A small molecule, Odanacatib, inhibits inflammation and bone loss caused by endodontic disease

Liang Hao[1,2,#], Wei Chen[2,#,*], Matthew McConnell[2], Zheng Zhu[2], Sheng Li[2], Michael Reddy[3], Paul D. Eleazer[4], Min Wang[1,*], Yi-Ping Li[2]

[1]The State Key Laboratory of Oral Diseases, West China College of Stomatology, Sichuan University, Sichuan, People's Republic of China;

[2]Department of Pathology, University of Alabama at Birmingham, SHEL 810, 1825 University Blvd, Birmingham AL 35294-2182, United States of America;

[3]Department of Periodontology, University of Alabama at Birmingham School of Dentistry, Birmingham, Alabama, United States of America;

[4]Department of Endodontics, University of Alabama at Birmingham, Birmingham, Alabama, United States of America;

[#]Authors contributed equally as first authors.

*Address correspondence to: Wei Chen, M.D, Department of Pathology, University of Alabama at Birmingham, SHEL 810, 1825 University Blvd, Birmingham AL 35294-2182, USA; Tel: 205-975-2606, Fax: 205-975-4919, E-mail: wechen@uab.edu; and to Min Wang, D.D.S, The State Key Laboratory of

Oral Diseases, West China College of Stomatology, Sichuan University, Sichuan, People's Republic of China; hxkqwangm@163.com;

Running Head: Inhibition of Ctsk prevents periapical tissue damage

Abstract

Periapical disease, an inflammation disease mainly caused by dental caries, is one of the most prevalent infectious diseases of mankind, affecting both children and adults. The infection travels through the root leading to inflammation, bone destruction, and severe pain for the patients. Therefore, the development of a new class of anti-periapical disease therapies is necessary and critical for treatment and prevention. A small molecule, Odanacatib (ODN), which is a Cathepsin K (Ctsk) inhibitor, was investigated to determine its ability to treat this disease in a mouse model of periapical disease. While Ctsk was originally found in osteoclasts as an osteoclast-specific lysosomal protease, we were surprised to find that ODN can suppress bacteria-induced immune response as well as bone destruction in the lesion area. The X-ray and MicroCT (μCT) showed that ODN treatment had significant bone protection effects at different time points. Immunohistochemical and immunofluorescent staining show that ODN treatment dramatically decreased $F4/80^+$ macrophages and $CD3^+$ T-cells in the lesion areas 42 days after infection. Consistent with these findings, qRT-PCR and ELISA analysis showed low levels of pro-inflammatory mRNA (*Tumor necrosis α*, *Interleukin 6* and *Interleukin 23α*) and correspondent cytokines expression in ODN-treated disease group. The mRNA level of *Toll-like receptor 4*, *5,* and *9* also

largely decreased in the ODN-treated disease group. Our results demonstrated that ODN can inhibit endodontic disease development, bone erosion, and immune response. These results indicate that application of this small molecule offers a new opportunity to design effective therapies that could prevent periapical inflammation and revolutionize current treatment options.

Keywords

Endodontic disease, Cathepsin K, Inflammation, Bone resorption, Dental caries, Osteoclast

Introduction

Periapical lesions result from microbial infection of the dental pulp tissue by autogenous oral microflora, which induce inflammation in the pulp tissue (1). This inflammatory process, much like periodontitis, increases in magnitude in the apical region of the root canal system and subsequently in the periapical area, leading to periapical bone resorption as the infection spreads throughout the canal system towards the apical foramen and into the adjacent bone (2). Furthermore, periapical lesions that present with radiographic bone lesions are preceded by necrotic pulp tissues. Unlike periodontal disease inflammation (3, 4), endodontic lesions exhibit differences in the character of the immune response (5).

Despite many studies aimed to alleviate the effects of oral disease, there is still an urgent need to improve the health of millions with periapical disease who suffer from oral bacterial infection-induced periapical inflammation, oral bone erosion and the potential loss of teeth. The discovery of the critical roles of Cathepsin K (Ctsk) in osteoclastic bone resorption is the fruit of decades of investigation on bone biology and disease (6, 7). Ctsk is the only gene for which an essential role in bone resorption has been clearly demonstrated in both mice and humans, and current research data shows that Ctsk is also indispensible in the immune system (8). Recent studies demonstrated that cathepsins are required for toll-like receptor 9 expressed in dendritic cells, which plays an essential role in innate recognition of microbial products and activation of defense responses (9). Our research led to the surprising and pivotal realization that Ctsk also has significant functions in the immune system (10, 11), so we termed Ctsk as an “osteoimmune gene”. The study of bone and the immune system, which are termed osteoimmunology, represents a new perspective reconsidering the multiple biological events in bone and the immune system in recent years (12, 13).

The success of the novel treatment of inflammatory diseases like periodontitis and rheumatoid arthritis (RA) has impressively demonstrated the clinical benefit can be gained from therapeutic intervention of specific protein by using small molecules (14). Odanacatib (ODN) is a potent, orally active selective inhibitor of Ctsk being developed for the treatment of postmenopausal osteoporosis.

Treatment with ODN decreases bone resorption by selectively inhibiting proteolysis of matrix protein by Ctsk without affecting other osteoclast activities or osteoclast viability (15). However, there is still no research activity using Ctsk inhibitors applied for oral inflammatory diseases considering its dual functions in bone and immune system. In addition, according to the knowledge of the investigators of current studies, there are still questions of what functions Ctsk has in activating immune cells, such as macrophages or dendritic cells (Antigen-presenting cells, APCs), and what leads to T cell activation and autoimmunity causing the subsequent production of inflammatory cytokines. Therefore, based on the cited research and our previous results, the current investigation was conducted with respect to the likelihood that Ctsk has a significant role in the activation of macrophages and TLRs-mediated innate and adaptive immune responses in the pathogenesis of periapical lesions by using a specific Ctsk small molecule inhibitor ODN.

Materials and Methods

Animals. 105 seven- to eight-week-old male wild-type (WT) BALB/cJ mice, purchased from the Jackson Laboratory were used for introducing a periapical mouse model. Mice were divided into 4 groups at each time point (7, 21 and 42 days): (1) normal group with Ctsk inhibitor (no bacterial infection) (3.606 mg/kg/wk, n=7 mice); (2) normal group without Ctsk inhibitor (no bacterial infection) (n=7 mice); (3) bacterial infection group treated with Ctsk inhibitor (3.606 mg/kg/wk, n=7 mice) or (0.7212 mg/kg/wk, n=7 mice); (4) bacterial

infection group without Ctsk inhibitor (n=7 mice). The animals were maintained in the University of Alabama at Birmingham animal facility and were given distilled water and allowed to feed freely. All experimental protocols were approved by the NIH and the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and completed within 16 weeks after birth. Approval for the animal protocol related to this study (Animal Protocol Number 131209236) was renewed by the University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee (IACUC) on August 27, 2013.

Administration of Cathepsin K (Ctsk) inhibitor. Male mice in the normal and periapical disease group were treated with ODN and compared with the untreated normal and disease groups. Treated mice were administrated orally at 3.606 mg/kg/wk or 0.7212 mg/kg/wk (5-fold lower dose) of pharmacological grade ODN (selleckChem, USA) in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) from one week before the establishment of the disease model to the end of sample harvest. The ODN dose was chosen to match the treatment of human by convention to a proper dose for mouse (16). The control mice were administrated orally with DMSO.

Pulp exposure and bacterial infection. The left and right first molar pulp exposure was performed as described (10, 17). In brief, mice were anesthetized with peritoneal injection of 62.5mg/kg ketamine and 12.5mg/kg xylazine according to body weight. The dental pulps of the mandibular first molars were exposed

with ¼ round carbide bur powered by a variable speed electric rotary hand piece (Osada Electric, Los Angeles, CA) under a surgical microscope (model MC-M92; Seiler, St Louis, MO). After removing the roof of the pulp chamber, the size of the exposure was approximately 0.5-1.0mm in diameter. We used stainless steel hand files #8 (Dentsply/Maillefer, Johnson City, TN) and stainless steel rotary files #15 (Dentsply/Maillefer, Johnson City, TN) to establish canal patency. Eradication of infected microorganisms was not completed in this therapy.

Bacterial culture and infection procedure protocols were conducted as described (10, 17). In brief, exposed pulps were infected with a mixture of four common human endodontic pathogens, including *Prevotella intermedia* [American Type Culture Collection (ATCC) 25611; Manassas, VA], *Fusobacterium nucleatum* (ATCC 25586), *Peptostreptococcus micros* (ATCC 33270), and *Streptococcus intermedius* (ATCC 27335). All four species of bacteria were cultured under strict anaerobic conditions (80% N_2, 10% H_2, and 10% CO_2), inoculated to broth, and cultivated over seven days. Microbes were harvested, resuspended, and evaluated for cell concentration of each species via optical density reading. The wave length used for cell number counting is 600nm (One OD unite equals $6.67*10^8$ particles), the cell density per ml can be obtained by calculation. Then the cell density of each species was adjusted to 10^{10} per ml for bacterial inoculation. The four organisms were then mixed for a total of 10^{10} cells of each bacterial species/ml PBS in 3% methylcellulose. Ten microliters of the polymicrobial solution was placed inside the access opening of each molar and

carried to the periapical tissues using a #8 endodontic file. After exposed pulps were infected with the mixture of four common human endodontic pathogens, access openings were left open to the oral environment for 24 hours. All exposure sites were then filled with self-cure composite temporary filling.

Harvest and preparation of samples. Animals were sacrificed by CO_2 inhalation on day 7, 21 and 42 after the initial infection. The mandibles were removed and hemisected. After removal of soft tissue, the jaw samples from the left side were fixed in 4% formaldehyde for 24 hours then stored in 70% ethanol prior to X-ray and μCT exposure and measurement analysis of bone loss. The jaw samples from the right side were fixed in 4% paraformaldehyde and prepared for histological analysis. The right sections were prepared for histology analysis according to standard protocol with modification for samples that were prepared for paraffin sectioning. In brief, samples specified for paraffin section were fixed in 4% formaldehyde for 24 hours, washed with PBS, and decalcified in 10% EDTA for 25 days (EDTA replenished each day). For the samples applied for RNA and protein extraction, the periapical bone tissues surrounding the mesial and distal root were extracted from the mandible together with surrounding bone in a block specimen using a surgical microscope for total RNA and cytokine enzyme linked immunosorbent assays (ELISA). For the RNA extraction, periapical tissues were rinsed in chilled PBS, weighed (3-5mg/tissue), and immediately put into RNA*later*® ICE (Invitrogen, USA) overnight at 4 °C and then stored at -80 °C until applied for RNA extraction. For ELISA assay, the periapical

tissues were rinsed in chilled PBS, weighed (3-5mg/tissue) and immediately frozen at -80 °C until applied for protein extraction.

Micro-computed tomography (μCT) analysis. Micro-computed tomography (μCT) scans were evaluated for bone loss as described with modification (10, 17). Briefly, the two-dimensional picture that showed the largest target root canal was selected as the baseline image. For each sample, an approximate total of 101 micro-tomographic slices with an increment of 12 μm were acquired in front of and behind the baseline image. From the three-dimensional stack of μCT images, a “pivot” section was the periapical area including root area and furcation area, where the inflammation may cause the bone resorption around the apex of the tooth. The segmentation parameter is 0.7/1/210.

Histological analysis. Samples were fixed by 4% paraformaldehyde for 24 hours, decalcified by 10% EDTA for 25 days with a new solution each day, dehydrated by 50%, 70%, 95%, 100% alcohol for 2 hours each, washed in paraffin acetone for 30 min in a 60 ºC incubator, washed in chloroform for 30 min in 60 ºC incubator, infiltrated with paraffin in a 60 ºC incubator 3x for 1hr each and embedded in paraffin. To detect bone-resorbing osteoclasts, Tartrate-resistant acid phosphatase (TRAP) staining was performed. Tissue sections were de-paraffinized and hydrated through xylenes and graded alcohol series, preincubated with 50 mM sodium acetate and 40 mM potassium sodium tartrate buffer for 20 min, and incubated with TRAP substrate solution.

206

207 **Immunofluorescence analysis.** We performed immunofluorescence analysis as
208 we have previously described (18), with the exception that we used rat polyclonal
209 anti-CD3 (Abcam, Cambridge, MA) as the primary antibodies and observations
210 were performed by epifluorescence in a Zeiss Axioplan microscope (Carl Zeiss
211 Microscopy, LLC, USA). Nuclei were visualized with 1 µg/ml DAPI (4',6-
212 diamidino-2-phenylindole) (Sigma Aldrich, USA). The experiments were set in
213 triplicate on three independent occasions.

214

215 **Immunohistochemistry analysis.** We performed immunohistochemistry using
216 corresponding sections of mandibular tissue from the normal group (with or
217 without inhibitor) and the disease group (with or without inhibitor), then the slides
218 were analyzed by immunohistochemistry for expression and localization of
219 proteins of the macrophage marker F4/80 (Rat monoclonal, 1:200) (eBioscience,
220 San Diego, CA), TLR4 (Goat polyclonal, 1:200), TLR5 (Rabbit polyclonal, 1:200)
221 (Santa Cruz, USA) and TLR9 (Rabbit monoclonal, 1:500) (Sigma-Aldrich, USA).
222 Vector stain ABC kit Anti-Rat, Goat and Rabbit IgG Peroxidase Polymer
223 Detection Systems along with a DAB kit (Vector Laboratories, Burlingame, CA)
224 as a substrate were used for the peroxidase-mediated reaction. The experiments
225 were set in triplicate on three independent occasions.

226

227 **RNA extraction and Real-time quantitative PCR (qRT-PCR**). For RNA
228 extraction, the prepared samples were transferred to the tube pre-filled with

beads (Nextadvance Company, USA) and homogenized using a Blender (Bullet Blender®, Nextadvance Company, USA). The RNA extraction was performed with the standard procedure using Trizol reagent (Invitrogen, USA). The extracted RNA was applied for reverse transcription using Vilo® master kit (Invitrogen). Real-Time quantitative PCR (qPCR) was performed as described (10, 17) using primers purchased from Invitrogen as listed (**see Table S1 in supplemental material**). Briefly, cDNA fragments were amplified by Sybrgreen® Fast Advanced Master Mix (Applied Biosystems, Foster City) and detected by a Step-One real-time PCR system (Applied Biosystems, Foster City). The mRNA expression level of the housekeeping gene *β-actin* was used as an endogenous control and enabled calculation of specific mRNA expression levels as a ratio of *β-actin*. Experiments were repeated at least three times.

Protein extraction and cytokine for Enzyme-linked immunosorbent assay (ELISA). For protein extraction, the frozen periapical tissue samples were transferred to a tube prefilled with beads (Nextadvance Company, USA) and homogenized using a Blender (Bullet Blender®, Nextadvance Company, USA). The tissue fragments were dispersed in 300ul of lysis buffer. The mixture was centrifuged at 1,2000 RPM for 10 min, and the supernatant was collected and stored at -80 °C until assay. ELISA was used as described (19, 20) to evaluate the effect of inhibition of Ctsk on the levels of TNF-α, IL-6 and IL-23α in inflammatory periapical tissues extracted from the normal group (with or without inhibitor) and the disease group (with or without inhibitor). Briefly, assays for

cytokines in extracts employed commercially available ELISA kits that are obtained from the following sources: TNF-α (eBioscience, USA), IL-6 (eBioscience, USA) and IL-23α (Biolegend, USA). All assays were conducted in accordance with the manufacturer's instructions. Results were expressed as pg cytokine/ml tissue.

Statistical analysis and data quantification analysis. Experimental data are reported as mean ± SD of triplicate independent samples. All experiments were performed in triplicate on three independent occasions. The figures are representative of the data. Data were analyzed with the two-tailed Student's t-test. Mann-Whitney U test was used for the non-parametric test. P values <0.05 or U values > 1.96 were considered significant. Data quantification analysis were performed using the NIH Image J Program as described (10, 17).

Results

Inhibition of Ctsk exhibits bone protective effects of the endodontic lesion area at different time points. Our previous study on AAV-mediated Ctsk inhibition in our periapical lesion mouse model revealed that Ctsk is important for bone resorption and inflammation in periapical lesions, but not for exploring the possible mechanism (10). Thus, it is important to determine the trend change of osteoclasts and inflammation in the periapical lesion area by inhibition of Ctsk and its possible mechanism at different time points. To test the possible role during the progress of periapical lesions mediated by Ctsk, we applied the

periapical mouse model established by our previous study (10, 17). Samples from the normal group with or without inhibitor (only images of day 42 showed in **Fig. 1**) and from the bacterial infected group with or without inhibitor (3.606 mg/kg/wk) were analyzed by X-ray and μCT at days 7, 21 and 42 after infection (**Fig. 1**). X-ray imaging of the first molar tooth of the normal group with and without inhibitor at different time points showed that there was no reduced bone density area (**see Fig. S1 in the supplemental material**). The X-ray and μCT test showed low bone density area at furcation and periapical area in the bacterial infection group without inhibitor at 7, 21 and 42 days (**Fig. 1B-D**). As the time increased, the low density of bone regions in the disease group without inhibitor was widening (red arrows). On the contrary, there was no obvious reduced bone density area in the infected disease group with inhibitor (3.606 mg/kg/wk) at different time points (**Fig. 1B-D**). To further quantify the bone resorption in the periapical lesion area, we applied quantification of μCT to compare the difference between different groups (**Fig. 1E**). The quantification analysis showed a significant difference in the disease group with or without inhibitor at 7, 21 and 42 days (**Fig. 1E**). The different dose of Ctsk inhibitor (3.606 mg/kg/wk) or 5-fold lower (0.7212 mg/kg/wk) seemed to have a dose dependent bone protective effect by μCT analysis (**see Fig. S2 in the supplemental material**). We also conducted histological analysis by Hematoxylin and Eosin (H&E) for the periapical lesion area for the different groups (**Fig. 2**). The H&E stain showed that the remaining bone area in mesio, distal and furcation areas in the disease group with inhibitor (3.606 mg/kg/wk) is much higher than that in the

disease group without inhibitor at 7, 21 and 42 days (**Fig. 2A** and **B**). We also found that the distribution of mononuclear inflammatory cells was evident in the disease group without inhibitor at different time points (red arrows) (**Fig. 2A**). Although the bone area decreased in the disease group without inhibitor, there was no significant change at the first 21 days. However, the remaining bone area in the disease group without inhibitor was still significantly less than the inhibitor treated group at 42 days (**Fig. 2C**). There was no significant change in the normal group with or without inhibitor (**see Fig. S3A, C in the supplemental material**). Above all, the results showed that Ctsk is critical for bone resorption during the progress of periapical lesions.

Inhibition of Ctsk exhibits decreased osteoclast numbers at endodontic and furcation areas at different time points. By applying TRAP staining, we found that the number of osteoclasts decreased in the disease group with inhibitor (3.606 mg/kg/wk) compared to the disease group without inhibitor at different time points (**Fig. 3A**, **B**). We also noticed that the number of osteoclasts increased significantly in the disease group without inhibitor compared to the inhibitor group at 7 days (**Fig. 3C**), and the osteoclast numbers remained at a high level during the progress of periapical lesion in our current study. There was no significant difference of osteoclast number in the normal group with or without inhibitors at 42 days (**see Fig. S3B**, **D in the supplemental material**).

Ctsk may have effects on immune response and have functions in immune cells at the periapical lesion area. The macrophage is important for the host immune response during the periapical bacterial infection (2). In order to determine whether inhibition of Ctsk has effects on the expression of macrophages, we conducted IHC for F4/80, a specific marker of macrophages (**Fig. 4**). The results showed that the F4/80 positive macrophages increased significantly in the disease group without inhibitor (**Fig. 4C**). We also conducted the immunofluorescence analysis for the periapical lesion area for the CD3 positive T cells in different groups (**Fig. 5**). We then merged each set of images for comparison and location (**Fig. 5A**). These images showed significantly less visible CD3 positive T cells (white arrow) in the disease group with inhibitor (3.606 mg/kg/wk) than that of the disease group without inhibitor, which indicated that inhibition of Ctsk decreased the number of T cells in the periapical lesion area (**Fig. 5C**).

Inhibition of Ctsk reduced the expression of pro-inflammatory and osteoclast marker genes as well as TLRs and cytokines in the periapical lesion at different time points. To evaluate the effect of Ctsk inhibition on the mRNA levels of *Tumor Necrosis Factor-α* (*TNF-*α), *Interleukin 6* (*IL-6*), *Interleukin 6* (*IL-23*α) and *Cathepsin K* (*Ctsk*), which are related to inflammatory and osteoclast status in periapical tissues, qRT-PCR was used as described (10, 17, 19). The relative mRNA expression levels showed that the expression of Ctsk was inhibited at different time points in the inhibitor (3.606 mg/kg/wk) treated disease

group, but not in the untreated disease group (**Fig. 6A**). Proinflammatory gene expression also increased in the disease group at 7, 21 and 42 days (**Fig. 6A**). The result of TLRs expression showed that *TLR9* increased significantly starting from day 21 in the disease group (**Fig. 6A**). The expression of *TLR4* and *TLR5* was also detected at different time points since *TLR4* and *TLR5* are responsible for recognition of bacteria pathogen lipopolysaccharide (LPS) and flagellin (21). Although *TLR4* increased at disease group without inhibitor at 7 and 21 days, there was no significant difference between disease group and inhibitor treated group at 42 days (**Fig. 6A**). The IHC stain confirmed the qRT-PCR result (**see Fig. S4 in the supplemental material**). The expression of *TLR5* decreased significantly in inhibitor treated disease group starting from 21 days (**Fig. 6A**). IHC stain of TLR5 showed TLR5 expression decreased significantly in the inhibitor treated disease group at 42 days (**see Fig. S5 in the supplemental material**), which was consistent with the qRT-PCR result. Although the gene expression level of *IL-6* and *IL-23*α decreased in the disease group treated with or without inhibitor (3.606 mg/kg/wk) at 42 days, cytokine expression was still higher when compared to the normal group. The ELISA result showed that TNF-α, IL-6 and IL-23α reached the highest levels in the disease group without inhibitor at 42 days (**Fig. 6B**). Since Ctsk has been shown to possibly play a critical role in the TLR9 related pathway (9), we also conducted IHC of TLR9 to explore whether Ctsk affects the TLR9 expression in periapical disease at 42 days after infection. The results showed that TLR9 was expressed abundantly in

the periapical lesion area in the disease group without inhibitor but not in the inhibitor treated group (**Fig. 7**).

Discussion

Periapical lesions result from pulp necrosis induced by bacterial infection. The infection produces an inflammatory reaction first within the pulp and, subsequently, in the periapical region after egress of microorganisms, their by-products and/or altered pulp tissues from the infected root canal. The periapical lesion is characterized by the presence of numerous inflammatory cells, which then lead to immune inflammation of periapical tissue. Generally, the body's immune defense system gradually removes the pathogens after the removal of bacterial infections, but during the process of obliterating foreign microorganisms, inflammatory factors produced by the body could also damage the surrounding normal tissue and cause periapical bone tissue absorption. If this process continues, teeth will eventually become loose or fall out. Bone resorption is mediated by bone resorptive cytokines produced by the inflammatory response. Consequently, the question is particularly important of how to control an excessive inflammatory response in the inflammatory process while inhibiting corresponding bone resorption.

Cathepsin K, a lysosomal cysteine protease, is abundantly expressed in osteoclasts. This fact, as well as its enzymatic properties, implies that it is a key factor in normal bone remodeling and in pathological processes, such as

388 osteoporosis, osteoarthritis and inflammatory mediated bone resorption disease
389 such as periapical disease (10, 11). Recent studies showed Ctsk also has
390 important functions in immune response (9, 22, 23). Our previous study has
391 already demonstrated that knocking down *Ctsk* by AAV gene therapy can
392 significantly reduce the bone resorption and inflammation (10). However, the
393 main drawback of using AAV as vectors is that the small size of their genome
394 significantly limits the amount of genetic material it can carry (24). Small
395 molecule can overcome this by having several advantages: a greater universe of
396 treatable diseases, lower cost with greater ease of manufacturing, and patient
397 preference for an oral availability (14, 25). ODN is a highly selective, potent, and
398 reversible inhibitor of Ctsk and inhibits OC-mediated bone resorption in vitro, but
399 does not reduce the number of osteoclasts, and appears to reduce bone
400 resorption while preserving bone formation (26, 27). ODN is currently being
401 developed as an orally bioavailable therapeutic for the treatment of
402 postmenopausal osteoporosis. In a clinical trial, 36 months of once weekly ODN
403 treatment increased lumbar spine and total hip bone mineral density (BMD) and
404 reduced bone turnover markers in postmenopausal women with low BMD (15).
405
406 Since Ctsk has been shown to have functions in both osteoclast and immune
407 cells such as macrophages and dendritic cells (9, 11, 28), whether inhibition of
408 Ctsk can affect not only osteoclasts but also immune response is still unclear.
409 This study proposed its novel role in endodontic inflammation lesions. Patients
410 with endodontic disease suffer from both inflammation-induced tissue damage

and bone loss (2), a single target that can dramatically improve both conditions is ideal. In current study, we tested the inhibition of Ctsk using this specific small molecule inhibitor ODN by oral administration in periapical lesion mouse model to determine whether Ctsk plays dual roles in periapical disease to simultaneously reduce inflammation and bone resorption at different time points.

Due to the nature and progression of endodontic infection, changing micro-environmental conditions are selected for more anaerobic and virulent species of bacteria (29). We induced periapical disease by inoculating the root canal system of the mandibular first molar with a mixture of four common endodontic pathogens: *Prevotella intermedia* [American Type Culture Collection (ATCC) 25611; Manassas, VA], *Fusobacterium nucleatum* (ATCC 25586), *Peptostreptococcus micros* (ATCC 33270), and *Streptococcus intermedius* (ATCC 27335) as described (10, 17). Our results showed that bone resorption at different time points was much more advanced in the periapical periodontitis group without inhibitor than that in the periapical periodontitis group with inhibitor, and meanwhile we found that bone resorption increased as time increased in different groups, but this trend was more obvious in the periapical disease group without inhibitor (**Fig. 1**). Further quantitative analysis by μCT confirmed the bone protective effects at 7, 21 and 42 days by inhibition of Ctsk (red arrow) (**Fig. 1**). To determine the trends of bone resorption and the infiltration of inflammatory cells in the periapical lesion area, we also conducted H&E staining to detect the changes of bone resorption in different groups at 7, 21 and 42 days after

infection (**Fig. 2**), which was consistent with the results of X-ray and μCT. We also want to explore the proper dose used for treatment of periapical disease in mice, so we chose two different dose 3.606 mg/kg/wk and 0.7212 mg/kg/wk, which was recalculated from the dose used for clinical trial on human by considering the difference of body surface area between human and mice (16). The result showed that the 3.606 mg/kg/wk had a better bone protection effect at 42 days after infection (**see Fig. S2 in the supplemental material**). These results showed that the bone resorption was more notable in the periapical disease group without inhibitor than that in the inhibitor treated disease group at different time points. Inflammatory cell infiltration in the periapical disease group without inhibitor was increasingly more serious at 7, 21 and 42 days, from which we can see a large number of blue-stained monocytes distributed in the apical, mesial/distal and furcation area (red arrow) (**Fig. 2A**). In the inhibitor treated periapical disease group we can see that the trabecular bone area was basically unchanged and infiltration of inflammatory cells was significantly reduced. Above all, inhibition of Ctsk has significant bone protection effects and prevents inflammatory cells infiltration.

To investigate the mechanism of the effect of Ctsk inhibition *in vivo*, TRAP staining and immunohistochemistry (IHC) staining was applied. Studies showed that periapical inflammatory cells play an important role in the development of periapical periodontitis, which secrete a variety of cytokines (IL-1α, TNF-α, IL-6 and IL-11), and are involved in the adjustment of the activation and apoptosis of

corresponding effector cells such as osteoclast, thus controlling the bone resorption (30-32). In our previous study, we showed that Ctsk knockdown can abolish the extra-cellular acidification and then inhibit bone resorption, while osteoclast differentiation is only slightly affected *in vitro* (10, 11). ODN has also been shown not to affect the osteoclast numbers *in vivo* (33). Therefore, the number of osteoclasts of various groups *in vivo* at different time points was determined in this experiment to clarify the possible mechanism. We found that the number of osteoclasts did not change in the normal control group (**see Fig. S3 in the supplemental material**), but significantly reduced at different time points in the lesion area of the inhibitor treated periapical periodontitis group compared to that of the untreated periapical disease group (**Fig. 3**). This indicated that the immune response may also be affected by inhibition of Ctsk since pre-osteoclast activation is due to RANKL secreted by osteoblasts, T cells and B cells, and these cells can be activated by proinflammatory cytokines and chemokines such as IL-1, IL-6, TNFα and IL-17 (34). Studies indicated that activated macrophages are the source of bone-resorbing cytokines in periapical disease (35), which can be activated by T-lymphocytes or by bacterial endotoxin, as part of innate immunity. In order to fully demonstrate the function of Ctsk in periapical disease immune response, the present study also examined whether inhibition of Ctsk affects the expression of macrophages by detecting the macrophage marker F4/80 (**Fig. 4**). The results showed that the number of macrophages increased significantly in the periapical disease group without inhibitor at 42 days, but not in the inhibitor treated group (**Fig. 4**), which indicated

that Ctsk may play an important role in both osteoclasts and immune responses (**Fig. 3, 4**).

Since macrophage expression decreased in the inhibitor treated disease group and to further investigate the effect of Ctsk function in adaptive immune response, immune fluorescence analysis was applied. We determined the number of CD3 positive T cells in the periapical disease area from different groups at 42 days (**Fig. 5**). The results showed that the CD3 positive T cells decreased significantly in the inhibitor treated infection group, which indicated that the adaptive immune response has been impaired by inhibition of Ctsk. As the effecter cell of inflammation, T cells can produce a variety of effecter molecules (36, 37). Therefore, the number of T cells is an important indicator of the severity of local inflammatory response. As a T-cell surface marker, CD3 is commonly used to detect T cell population including αβ T cells and γδ T cells. The results showed that the number of $CD3^+$ T cells was significantly reduced in the inhibitor treated periapical disease group than that in the disease group (**Fig. 5A**). T cells showed aggregated distribution in the periapical periodontitis group, while the inhibitor treated periapical periodontitis group only showed minor T cell distribution, which indicated that the immune system-mediated inflammatory reaction is suppressed in Ctsk inhibited disease groups. The result can be an explanation of the reduction of the number of osteoclasts from another aspect (**Fig. 3**). T cells and macrophages decreased, which might result in a lower

proinflammatory cytokine production, which might finally cause lower osteoclasts' activation and differentiation.

In order to confirm this hypothesis, the relative mRNA level was quantified by Sybrgreen based qRT-PCR by using the primer set as listed (**see Table S1 in the supplemental material**). Our results showed that the Ctsk gene reached the highest level at 42 days in the periapical disease group *in vivo*, but not in the inhibitor treated disease group, which showed no obvious change of Ctsk expression (**Fig. 6A**). To further investigate the mechanism of the protective effect of inhibition of Ctsk in the mouse model of periapical disease, we tested the expression level of pro-inflammatory maker genes including *TNF-α*, *IL-6* and *IL-23α*. The *TNF-α* and *IL-6* genes' expression levels increased significantly in the periapical disease group at day 7 compared to that in the inhibitor treated disease group (**Fig. 6A**). The expression of *IL-23α* reached the highest level at day 21. The protein level expression of cytokines, including TNF-α, IL-6 and IL-23, all increased in the periapical disease group at different time points. Although the proinflammatory cytokine also increased in the inhibitor treated disease group, the level is much lower when compared to the disease group (**Fig. 6B**). Cytokines such as TNF-α, IL-6 and IL-23α are soluble mediators and released from immunocompetent cells in the periapical inflammatory processes. These cytokines can stimulate bone resorption in periapical lesion areas (38). The proinflammatory cytokines such as TNF-α has been reported to have a key role in regulating the inflammatory response (39). IL-6 is produced at the site of

525 inflammation and also plays a key role in the acute phase response, which is
526 also shown by our qRT-PCR results (**Fig. 6A**) (40). As receptors recognize
527 several major bacterial pathogenics, Toll-like receptors (TLRs) are important in
528 immune response. Currently 12 TLR family members have been found in
529 humans and other mammals, but not all TLRs are expressed in mammals. For
530 example, TLR11 and 12 are expressed in mice, but not in humans (41). TLR4, 5
531 and 9 can specifically identify different types of bacterial antigens, which play an
532 important role in bacterial infection. The study from Beklen et al. showed that
533 except TLR7 and TLR8, expression levels of TLR1 to TLR10 in human gingival
534 surface is higher in periodontitis than healthy people (42), suggesting that TLRs
535 play a more significant role in the activation and development of periodontitis.
536 Therefore, TLR4, 5 and 9 were selected as the research object in this experiment
537 to detect the changes in the inhibition of Ctsk in the periapical disease model.
538 The relationship between Ctsk and TLRs has not been fully clarified yet. The
539 recent studies demonstrate that Ctsk has critical role in TLRs functions. In
540 rheumatoid arthritis disease, Asagiri et al. demonstrated that Ctsk might have
541 effect on DCs cytokine expression through TLR9 in vitro (9). Hirabara et al.
542 showed Ctsk inhibitor might inhibit TLR-4 dependent Ctsk expression in human
543 fibroblast *in vitro* (43). Ctsk has also been shown to be involved in development
544 of psoriasis-like skin lesions through TLR7 activation (44). In our current study,
545 we showed that TLR5 and 9 all decreased in the inhibitor treated disease group
546 compared to the disease group at different time points (**Fig. 6A**). The IHC stain of
547 TLR5 and 9 further confirmed the qRT-PCR result (**see Fig. S5 in supplemental**

material and Fig. 7). The TLR4 expression increased in the disease group at 7 and 21 days, however, decreased at 42 days. There was no significant difference at 42 days between disease group and inhibitor treated disease group (**see Fig. S4 in supplemental material and Fig. 6**).

In summary, we investigated the possible mechanism of the effect of Ctsk in simultaneously targeting osteoclasts and immune cells. The inhibition of Ctsk by the specific small ODN molecule reduced bone resorption by inhibition of the osteoclast function and inflammation simultaneously and in turn decreased osteoclast activation and differentiation, which exhibits a promising therapeutic method on periapical inflammation disease.

Acknowledgments

We thank Ms. Christie Paulson for her excellent assistance with this manuscript. We appreciate the assistance of the Center for Metabolic Bone Disease at the University of Alabama at Birmingham (P30 AR046031). We are also grateful for the assistance of the Small Animal Phenotyping Core (P30 DK079626), Metabolism Core, and Neuroscience Molecular Detection Core Laboratory at the University of Alabama at Birmingham (P30 NS0474666). This work was supported by NIH grant RC1-DE-020533-01 (Y.P.L.). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Reference

1. **Wang QQ, Zhang CF, Chu CH, Zhu XF.** 2012. Prevalence of Enterococcus faecalis in saliva and filled root canals of teeth associated with apical periodontitis. International journal of oral science **4:**19-23.
2. **Stashenko P, Teles R, D'Souza R.** 1998. Periapical Inflammatory Responses and Their Modulation. Critical Reviews in Oral Biology & Medicine **9:**498-521.
3. **Jiang H, Chen W, Zhu G, Zhang L, Tucker B, Hao L, Feng S, Ci H, Ma J, Wang L, Stashenko P, Li YP.** 2013. RNAi-mediated silencing of Atp6i and Atp6i haploinsufficiency prevents both bone loss and inflammation in a mouse model of periodontal disease. PloS one **8:**e58599.
4. **Yang S, Hao L, McConnell M, Zhou X, Wang M, Zhang Y, Mountz JD, Reddy M, Eleazer PD, Li YP, Chen W.** 2013. Inhibition of Rgs10 Expression Prevents Immune Cell Infiltration in Bacteria-induced Inflammatory Lesions and Osteoclast-mediated Bone Destruction. Bone research **1:**267-281.
5. **Eleazer PD, Farber PA, Seltzer S.** 1975. Lack of lymphocyte stimulation by root canal products. J Endod **1:**388-394.
6. **Duong LT.** 2013. Inhibition of cathepsin K: blocking osteoclast bone resorption and more. IBMS BoneKEy **10**.
7. **Troen BR.** 2004. The role of cathepsin K in normal bone resorption. Drug news & perspectives **17:**19-28.
8. **Matsumoto F, Saitoh S-i, Fukui R, Kobayashi T, Tanimura N, Konno K, Kusumoto Y, Akashi-Takamura S, Miyake K.** 2008. Cathepsins are required for Toll-like receptor 9 responses. Biochemical and Biophysical Research Communications **367:**693-699.
9. **Asagiri M, Hirai T, Kunigami T, Kamano S, Gober H-J, Okamoto K, Nishikawa K, Latz E, Golenbock DT, Aoki K, Ohya K, Imai Y, Morishita Y, Miyazono K, Kato S, Saftig P, Takayanagi H.** 2008. Cathepsin K-Dependent Toll-Like Receptor 9 Signaling Revealed in Experimental Arthritis. Science **319:**624-627.
10. **Gao B, Chen W, Hao L, Zhu G, Feng S, Ci H, Zhou X, Stashenko P, Li YP.** 2013. Inhibiting periapical lesions through AAV-RNAi silencing of cathepsin K. Journal of dental research **92:**180-186.
11. **Li YP, Chen W.** 1999. Characterization of mouse cathepsin K gene, the gene promoter, and the gene expression. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research **14:**487-499.
12. **Walsh MC, Kim N, Kadono Y, Rho J, Lee SY, Lorenzo J, Choi Y.** 2006. OSTEOIMMUNOLOGY: Interplay Between the Immune System and Bone Metabolism. Annual Review of Immunology **24:**33-63.
13. **Takayanagi H.** 2007. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat Rev Immunol **7:**292-304.
14. **Fleischmann R.** 2012. Novel small-molecular therapeutics for rheumatoid arthritis. Current opinion in rheumatology **24:**335-341.
15. **Eisman JA, Bone HG, Hosking DJ, McClung MR, Reid IR, Rizzoli R, Resch H, Verbruggen N, Hustad CM, DaSilva C, Petrovic R, Santora AC, Ince BA,**

Lombardi A. 2011. Odanacatib in the treatment of postmenopausal women with low bone mineral density: three-year continued therapy and resolution of effect. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research **26:**242-251.

16. **Stoch SA, Zajic S, Stone J, Miller DL, Van Dyck K, Gutierrez MJ, De Decker M, Liu L, Liu Q, Scott BB, Panebianco D, Jin B, Duong LT, Gottesdiener K, Wagner JA.** 2009. Effect of the Cathepsin K Inhibitor Odanacatib on Bone Resorption Biomarkers in Healthy Postmenopausal Women: Two Double-Blind, Randomized, Placebo-Controlled Phase I Studies. Clin Pharmacol Ther **86:**175-182.
17. **Ma J, Chen W, Zhang L, Tucker B, Zhu G, Sasaki H, Hao L, Wang L, Ci H, Jiang H, Stashenko P, Li Y-P.** 2013. RNA Interference-Mediated Silencing of Atp6i Prevents Both Periapical Bone Erosion and Inflammation in the Mouse Model of Endodontic Disease. Infection and immunity **81:**1021-1030.
18. **Feng S, Deng L, Chen W, Shao J, Xu G, Li YP.** 2009. Atp6v1c1 is an essential component of the osteoclast proton pump and in F-actin ring formation in osteoclasts. The Biochemical journal **417:**195-203.
19. **Sasaki H, Hou L, Belani A, Wang CY, Uchiyama T, Muller R, Stashenko P.** 2000. IL-10, but not IL-4, suppresses infection-stimulated bone resorption in vivo. Journal of immunology (Baltimore, Md. : 1950) **165:**3626-3630.
20. **Sasaki H, Okamatsu Y, Kawai T, Kent R, Taubman M, Stashenko P.** 2004. The interleukin-10 knockout mouse is highly susceptible to Porphyromonas gingivalis-induced alveolar bone loss. Journal of periodontal research **39:**432-441.
21. **O'Neill LAJ, Golenbock D, Bowie AG.** 2013. The history of Toll-like receptors [mdash] redefining innate immunity. Nat Rev Immunol **13:**453-460.
22. **Conus S SH.** 2010. Cathepsins and their involvement in immune responses. Swiss Med Wkly. **140:w13042.:**10.4414/smw.2010.13042.
23. **Krieg AM, Lipford GB.** 2008. IMMUNOLOGY: The Toll of Cathepsin K Deficiency. Science **319:**576-577.
24. **Owens RA.** 2002. Second generation adeno-associated virus type 2-based gene therapy systems with the potential for preferential integration into AAVS1. Current gene therapy **2:**145-159.
25. **Spring DR.** 2005. Chemical genetics to chemical genomics: small molecules offer big insights. Chemical Society reviews **34:**472-482.
26. **Yasuda Y, Kaleta J, Bromme D.** 2005. The role of cathepsins in osteoporosis and arthritis: rationale for the design of new therapeutics. Advanced drug delivery reviews **57:**973-993.
27. **Vasiljeva O, Reinheckel T, Peters C, Turk D, Turk V, Turk B.** 2007. Emerging roles of cysteine cathepsins in disease and their potential as drug targets. Current pharmaceutical design **13:**387-403.
28. **Herroon MK, Rajagurubandara E, Rudy DL, Chalasani A, Hardaway AL, Podgorski I.** 2013. Macrophage cathepsin K promotes prostate tumor progression in bone. Oncogene **32:**1580-1593.
29. **Moller AJ.** 1966. Microbiological examination of root canals and periapical tissues of human teeth. Methodological studies. Odontologisk tidskrift **74:**Suppl:1-380.

30. **Strand V, Kavanaugh AF.** 2004. The role of interleukin-1 in bone resorption in rheumatoid arthritis. Rheumatology (Oxford, England) **43 Suppl 3:**iii10-iii16.
31. **de la Mata J, Uy HL, Guise TA, Story B, Boyce BF, Mundy GR, Roodman GD.** 1995. Interleukin-6 enhances hypercalcemia and bone resorption mediated by parathyroid hormone-related protein in vivo. The Journal of clinical investigation **95:**2846-2852.
32. **Schett G.** 2011. Effects of inflammatory and anti-inflammatory cytokines on the bone. European Journal of Clinical Investigation **41:**1361-1366.
33. **Cusick T, Chen CM, Pennypacker BL, Pickarski M, Kimmel DB, Scott BB, Duong LT.** 2012. Odanacatib treatment increases hip bone mass and cortical thickness by preserving endocortical bone formation and stimulating periosteal bone formation in the ovariectomized adult rhesus monkey. Journal of Bone and Mineral Research **27:**524-537.
34. **Cochran DL.** 2008. Inflammation and Bone Loss in Periodontal Disease. Journal of Periodontology **79:**1569-1576.
35. **Metzger Z.** 2000. Macrophages in periapical lesions. Endodontics & dental traumatology **16:**1-8.
36. **Ueda H, Howson JMM, Esposito L, Heward J, Snook, Chamberlain G, Rainbow DB, Hunter KMD, Smith AN, Di Genova G, Herr MH, Dahlman I, Payne F, Smyth D, Lowe C, Twells RCJ, Howlett S, Healy B, Nutland S, Rance HE, Everett V, Smink LJ, Lam AC, Cordell HJ, Walker NM, Bordin C, Hulme J, Motzo C, Cucca F, Hess JF, Metzker ML, Rogers J, Gregory S, Allahabadia A, Nithiyananthan R, Tuomilehto-Wolf E, Tuomilehto J, Bingley P, Gillespie KM, Undlien DE, Ronningen KS, Guja C, Ionescu-Tirgoviste C, Savage DA, Maxwell AP, Carson DJ, Patterson CC, Franklyn JA, Clayton DG, Peterson LB, Wicker LS, Todd JA, Gough SCL.** 2003. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature **423:**506-511.
37. **Marçal JRB, Samuel RO, Fernandes D, de Araujo MS, Napimoga MH, Pereira SAL, Clemente-Napimoga JT, Alves PM, Mattar R, Rodrigues Jr V, Rodrigues DBR.** 2010. T-Helper Cell Type 17/Regulatory T-Cell Immunoregulatory Balance in Human Radicular Cysts and Periapical Granulomas. Journal of Endodontics **36:**995-999.
38. **Ataoglu T, Ungor M, Serpek B, Haliloglu S, Ataoglu H, Ari H.** 2002. Interleukin-1beta and tumour necrosis factor-alpha levels in periapical exudates. International endodontic journal **35:**181-185.
39. **Beutler B, Cerami A.** 1989. The biology of cachectin/TNF--a primary mediator of the host response. Annual review of immunology **7:**625-655.
40. **Gabay C.** 2006. Interleukin-6 and chronic inflammation. Arthritis research & therapy **8 Suppl 2:**S3.
41. **Beutler B.** 2004. Inferences, questions and possibilities in Toll-like receptor signalling. Nature **430:**257-263.
42. **Beklen A, Hukkanen M, Richardson R, Konttinen YT.** 2008. Immunohistochemical localization of Toll-like receptors 1-10 in periodontitis. Oral microbiology and immunology **23:**425-431.

43. **Hirabara S, Kojima T, Takahashi N, Hanabayashi M, Ishiguro N.** 2013. Hyaluronan inhibits TLR-4 dependent cathepsin K and matrix metalloproteinase 1 expression in human fibroblasts. Biochem Biophys Res Commun **430:**519-522.
44. **Hirai T, Kanda T, Sato K, Takaishi M, Nakajima K, Yamamoto M, Kamijima R, Digiovanni J, Sano S.** 2013. Cathepsin K is involved in development of psoriasis-like skin lesions through TLR-dependent Th17 activation. Journal of immunology (Baltimore, Md. : 1950) **190:**4805-4811.

Figure Legends

Figure 1. Inhibition of Ctsk by specific inhibitor reduced infection-stimulated endodontic bone resorption by Micro-computed tomography (μCT) analysis. (**A**) X-ray and μCT analysis of endodontic lesion area of the normal group in natural course with or without inhibitor (3.606 mg/kg/wk) at 42 days. (**B-D**) X-ray and μCT analysis of endodontic lesion area of bacterial infected group with or without inhibitor (3.606 mg/kg/wk) at 7, 21 and 42 days. There was an obvious reduced bone density area compared to inhibitor treated disease group at different time points (Black arrows). The X-ray and μCT analysis showed increased low bone density area at furcation and periapical area at 42 days compared with 7 days and 21 days. (**E**) Quantification of bone volume/tissue volume (BV/TV) measured for periapical lesion area of different groups. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$. N = 3, repeated three times. N.S: No Significance. Left column of panel **A-D** are X-ray images. Middle column of panel **A-D** are typical slice 2D μCT images, while the right column of panel **A-D** are 3D Reconstitutions images.

Figure 2. Inhibition of Ctsk by specific inhibitor showed bone protection effects at different time points by histology analysis. (**A**) H&E staining of the periapical mesio, distal root sections and furcation area sections from the bacterial infected group with or without inhibitor (3.606 mg/kg/wk) at different time points (7, 21 and 42 days). Red arrows indicated the inflammatory monocytes infiltration. The red dot area indicated the remaining bone in the lesion area. (**B**) Quantification of remaining bone area at 7, 21 and 42 days of these three areas. (**C**) Time course analysis of the bone resorption at bacterial infected group with or without inhibitor (3.606 mg/kg/wk). *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$. N.S: No Significance. N = 3, repeated three times.

764 **Figure 3. Inhibition of Ctsk by specific inhibitor showed reduced TRAP$^+$ cells in**
765 **periapical lesion area at different time points by histology analysis.** (**A**) TRAP
766 staining of the periapical mesio, distal root sections and furcation area sections
767 from the bacterial infected group with or without inhibitor (3.606 mg/kg/wk) at
768 different time points (7, 21 and 42 days). (**B**) Quantification of TRAP$^+$ cell
769 numbers at 7, 21 and 42 days of these three areas. (**C**) Time course analysis of
770 TRAP$^+$ cell numbers at bacterial infected group with or without inhibitor (3.606
771 mg/kg/wk). The TRAP$^+$ cell number increased in bacterial infected group without
772 inhibitor at different time points, and the TRAP$^+$ cell number in infected group
773 with inhibitor (3.606 mg/kg/wk) was significant lower compared to infected group
774 without inhibitor. *: P<0.05, ***: P<0.001. N.S: No Significance. N = 3, repeated
775 three times.
776

777

778 **Figure 4. Inhibition of Ctsk by specific inhibitor showed reduced F4/80$^+$ cells of**
779 **periapical lesion area at 42 days by immunohistological analysis. (A)**
780 Immunohistochemistry stains of F4/80$^+$ (Brown) macrophages in periapical lesion
781 area in normal group and bacterial infected group with or without inhibitor (3.606
782 mg/kg/wk) at 42 days. Normal serum served as negative control. **(B)** Higher
783 magnification view of infection disease group at 42 days. Red arrows indicated
784 F4/80$^+$ macrophages. **(C)** Quantification of F4/80$^+$ macrophages in alveolar area
785 of normal group and bacterial infected group with or without inhibitor (3.606
786 mg/kg/wk) at 42 days. **: P<0.01, ***: P<0.001. N = 3, repeated three times.
787

788

789 **Figure 5. Inhibition of Ctsk by specific inhibitor decreased the number of T**
790 **cells in periapical lesion area at 42 days.** (**A**) Immunofluorescence staining of
791 CD3$^+$ (red) T cells in periapical lesion area in the normal group and the bacterial
792 infected group with or without inhibitor (3.606 mg/kg/wk) at 42 days. (**B**) Normal
793 serum served as negative control at the same area (Without primary antibody).

(**C**) Quantification of $CD3^+$ cells analysis demonstrated that inhibition of Ctsk has significantly reduced expression of $CD3^+$ T cells in bacterial infected group with inhibitor (3.606 mg/kg/wk) at 42 days. ***: *P*<0.001. N.S: No Significance. N = 3, repeated three times.

Figure 6. Inhibition of Ctsk by specific inhibitor reduced the expression of pro-inflammatory genes as well as TLRs genes and cytokines in the periapical lesion at different time points. (**A**) qRT-PCR of osteoclast genes (i.e. *Ctsk*) and pro-inflammatory genes (i.e. *TNF-α*, *IL-6* and *IL-23 α*) as well as TLRs genes (i.e. *TLR4*, *5* and *9*) in the periapical lesion from normal group and bacterial infected group with or without inhibitor (3.606 mg/kg/wk) at 7, 21 and 42 days. *β-actin* was used as an endogenous control. (**B**) Expression of TNF-α, IL-6 and IL-23α in the periapical lesion at 7, 21 and 42 days detected by ELISA. Significance was compared between disease group with and without inhibitor at different time points. *: *P*<0.05, **: *P*<0.01, ***: *P*<0.001. N.S: No Significance. N = 4, repeated three times.

Figure 7. Inhibition of Ctsk by specific inhibitor shows reduced expression of TLR9 in periapical lesion area at 42 days by immunohistological analysis. (A) Immunohistochemistry stains of $TLR9^+$ (Brown) cells areas in normal group and bacterial infected group with or without inhibitor (3.606 mg/kg/wk) at 42 days. Normal serum served as negative control. **(B)** Higher magnification view of infection disease group at 42 days. Red arrows indicated $TLR9^+$ cells. **(C)** Quantification of $TLR9^+$ cells in frucation, mesio and distal periapical area of normal group and bacterial infected group with or without inhibitor (3.606 mg/kg/wk) at 42 days. *: *P*<0.05, **: *P*<0.01, ***: *P*<0.001. N.S: No Significance. N = 3, repeated three times.