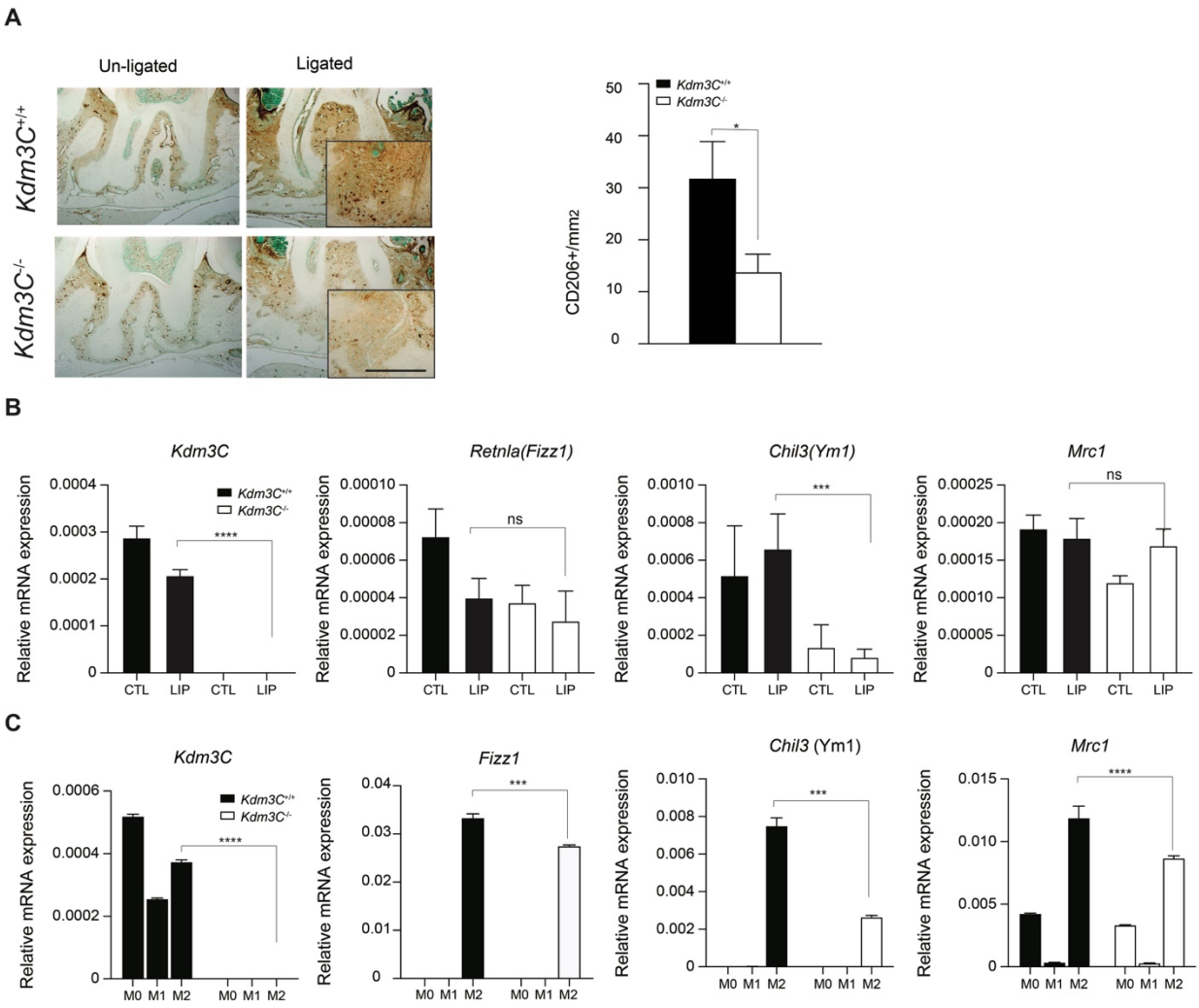


Figure 6. Loss of KDM3C suppress M2 markers

(A) CD206 staining was analyzed in histological section of maxilla with and with ligature. Expression levels (determined by qRT-PCR) of KDM3C and M2 markers (Retnla, Chil3, and Mrc1) in (B) gingival tissue of second molars and, in (C) BMDM. Expressions values were normalized by 18S. $*P < 0.05$, and $***P < 0.001$, $****P < 0.0001$. Results represent the means ± SEM performed in triplicate.

DISCUSSION

In this project we provide an insight on how epigenetic changes in aging affect oral and systemic inflammation. We utilized in vivo models to demonstrate evidence of increased systemic and oral inflammation upon exposure to bacterial toxins in aged mice. Initially, we demonstrated an increase in oral and systemic inflammation in aged mice when they were exposed to bacteria and bacterial toxins (Figure 1,2). We also provided evidence of increase of inflammatory cytokines in aged macrophage when they were challenged with LPS (Figure 2). Additionally, we identified a KDM3C among other KDM as a negative regulator in aging in various organs (Figure 3). Moreover, we demonstrated that loss of KDM3C exacerbate the inflammatory response in oral inflammation models in mice (Figure 4). Lastly, we were able to identify that loss of KDM3C did affect macrophage polarization but did not have any role in T-cell differentiation nor activation (Figure 5,6). Collectively, our data reveals insight into how epigenetic changes in aging affect macrophage changes and thus exacerbate the inflammatory response to external toxins.

In this project, we established an oral chronic inflammation where ligature was placed to induce periodontitis up to 21 days (Appendix Figure 1). In addition, we noticed changes in KDM3C during different time points of ligature-induced periodontitis (Appendix Figure 1). In our model, we observed a rapid increase in the bone loss during the early stage of inflammation from 0 to 7 days. Followed by a steady slow progression and then a plateau from 10-21 days. This observation was similar to previous reports [72], that usually there is a rapid increase in the severity of bone loss representing the acute phase of inflammation. Followed by a chronic phase, where bone loss levels are not changed. Since we were interested in studying the chronic inflammation in aging, we decided to choose 21 days as the model for later experiments. Previous reports regarding aged BMDM macrophage were controversial, some reported that there was no difference in the inflammatory cytokines levels between young and old mice after LPS change [73], where others

did demonstrate that BMDM from old mice exhibited increase in the levels of pro-inflammatory cytokines [74]. In our study, we tested BMDM and splenocyte in aged mice and both demonstrated an increase in the level of inflammatory cytokines such as IL-6, TNF-α, and IL-1β after LPS exposure.

Aging is associated with low-grade systemic inflammation in the absences of stimulus (inflammaging) [4]. Inflamamging leads to changes in the immune system including overstimulation or exacerbation of the immune system when exposed to an external or internal toxin [4]. Although several studies have investigated the immune response in aging, the role of aging and its impact on the oral environment remains unclear. In our study, we considered 18-20 months old mice to be aged based on previous literature that the average mouse lifespan is 24 months. In addition, 18 months old and later mice demonstrate aged phenotypes including loss and change of hair color, increase in size and they become less active. Our data indicate that chronic exposure to inflammatory stimulus in old mice, either through ligature induced periodontitis or pulp-exposure induced apical periodontitis, lead to more local inflammation presented by bone loss in both models suggesting that the immune system is overstimulating the pro-inflammatory factors or/and lacking the anti-inflammatory factors to suppress and control the inflammatory response. Both phenotypes were previously discussed in the literature and remains inconclusive which of these mechanisms is affected in aging, the pro-inflammatory pathway or the anti-inflammatory pathway[17, 73, 75]. Systemic organs from aged mice including liver, without any stimulation, reveled some level of inflammation and accumulation of immune cells histologically, this phenotype is in accordance with previous reports [76]. However, the increase in the pro-inflammatory cytokines at the basal level (with no stimulation) was not observed in the circulating blood serum, suggesting the inflammation was observed in these organs was due to a local reaction

from the resident immune cells. However, after inducing systemic inflammation by LPS, we noticed significant elevation in inflammatory cytokines in blood serum and splenocyte from young mice, the level of cytokines was significantly higher in old mice. This supports the hypothesis that aged immune system overstimulates the pro-inflammatory response and secrete more cytokines upon exposure to pathogens or toxins leading to more tissue damage.

Our screening reveled that KDM3C and KDM2A among others KDM that were downregulated, our laboratory previously identified KDM3C as an important epigenetic regulator of inflammatory response. Specifically, we found that KDM3C regulate inflammatory cytokine expression in macrophage by demethylating mono- and di-methylated histone 3 and lysine 9 (H3K9me1/2) [19]. Our data in (Figure 3) demonstrate that the loss of KDM3C is more likely affecting the adaptive immunity, since most of the organs that had KDM3C expression reduced are tissues that mainly populated with T and B cells. However, flowcytometry results from T-cells provided evidence that KDM3C has no role in T-cell maturation, activation and differentiation. On the other hand, our previous work demonstrates the effect of KDM3C on macrophage [19], we demonstrated that KDM3C negatively affect the pro-inflammatory cytokine through the regulation of NF-kB[19]. In addition, we provided evidence that KDM3C is enriched at the promoter regions of the target genes that elicit negative effects on NF-kB signaling [19]. Thus, we hypothesized that loss of KDM3C in aging affect macrophage and the changes in cytokines expression due to the loss of KDM3C may affect macrophage polarization.

In this study, we were able to successfully polarize mouse macrophage in vitro to M1 and M2 utilizing specific protocol that we previously published[77]. Our results indicate that there is a shift in macrophage phenotype in KDM3C-KO mice. Loss of KDM3C resulted in more pro-inflammatory macrophage and decrease in the anti-inflammatory cytokines (Appendix figure2).

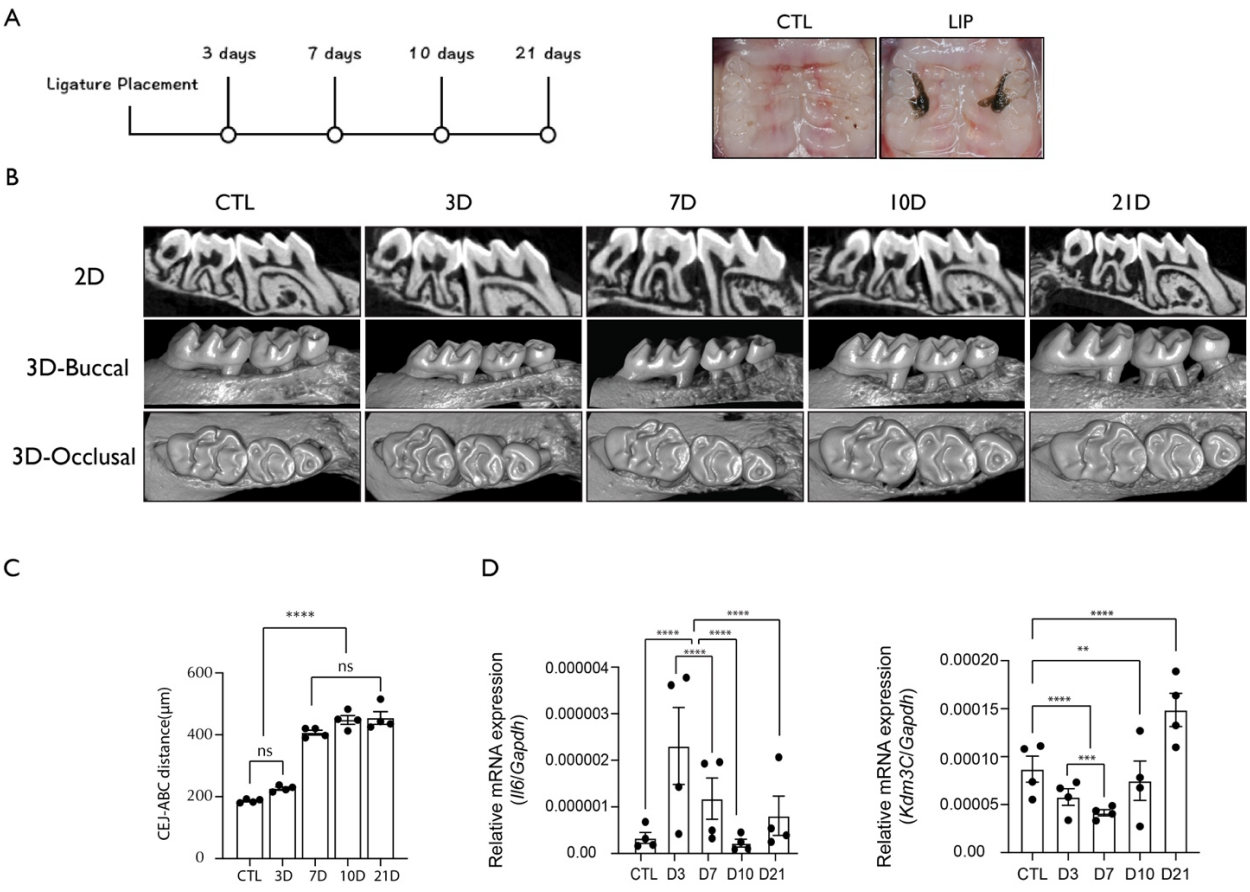
In addition, we demonstrate that less anti-inflammatory macrophages (M2) were present in oral tissue using CD206 as an M2 marker.

The ligature-induced experimental periodontitis model is one of the widely used preclinical models to initiate experimental periodontitis in different animals, ranging from mice to nonhuman primates[78-80]. However, there are limitations with this model that should be considered with data interpretation. Although the ligature aids in facilitating bacterial colonization and induced periodontal bone loss, it does not precisely reflect the human microbiome of periodontal disease. In addition, the structure of the mice periodontal apparatus does not precisely reflect that of humans. Despite these limitations, the ligature model remains one of the versatile systems with which to study the pathogenic events of periodontitis, such as gingival inflammatory responses to bacterial infection.

In this study, we utilized global KDM3C-KO mice to study the phenotype in mice upon the loss of KDM3C. To confirm that intrinsic loss of KDM3C have a specific effect on macrophages, we utilized , in our previous study , differentiated THP-1 cells and knock-down KDM3C in these cells using siRNA[19]. Knockdown of KDM3C resulted in upregulation of pro-inflammatory cytokines including TNF-α, IL-6, IL-1β[19].

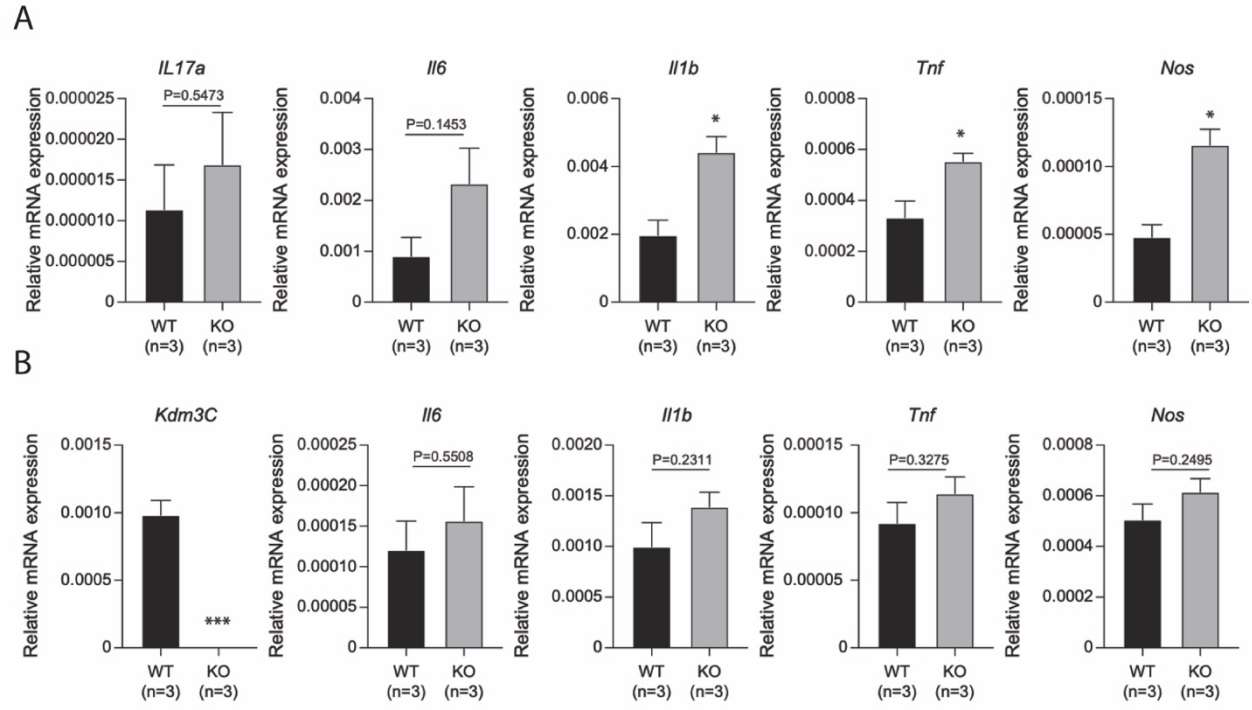
The current study utilized the ligature model to study the mechanisms of host-tissue responses to microbial challenges in the periodontium and to determine the role of KDM3C in the inflammatory response in vivo rather than focus on the microbiota associated with the disease progress. KDM3C-KO mice demonstrate significant bone loss as well as an increase in osteoclast activation LIP model similar to what was observed in the apical periodontitis model. Its more likely that KDM3C affect osteoclast activation, which we have demonstrated previously [19], either by affecting 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 [59], or indirectly through cytokines that play a key role in osteoclast differentiation including IL-1, IL-6 and TNF-α.



Appendix Figure 1.

Ligature was placed around the maxillary second molars. Mice were sacrificed after 3, 7, 10, and 21 days. (**A**) Experimental schematic of the time points and clinical photograph depicting ligature and non-ligature maxilla. (B) Representative two dimensional or three dimensional μCT images of mouse maxilla. (C) Alveolar bone loss measured at the distobuccal (DB) and mesiobuccal (MB) roots of the maxillary second molars from cemento-enamel junction (CEJ) to alveolar bone crest (ABC). (D) Expression levels (determined by qRT-PCR) of IL-6 and KDM3C at the palatal tissue of second molars. 18S served as loading control. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, $****P < 0.0001$. Results represent the means ± SEM performed in triplicate.



Appendix Figure 2.

Deletion of KDM3C resulted in increase of the inflammatory cytokines in liver and spleen. *Kdm3C* WT (*Kdm3C*+/+) and *Kdm3C* KO (*Kdm3C*–/–) mice received an intraperitoneal injection with E-coli lipopolysaccharide (Ec-LPS). After 4 hours, tissues were harvested and analyzed. (A) Expression levels (determined by qRT-PCR) of inflammatory cytokines in spleen . (B) Expression levels (determined by qRT-PCR) of inflammatory cytokines and KDM3C in liver $*P < 0.05$, and $***P < 0.001$. Results represent the means ± SEM performed in triplicate

CONCLUSION

Inflammaging is considered as a predictor of age-associated pathologies, including atherosclerosis, type II diabetes, and dementia, obesity, and cancer . Several factors have been proposed as contributors of inflammaging, *e.g.*, endogenous reactive oxygen species (ROS), mitochondrial damage, age-related decline of sex-hormones, and failure of organ systems . However, detailed molecular mechanism of inflammaging that can be a potential target for therapeutic intervention is largely unknown. Here we investigated the molecular mechanism that regulates inflammaging in the context of oral and systemic inflammatory conditions, *e.g.*, pulpal infection, periodontitis, and sepsis and to identify the potential markers and therapeutic targets in aging-associated diseases.

Our findings provide insight into the epigenetic mechanisms by which KDM3C regulates inflammatory response and macrophage polarization markers in aging. 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 in aged mice can serve to abrogate inflammatory lesion development found in periodontitis and apical periodontitis as a possible treatment modality for these disease processes. Lastly, Our finding highlight the importance of regular dental check-up for old individuals to prevent developing severe peri-radicular inflammation and periodontitis due to their immune changes.

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