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Gene Therapy of Periodontal Disease by AAV-shRNA Silencing of Cathepsin K by inhibiting inflammation in periodontal lesion area

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Abstract

Background and Objective—Periodontitis is a serious disease that affects the majority of adult population around the world. While great efforts have been devoted toward understanding the pathogenesis of periodontitis, there remains a pressing need for developing potent therapeutic strategies for targeting this dreadful disease. In this study, we utilized adeno-associated virus (AAV) expressing Cathepsin K (Ctsk) shRNA (AAV-sh-Ctsk) to silence Ctsk *in vivo* and subsequently evaluated its impact in periodontitis as a potential therapeutic strategy for this disease.

Material and Methods—We used a known mouse model of periodontitis, in which wild-type BALB/cJ mice were infected with *Porphyromonas gingivalis* W50 (*P. gingivalis*) in the maxillary and mandibular periodontium to induce the disease. AAV-sh-Ctsk was then administrated locally into the periodontal tissues *in vivo*, followed by analyses to assess the progression of the disease.

Results—AAV-mediated Ctsk silencing drastically protected mice (>80%) from *P. gingivalis*-induced bone resorption by osteoclasts. Also, AAV-sh-Ctsk administration drastically reduced inflammation by impacting the expression of many inflammatory cytokines as well as T cell and dendritic cell numbers in periodontal lesions.

Conclusion—AAV-mediated Ctsk silencing can simultaneously target both the inflammation and bone resorption associated with periodontitis through its inhibitory effect on immune cells and

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osteoclast function. Thereby, AAV-sh-Ctsk administration can efficiently protect against periodontal tissue damage and alveolar bone loss, establishing this AAV-mediated local silencing of Ctsk as an important therapeutic strategy for potentially treating periodontal disease.

Keywords

Adeno-associated virus (AAV); Gene therapy; Periodontal diseases; Cathepsin K; Alveolar bone loss

Introduction

Periodontitis, affecting the oral health of most adult Americans, is a chronic inflammatory disease that damages the soft tissue and destroys the alveolar bone that supports the teeth, which ultimately lead to tooth loss. Whereas most studies indicate that periodontitis is caused by polymicrobial infection, there is evidence that the extent of the inflammation in periodontitis may also result from interaction between select species of the oral micro-flora and the host immune response (1–3). Nevertheless, *Porphyromonas gingivalis* W50 (*P. gingivalis*) has been appointed as one of the most prominent pathogens that are highly associated with periodontitis (4, 5). Notably, the inflammatory spectrum associated with the host response in the gingival tissues trigger enhanced bone resorption by osteoclasts, the body sole bone-resorbing polykaryons, for the resulted tooth loss. Osteoclasts originate from monocytic precursors of the hematopoietic lineage by stimulation with receptor activator of NF κ B ligand (RANKL) and macrophage-colony stimulation factor (M-CSF). Moreover, numerous inflammatory cytokines including tumor necrosis factor alpha α (TNF α), interleukin 1 (IL-1), and IL-6 can also promote osteoclast formation and/or function.

Cathepsin K (Ctsk) is a lysosomal cysteine protease that belongs to the peptidase C1 protein gene family (6). Ctsk is strongly expressed by osteoclasts and specifically induced during osteoclast differentiation through stimulation of osteoclast precursors with M-CSF and RANKL. As such, Ctsk is highly considered a good marker gene for osteoclasts. In particular, Ctsk functions by degrading the protein components of the bone matrix which is critical for osteoclastic bone resorption. Hence, inactivating mutations which affect Ctsk function have resulted in pycnodysostosis in humans, a disease that is characterized by impaired bone resorption (7). Given its crucial role and specific expression in osteoclasts, Ctsk is often considered as an important therapeutic target for targeting bone loss in many bone diseases of excessive osteoclast formation and/or functions including rheumatoid arthritis, postmenopausal osteoporosis, and periodontal disease.

Adeno-associated virus (AAV) gene silencing is a new and potent strategy that has been utilized in many clinical studies to target many pathological disorders (8). Specifically, AAV therapy can insert specific genes with great certainty into the genome to maintain long-term and sustainable gene expression in diseases resulted from defective gene expression and/or function. Furthermore, novel strategies have been devised to employ AAV *in vivo* to induce long-term and potent silencing of specific genes associated with disease outcomes through local administration (9). Importantly, aside from mild immune response elicited, numerous studies have demonstrated that AAV administration is not only safe but is also well tolerated

by patients (10). Hence, given the essential role of Ctsk in osteoclast function and the favorable characteristics of AAV-mediated gene therapy, we hypothesized that targeting Ctsk inhibits the inflammation and bone loss caused by bacterial infection in periodontitis. In order to test our hypothesis, we employed AAV-mediated RNAi to investigate the effects of Ctsk silencing in a mouse model of periodontitis. This study was aimed at examining conclusively the therapeutic potential of Ctsk depletion *in vivo* via local administration of AAV-sh-Ctsk to reduce tissue damage from inflammation and bone resorption in periodontitis induced by *P. gingivalis* W50.

Materials and Methods

Animals

Wild-type (WT) female BALB/cJ mice were obtained from the Jackson Laboratory for the study. The animals were housed in The University of Alabama at Birmingham (UAB) animal facility and were given food and water ad libitum. All experimental protocols were approved by the NIH and the UAB Institutional Animal Care and Use Committee. Animal protocol related to this study is (Animal Protocol Number 131209236). Please refer to Supplemental Materials and Methods Table S1 for detailed information.

Design and construction of shRNA

To design a shRNA construct that can effectively knockdown Ctsk, we used the Dharmacon siDESIGN center (<http://www.dharmacon.com>) as described in our recent publication (11). The Ctsk shRNA oligonucleotide (5'-GATCCCC-GAGGTGTGTACTATGATGAAA-TTCAAGAGA-TTTCATCATAGTACACACCTCTTTTGGGAAT-3') was annealed and cloned downstream of the H1 promoter in the AAV-H1 vector (gift from Dr. Sergei Musatov) into BglII and HindIII sites to generate the AAV-H1-shRNA-Ctsk construct. The AAV-H1-shRNA-luc construct (gift from Dr. Sergei Musatov) contains a luciferase gene and a yellow fluorescent protein (YFP) expression cassette and was used as a control vector for both shRNA and AAV as used in previous studies (12). BLAST homology search predicted that this control shRNAs would not affect Ctsk or any other known mouse gene (12).

AAV-ShRNA viral production and purification

We utilized the AAV Helper-Free System (AAV Helper-Free System, Stratagene) for viral production using a triple-transfection, helper-free method, and purified as described in a previous study but with slight modification (13). Briefly, HEK 293 cells were transduced with pAAV-shRNA, pHelper and pAAV-RC plasmids (Stratagene) using the calcium phosphate method. Cells are collected after 60–72 hours and lysed with chloroform at 37° for 1 hour. Sodium chloride was then added before shaking the cells room temperature for 30 minutes. The stock was then spun at $10,000 \times g$ for 15 minutes and the supernatant was collected and cooled on ice for 1 hour with PEG8000. The solution was spun at $9,000 \times g$ for 15 minutes, and then the pellet was treated with DNase and RNase. After the addition of chloroform and centrifugation at $10,000 \times g$ for 5 minutes, the purified virus was in the aqueous phase at viral particle numbers of $1 \times 10^{10}/\text{ml}$. The AAV particle titer was determined by AAV Quantitation Titer Kit (Cell Biolabs, San Diego, CA, USA).

The viral vectors were evaluated for their ability to infect target tissue as previously described (14). Briefly, optimal viral particle numbers for infection were determined from the percentage of target cells expressing YFP. To confirm the effect of Ctsk silencing, we examined the expression of Ctsk in osteoclasts using Western blot and immunofluorescence. Luciferase expression vector AAV2-Luc was purchased from the University of North Carolina (Chapel Hill, NC). The AAV2-Luc was injected into the right side of the lower jaw one time and luciferase expression was measured by the IVIS Imaging System 100 Series (Xenogen Corporation, Alameda, CA) after 2 or 5 weeks.

***In vitro* bone resorption assays**

Bone resorption pits was assessed as described in previous studies (15, 16). Mouse bone marrow (MBM) cells seeded on bovine cortical bone slices plated in 24-well dishes were transduced with viral vectors after 72 hour of RANKL and M-CSF. Bone slices were harvested after stimulation of infected MBM cells with M-CSF and RANKL for three additional days, and culture media was also collected. Cells were removed from the bone slices with 0.25 M ammonium hydroxide and mechanical agitation. Bone slices were subjected to scanning electron microscopy (SEM) using a Philips 515 SEM (Department Materials Engineering, UAB). Bone resorption was also assessed using wheat germ agglutinin (WGA) to stain exposed bone matrix proteins. Assays were quantified by measuring the percentage of the areas resorbed in three randomly selected sites using the ImageJ analysis software.

Cells and cell culture

Pre-osteoclasts and mature osteoclasts were generated from MBM as previously described (11, 17). Briefly, MBM was obtained from tibiae and femora from six-week-old female WT BALB/cJ mice. 1×10^5 and 1×10^6 MBM cells were seeded per well of 24-well plate and 6-well plate, respectively. MBM cells were cultured in α -modified Eagle's medium (α -MEM; Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY, USA) containing 10 ng/ml M-CSF (R&D Systems, Minneapolis, MN, USA) for 24 hours. Subsequently, cells were cultured with 10 ng/ml RANKL (R&D Systems, Minneapolis, MN, USA) and 10 ng/ml M-CSF for an additional 48 or 96 hours to generate pre-osteoclasts or mature osteoclasts.

Western blotting analysis

Western blotting was performed as previously (18, 19) and visualized and quantified using a Fluor-S Multi-Imager with Multi-Analyst software (Bio-Rad, Hercules, CA, USA). We utilized a rabbit anti-Ctsk antibody previously generated in our lab (11) and a goat anti-rabbit IgG-HRP (7074S, Cell signaling, Danvers, MA, USA) for this Western analysis.

Infection with *P. gingivalis* W50

P. gingivalis W50 (ATCC: 53978) was cultured on sheep's blood agar plates supplemented with Hemin and Vitamin K (BAPHK) for 3 days. Single clones were harvested and transferred to Trypticase Soy Broth supplemented with hemin and vitamin K. On day 4, bacteria were harvested, resuspended, and bacterial concentration was accessed at optical

density readings 600nm (One OD unit equals 6.67×10^8 bacteria). Cell density was then adjusted to 10^{10} cells/ml in 0.2 ml of phosphate buffered saline (PBS) containing 2% carboxymethylcellulose (CMC: Sigma-Aldrich, St. Louis, MO, USA) to prepare the infectious-bacterial solution. Periodontal infection was performed using 20 μ l of infectious-bacterial solution as described in previous studies (15, 16). In brief, all animals received antibiotic treatment for three days prior to the infection to reduce the original oral flora, followed by three days of an antibiotic-free period before submitted to oral inoculation with a dental micro-brush (Henry Schein, USA) once per day for four consecutive days. To monitor bacterial colonization, murine oral cavities were sampled under sterile condition on day 14 after infection for identification of *P. gingivalis* W50.

AAV-sh-Ctsk transduction of *P. gingivalis* W50 infected mice

We administrated AAV-sh-Ctsk in a site-specific manner as described in a previous study but with minor modifications (12). Briefly, mice were anesthetized via peritoneal injection with ketamine (62.5 mg/kg) and xylazine (12.5 mg/kg). Four days after the initial infection, mice were given either AAV-sh-Ctsk or AAV-sh-luc-YFP once per day for seven consecutive days. Specifically, mice were injected approximately 0.3–0.5 mm above the gingival margin of the maxillary molars on the right and left palatal aspects with 3 μ l containing of either AAV-sh-Ctsk or AAV-sh-luc-YFP viral vector using a Hamilton syringe attached to a microinfusion pump (World Precision Instruments, Sarasota, FL, USA). As negative control (normal), mice were not infected with *P. gingivalis* W50 or treated with viral vectors. As positive control (disease), *P. gingivalis* W50-infected mice were not injected with viral vector. Detailed experimental strategy is provided in Supplemental Diagram 1.

Harvest and preparation of tissue samples

Animals were sacrificed by CO₂ inhalation 56 days after first infection. The maxillae were removed and hemisected. The samples from the left side were defleshed in 2.6% sodium hypochlorite for bone measurement analysis as previously determined (15, 16). Subsequently, maxillae samples from the right side were immediately fixed in 4% paraformaldehyde (PFA) and prepared for histological analysis according to standard protocol but with minor modifications. In brief, samples for paraffin sections were fixed in 4% PFA for 24 hours, washed with PBS, decalcified in 10% of Ethylenediaminetetraacetic acid (EDTA) in 0.1M TRIS solution (pH 7.0) for 10 days with daily replenishment, washed with PBS, and embedded in paraffin after series dehydration. Low jaw samples from the right and left sides in independent experiment were collected for real-time quantitative PCR (qRT-PCR) analyses and enzyme-linked immunosorbent assays (ELISAs). Gingival tissues and/or alveolar bone were isolated under a surgical microscope. Gingival tissues and alveolar bone from right samples were pooled for qRT-PCR, and gingival tissues from right samples were pooled for ELISAs for cytokines.

Histological Analysis

For paraffin sections, tissue were fixed by 4% PFA for 24 hours, and then decalcified by 10% EDTA for 10 days with daily replenishment before embedding in paraffin. For osteoclast analysis, tartrate-resistant acid phosphatase (TRAP) staining was performed and TRAP positive osteoclasts were counted as described (16). Tissue sections were

deparaffinized and hydrated through xylene and graded alcohol series, preincubated with 50 mM sodium acetate and 40 mM potassium sodium tartrate buffer for 20 minutes and then incubated with TRAP substrate solution for glycerol jelly. The histological analysis was carried out as previously described with slight modification (16), multinucleated TRAP-positive cells (> 3 nuclei) were counted in two different areas (i.e. the alveolar bone and periodontal ligament areas). The measurements consisted of measuring 3 different parts in the periodontal ligament area: the coronal location, the middle location, and the apical location. To ensure accurate analysis, we used series section (at least 3 sections) for width calculation.

Bone loss measurements

Analysis of bone loss was performed as previously described (15, 16). Briefly, images of molar tooth roots and alveolar bone were captured using digital microscopy followed by analysis using Adobe Photoshop™ (Adobe Systems, USA). The polygonal area enclosed by the cemento-enamel junction, the lateral margins of the exposed tooth root, and the alveolar ridge was measured using the ImageJ analysis software (Wayne Rasband, NIH, USA). Measurements were expressed in mm².

Immunofluorescence (IF) staining

IFC analysis was carried as indicated previously (20, 21), except that anti-Ctsk rabbit polyclonal, anti-CD3 (Santa Cruz, Dallas, Texas, USA) and armenian hamster anti-mouse CD11c (Biolegend, San Diego, CA, USA) were used as primary antibody. Data was imaged using epifluorescence on a Zeiss Axioplan microscope in the Developmental Neurobiology Imaging and Tissue Processing Core at the UAB Intellectual and Disabilities Research Center. Nuclei were visualized with DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich, St. Louis, MO, USA).

qRT-PCR Analysis

qRT-PCR analysis was performed as described (15, 16) using TaqMan probes from Applied Biosystems according to manufacturer's instructions. Briefly, cDNA was amplified by TaqMan® Fast Advanced Master Mix (Applied Biosystems, Life Technologies, Grand Island, NY, USA) (Supplemental Table 2). Amplification reaction was carried via the Step-One Plus real-time PCR system (Applied Biosystems, Life Technologies, Grand Island, NY, USA). *Hprt* (hypoxanthine-guanine phosphoribosyl transferase) was used as endogenous control for gene expression analysis calculated as ratio of *Hprt* (deltaCT).

ELISA Analysis

ELISA was performed in tissue extracts as previously described (15, 16). These assays were carried using commercial kits for IL-1α and IL-10 (BioLegend, San Diego, CA, USA); TNFα, IL-12, IL-6, Interferon gamma (IFN-γ) and IL-17α (eBioscience, San Diego, CA, USA) according to manufacturer's instructions. Data were reported as pg cytokine/ml.

Data quantification and statistical analyses

All experiments were repeated 3 times. Results were reported as mean $\pm$ SD. All experiments were performed in triplicate on three independent occasions. For the parametric data: bone loss measurements, histological analyses, qRT-PCR, and ELISA data were analyzed with ANOVA, P values <0.05 were considered significant. For the non-parametric data: IF data were analyzed with the Mann-Whitney U test, $U>1.96$ was denoted as P values <0.05 .

Results

Ctsk silencing by AAV impairs osteoclastic bone resorption *in vitro*

In order to target bone resorption in periodontitis, we first used AAV-mediated gene therapy to silence Ctsk through an AAV-sh-Ctsk construct which contains the yellow fluorescent protein (YFP) expression cassette *in vitro*. We used the AAV-sh-luc-YFP vector which contains a luciferase gene and YFP expression cassette as a control for AAV infection. To confirm the feasibility of these constructs for gene therapy, we examined the ability of AAV-sh-luc-YFP or AAV-sh-Ctsk to infect target cells (osteoclasts) through analysis of YFP or Ctsk expression. Hence, WT MBM cells cultured with M-CSF and RANKL for osteoclastogenesis assays were then transduced with no vector (mock group), AAV-sh-luc-YFP (control group), or AAV-sh-Ctsk to generate pre-osteoclasts and mature osteoclasts. Fluorescence analysis showed that pre-osteoclasts and osteoclasts were efficiently transduced with the AAV-sh-Ctsk or AAV-sh-luc-YFP constructs at a titer of 6×10^{11} Dnase resistant Particles (DRP)/ml (Fig. 1A). Consistently, Western blot analysis showed an 80% reduction in Ctsk expression in cells transduced with AAV-sh-Ctsk compared those transduced with AAV-sh-luc-YFP (Fig. 1B, C). Furthermore, cells transduced with AAV-sh-Ctsk had no difference in extracellular acidification compared to cells transduced with AAV-sh-luc-YFP (control group) or non-infected cells from WT mice (mock control) (Fig. 1D). In order to establish the effect of AAV-mediated Ctsk knockdown on osteoclast function *in vitro*, bone resorption pits were assessed using WGA, which stains exposed bone matrix proteins, and SEM to visualize bone resorption pits. Data showed that bone resorption by osteoclasts was almost completely blocked by AAV-sh-Ctsk treatment (Fig. 1E, top panel), demonstrating the functional efficiency of AAV-sh-Ctsk as an agent for targeting bone loss (Fig. 1E, bottom panel). Hence, AAV-mediated Ctsk silencing resulted in a significant impairment of bone resorption compared to the control (Fig. 1F). Importantly, these results showed that AAV-sh-Ctsk can effectively inhibit osteoclastic bone resorption *in vitro*. Collectively, these data indicate that AAV-sh-Ctsk can efficiently infect osteoclasts to affect Ctsk expression *in vitro*.

AAV effectively transduces periodontal tissue and displays a local distribution pattern *in vivo*

To further evaluate the potential of AAV-mediated gene therapy as a tool for gene transfer to periodontal tissues, we assessed the ability of AAV-sh-luc-YFP (used as a negative control) to achieve sustained and localized luciferase expression *in vivo*. AAV was administrated into the gingival tissue on the right side of the lower jaw as indicated (Fig. 2A). Data showed that luciferase was not only expressed but also limited to the injection sites after two and five

weeks of injection (Fig. 2B). Importantly, luciferase expression was higher after five weeks of administration as compared to two weeks, demonstrating that AAV could induce sustained gene expression in gingival tissues. These data show that AAV-mediated gene therapy can effectively transduce periodontal tissues and that injection of AAV into gingival tissue can induce sustained and localized gene expression. Moreover, fluorescent analysis of infected mice treated with AAV-sh-luc-YFP and AAV-sh-Ctsk compared to uninfected control group further demonstrated that the AAV vectors could successfully infiltrated periodontal tissues (Fig. 2C). Consistent with the *in vitro* data, we showed that whereas TRAP-positive osteoclasts were present around the teeth in the AAV-sh-luc-YFP or AAV-sh-Ctsk treated groups, the number of osteoclasts present was decreased significantly in the periodontal lesion area in the AAV-sh-Ctsk group (Figs. 2D,F). Moreover, IFC analysis revealed that Ctsk expression was markedly decreased in the fibroblast and periodontal ligament area of the AAV-sh-Ctsk treated group compared to the AAV-sh-luc-YFP treated group (Fig. 2E,G), indicating that Ctsk may also have functions in fibroblasts.

Ctsk silencing by AAV protects mice from periodontitis-induced bone loss

Next, we examined whether Ctsk depletion by local administration of AAV could protect mice from bone loss stemming from *P. gingivalis*-induced periodontitis through analysis of alveolar bone loss in uninfected mice (normal) compared to *P. gingivalis*-infected mice treated with either AAV-sh-Ctsk, AAV-sh-luc-YFP (negative control) or PBS (disease group) (Fig. 3A). Data showed that the AAV-sh-luc-YFP or PBS group had significantly more bone loss compared to the AAV-sh-Ctsk or normal group (Fig. 3B). Moreover, to determine whether the periodontal ligament between the tooth root and the bone was normalized by AAV-sh-Ctsk treatment, we performed hematoxylin and eosin stain (H&E) analysis on infected mice treated with AAV-sh-Ctsk compared to AAV-sh-luc-YFP or PBS treated group and demonstrated that AAV-sh-Ctsk treatment caused a reduction in *P. gingivalis*-induced periodontal ligament widening and bone loss (Fig. 3C). Also, the distance between the tooth root surface and the alveolar bone was decreased in half in the AAV-sh-Ctsk and disease group as compared to the AAV-sh-luc-YFP group (Fig. 3C, D), demonstrating that AAV-sh-Ctsk can prevent periodontal ligament damage.

Ctsk silencing by AAV reduces T cells and dendritic cells in alveolar bone

To examine the effects of AAV-mediated Ctsk silencing on T-cells *in vivo*, tooth root sections were subjected to IFC staining using to analyze CD3-positive T-cells (Fig. 3E). Data revealed that T cells in the periodontal ligament were significantly decreased in the AAV-sh-Ctsk treatment group as compared to the other groups (Fig. 3E, F). Also, we performed IFC staining for analysis of dendritic cells (DCs) in uninfected mice (Normal) and *P. gingivalis*-infected mice treated with AAV-sh-Ctsk, PBS or AAV-sh-luc-YFP by co-expression of Ctsk and CD11c. Data showed that the number of CD11c-positive DCs in the AAV-sh-Ctsk treated group was decreased significantly, indicating an increase in DCs in the periodontal lesion area of AAV-sh-luc-YFP control group and disease control group (Fig. 4). Notably, our data also revealed that Ctsk was expressed in DCs, suggesting that Ctsk may also carry important functions in immune response cells. Collectively, the results indicate that Ctsk depletion by AAV not only protects mice from *P. gingivalis*-induced bone loss, but

also reduces immune response *in vivo* and protects against inflammation caused by periodontal disease.

Ctsk silencing by AAV attenuates the expression of osteoclast marker genes and cytokines in periodontal lesions

To further determine the impact of AAV-mediated Ctsk depletion on the inflammatory response, qRT-PCR was used to examine the expression level of the inflammatory markers *IL-1 α* and *IL-1 β* in the periodontal tissues of the different experiment groups. *IL-1 α* and *IL-1 β* expression was higher in the AAV-sh-luc-YFP group as compared to the AAV-sh-Ctsk treated group (Fig. 5A). Furthermore, when compared to the normal group, *IL-6* expression was increased in *P. gingivalis*-infected mice treated with the control AAV-sh-luc-YFP group. Further analysis showed that the expression of *acid phosphatase 5, tartrate resistant (Acp5)*, an osteoclast marker gene, was comparable in either the AAV-sh-luc-YFP group or the AAV-sh-Ctsk treated group (Fig. 5A). We noted that whereas the expression of osteoprotegerin (*OPG*), an inhibitor of osteoclastogenesis, was decreased in the AAV-sh-luc-YFP treated group, RANKL displayed an inversed expression pattern in these groups (Fig. 5A). The finding was further confirmed by analysis of *IL-1 α* by ELISA (Fig. 5B). The expression levels of the pro-inflammatory cytokines *IL-17*, *TNF α* , *IL-12* and Interferon-gamma (*INF- γ*) were also higher in the AAV-sh-luc-YFP group as compared to the AAV-sh-Ctsk control and uninfected groups (Fig. 5B). The anti-inflammatory cytokines *IL-10* was increased in the AAV-sh-Ctsk treated group but not in AAV-sh-luc-YFP treated group (Fig. 5B), suggesting an inhibition in the inflammation response in the AAV-sh-Ctsk treated group. These results indicate that Ctsk knockdown reduces expression of genes important for osteoclastic bone resorption and inflammation in periodontal tissues.

Discussion

Local administration of AAV-sh-Ctsk gene therapy is a promising therapeutic strategy for periodontal disease

Current therapies for periodontal disease are focused on anti-microbial treatments and surgery which bear limited efficacy (22). AAV-mediated gene therapy carries great potential for treating periodontitis through maintenance of sustainable endogenous gene in a non-invasive manner (23, 24). However, it remains to be determined which genes and AAV vectors that can be safely used to induce effective gene expression in local periodontal tissues. This is a fundamental issue that needs to be addressed in order to development potent and safe AAV-mediate gene therapy for periodontal disease (24). Notably, intra-articular administration of AAV2 has been shown to trigger sustainable gene expression for 120 days (25). Hence, we utilized the AAV2 serotype to subclone a shRNA construct which targets Ctsk into the AAV.H1 vector, which was successfully used *in vivo* to knockdown estrogen receptor- α expression in the brain (12). In line with this finding, we demonstrated that AAV can also effectively infect periodontal tissues to transfer a specific gene. After one local injection of AAV-sh-luc-YFP, which contains YFP and luciferase genes, luciferase expression was sustained and limited to the local injection sites for at least 5 weeks. A sustainable YFP expression was observed in gingival tissue, periodontal ligament, and dental pulp tissue. Most importantly, luciferase expression was limited to the local administrated

areas. It is well documented that AAV vectors do not contain viral genes that could elicit undesirable cellular immune responses and generally appear not to induce inflammatory responses (26). Consistent with the essential role of Ctsk in osteoclast function, local administration of AAV-sh-Ctsk gene therapy carries great potential for effectively and safely targeting bone loss that results from oral infectious diseases like periodontitis.

AAV-sh-Ctsk knockdown can inhibit Ctsk expression and bone resorption *in vitro* and *in vivo*

Our investigation of the effects of AAV-mediated Ctsk knockdown on osteoclast function *in vitro* was carried out through analysis of bone resorption using WGA and SEM. Our results revealed that AAV-mediated Ctsk depletion caused a drastic impairment in bone resorption *in vitro* ($P < 0.001$). Consistent with that posture, TRAP staining showed a significant decrease in TRAP-positive osteoclasts *in vivo* in AAV-sh-Ctsk treated group as compared to the AAV-sh-luc-YFP group. Periodontal ligament cells are fibroblast-like cells characterized by collagen production as well as cytokine and chemokine release, which can contribute to periodontal inflammation (27). Notably, we found that Ctsk was highly expressed in the periodontal ligament area in the AAV-sh-luc-YFP group by IFC staining, which suggest an important role for Ctsk in fibroblast-like cells. Osteoclasts are the only well studied cell type that can mediate the bone resorption (28, 29). Notably, recent studies have indicated that osteoclast activation is not only solely dependent on osteoblasts, but it may also dependent on other cells present in the local bone environment. Given that DCs, macrophages, and osteoclasts are all originated from the hematopoietic cell lineage, it is believed that osteoclasts are closely related to the immune cells which may affect osteoclast formation and/or function through formation of different types of terminal cells in their local environment (30–32). It was recently reported that whereas Ctsk knockout in mouse displayed normal osteoclast numbers but nonfunctional osteoclasts as assessed by their inability to resorb bone matrix, indicating that Ctsk is critical for osteoclast function but may play a dispensable role in osteoclast differentiation (33). Unlike the finding from Ctsk knockout mice, our data showed that osteoclast numbers were decreased in the AAV-sh-Ctsk treated group, suggesting that the knockdown of Ctsk may affect osteoclast activation through its effect on immune cells. Further studies are needed to assess the role of Ctsk in osteoclastogenesis in the context of the bone microenvironment under inflammatory stimulation.

AAV-mediated Ctsk by AAV can effectively target both inflammation and bone loss in periodontitis

Bone resorption and inflammation are two key challenges in oral health because oral bacteria can strongly stimulate T and B cell activation. The later can strongly promote osteoclast formation and function which can ultimately lead to bone loss around the teeth (34, 35). We previously characterized the role of Ctsk in osteoclasts and revealed that Ctsk is crucial for osteoclastic bone resorption (20, 36). It was recently reported that Ctsk inhibition could suppress autoimmune inflammation of the joints as well as bone resorption in autoimmune arthritis and that *Ctsk*^{-/-} rats were resistant to experimental autoimmune encephalomyelitis (37). Collectively, these findings suggest that Ctsk plays an important role in the immune system aside from its role in osteoclasts and may thus serve as an important

therapeutic target for targeting autoimmune diseases (37–39). Notably, these dual roles of Ctsk in osteoclast and immune functions make it an ideal candidate for targeting both inflammation and bone loss resorption in many inflammatory bone diseases. Indeed, local administration of AAV-sh-Ctsk can not only reduce bone loss and inflammation in periodontitis in our current study, but it can also inhibit periapical bone loss and inflammation in a mouse model of endodontic disease as we previously reported (40). Therefore, AAV-sh-Ctsk may be useful for targeting the interplays of osteoclastic bone resorption and activated immune functions in many inflammatory diseases. In this current study, we investigated the dual functions of Ctsk in relationship to periodontal disease. We revealed that treatment of periodontitis induced in mice by *P. gingivalis* infection with AAV-sh-Ctsk can protect mice from *P. gingivalis*-stimulated alveolar bone loss. Notably, AAV-sh-Ctsk treatment can also attenuate inflammation in *P. gingivalis*-induced periodontitis by in part decreasing T cells and DC numbers in the periodontal lesions.

The underlying mechanism of Ctsk functions is different in in periodontitis than periapical disease

Furthermore, we explored the functions of Ctsk using in this established mouse periodontitis model induced by infection with *P. gingivalis* W50, a known pathogen that is highly associated with periodontal disease progression by stimulating both inflammation and bone resorption, two key features of periodontitis (41). Also, the host immune and inflammatory response plays important roles periodontitis (1, 2). In our previous study, we applied four different bacterial strains to generate a periapical disease model to investigate the role of Ctsk in this disease (40). Whereas inflammation associated with periapical disease is mainly localized to the oral cavity, inflammation in periodontitis stems mostly from systemic reactions which can trigger other diseases (42). These findings indicate that the molecular mechanism by which Ctsk functions in periodontitis model is different than its role in periapical disease. We demonstrated that AAV-mediated Ctsk in periodontitis inhibit bone resorption and attenuate local inflammation. Specifically, AAV-sh-Ctsk caused down regulation of many inflammatory cytokines in the periodontal tissues as assessed by ELISA and qRT-PCR analyses. The reduction in inflammation in the AAV-sh-Ctsk treatment group may also be partially due to down regulation of IL-6 which can promote the activation and differentiation of the macrophages. In fact, IL-6 can be secreted by osteoblasts in response to bone resorbing agents such as IL-1 α , IL-1 β , and TNF α (43). Ctsk has been reported to have functions in DCs through Toll like receptors (37). Our results through co-expression of CD11c and Ctsk support that Ctsk may simultaneously stimulate the functions of the DCs and osteoclast function. Hence, our finding that expression of IL-6, IL-12 and TNF α was attenuated by AAV mediated Ctsk depletion may stem from the inhibition Ctsk functions in DCs, which should further be validated by *in vitro* study. In further understanding the molecular mechanisms underlying the protection from periodontitis-induced bone loss with Ctsk deletion, we revealed that while the expression of the osteoclast marker *Acp5* in the periodontitis lesion area was almost unaffected by AAV-mediated Ctsk knockdown, there was a block in bone turnover in the periodontitis which was due to an increase in OPG and a decrease in RANKL expression. This finding is in consistent with other reports demonstrating that RANKL is also expressed in inflammatory cells (mainly lymphocytes and macrophages) and proliferating epithelium in the vicinity of inflammatory cells (44).

Hence, the decrease in RANKL expression may be the result of attenuated inflammation by depletion of Ctsk in periodontitis lesions. Due to the limited tissue available for ELISA assays, we chose to examine the expression level of known inflammatory factors including IL-1 α , IL-1 β , TNF α , IL-6, IL-12, and IL-10 which can provide great mechanistic information on the functions of Ctsk in periodontitis. Furthermore, such analysis can help explain whether these expression changes can promote innate immune responses from tissue epithelia to limit the damage caused by viral and bacterial infections. These cytokines can also facilitate the tissue-healing process in injuries caused by infection or inflammation. TNF α and IL-1 α have been indicated as potent stimulators of bone resorption, and they have been found in the periodontitis lesion area (45, 46). IL-1 β is another inflammation factor that is connected with gingival inflammation (47). IL-6 may have a pro-inflammatory function by increasing its levels and reabsorbing bone in the presence of inflammatory factors like TNF α and IL-1 α (48). IL-12 can stimulate interferon-gamma (IFN γ) production through T helper type 1 (Th1) cells, which are considered to have a connection with the severity of periodontitis (49). All of these markers were downregulated after Ctsk was knocked down, which indicated that Ctsk deficiency might impair the immune response in the periodontitis local lesion area. Ctsk has been reported to be expressed in DCs (37). Notably, our immunofluorescence data showed that the number of DCs decreased significantly in the lesion area. Notably, IL-10 can also repress proinflammatory responses and limit tissue damage caused by inflammation, which support crucial roles for IL-10 family of cytokines in many infectious and inflammatory diseases (50). Collectively, our data demonstrate that AAV-mediated Ctsk silencing therapy can provide the unique opportunity to address the interconnected issues of inflammation and bone resorption in periodontitis.

Although, it is difficult to define the exact details of the mechanism at this point, we can determine that Ctsk knockdown results in defective Toll-like receptor 9 signaling in dendritic cells in response to bacterial infection as shown by the reduced mRNA level of proinflammatory cytokines (*e.g.* IL-12 and IL-6) that are regulated by TLR9 (Fig. 5). This finding is consistent with that reported by Asagiri *et al.* in a study using a mouse model of experimental arthritis in which Ctsk inhibitor reduced the TLR9-mediated immune response as shown by a reduction in cytokine (*e.g.* IL-12 and IL-6) mRNA (37). We further determined that Ctsk knockdown in periodontal lesions results in defective TLR4 signaling because the expression of TNF α was reduced in the lesion area. Our data indicates a dramatic reduction in Ctsk expression after Ctsk knockdown *in vitro* (Fig. 1F) and *in vivo* (Fig. 2F). Importantly, AAV-sh-Ctsk is administered after the immune response, inflammation, and bone resorption have already been initiated as would be the case in the clinic. Notably, the Asagiri *et al.* study did not examine bone resorption like our study (37).

In summary, our local administration of sh-Ctsk through AAV-mediated gene therapy can simultaneously and effectively inhibit both inflammation and osteoclastic bone resorption in *P. gingivalis*-induced periodontitis after these processes have already been initiated as would be the case in the clinic. Importantly, AAV gene therapy has been used in numerous studies to target many human diseases and thus carry great therapeutic potential for periodontal disease. Given the dual roles of Ctsk in promoting inflammation and osteoclastic bone resorption, utilization of this promising AAV technology to target Ctsk offers a unique tool

to simultaneously target bone resorption and inflammation with limited side-effects in numerous diseases including oral infectious diseases, rheumatoid arthritis, and bone metastases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Reference List

1. Sasaki H, Okamatsu Y, Kawai T, Kent R, Taubman M, Stashenko P. The interleukin-10 knockout mouse is highly susceptible to *Porphyromonas gingivalis*-induced alveolar bone loss. *J Periodontal Res.* 2004; 39:432–441. [PubMed: 15491348]
2. Wilensky A, Polak D, Awawdi S, Halabi A, Shapira L, Houri-Haddad Y. Strain-dependent activation of the mouse immune response is correlated with *Porphyromonas gingivalis*-induced experimental periodontitis. *Journal of clinical periodontology.* 2009; 36:915–921. [PubMed: 19735468]
3. Seckin Ertugrul A, Arslan U, Dursun R, Sezgin Hakki S. Periodontopathogen profile of healthy and oral lichen planus patients with gingivitis or periodontitis. *In J Oral Sci.* 2013; 5:92–97.
4. Darveau RP. Periodontitis: a polymicrobial disruption of host homeostasis. *Nature reviews Microbiology.* 2010; 8:481–490. [PubMed: 20514045]
5. Fischer CL, Walters KS, Drake DR, et al. Oral mucosal lipids are antibacterial against *Porphyromonas gingivalis*, induce ultrastructural damage, and alter bacterial lipid and protein compositions. *In J Oral Sci.* 2013; 5:130–140.
6. Li YP, Alexander M, Wucherpfennig AL, Yelick P, Chen W, Stashenko P. Cloning and complete coding sequence of a novel human cathepsin expressed in giant cells of osteoclastomas. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research.* 1995; 10:1197–1202.
7. Gelb BD, Shi GP, Chapman HA, Desnick RJ. Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency. *Science.* 1996; 273:1236–1238. [PubMed: 8703060]
8. Bankiewicz KS, Forsayeth J, Eberling JL, et al. Long-Term Clinical Improvement in MPTP-Lesioned Primates after Gene Therapy with AAV-hAADC. *Mol Ther.* 2006; 14:564–570. [PubMed: 16829205]
9. Wang L, Cao O, Swalm B, Dobrzynski E, Mingozzi F, Herzog RW. Major role of local immune responses in antibody formation to factor IX in AAV gene transfer. *Gene Ther.* 2005; 12:1453–1464. [PubMed: 15889137]
10. Kaplitt MG, Feigin A, Tang C, et al. Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial. *The Lancet.* 2007; 369:2097–2105.
11. Feng SM, Deng LF, Chen W, Shao JZ, Xu GL, Li YP. Atp6v1c1 is an essential component of the osteoclast proton pump and in F-actin ring formation in osteoclasts. *Biochem J.* 2009; 417:195–203. [PubMed: 18657050]
12. Musatov S, Chen W, Pfaff DW, Kaplitt MG, Ogawa S. RNAi-mediated silencing of estrogen receptor {alpha} in the ventromedial nucleus of hypothalamus abolishes female sexual behaviors.

- Proceedings of the National Academy of Sciences of the United States of America. 2006; 103:10456–10460. [PubMed: 16803960]
13. Hommel JD, Sears RM, Georgescu D, Simmons DL, DiLeone RJ. Local gene knockdown in the brain using viral-mediated RNA interference. *Nat Med.* 2003; 9:1539–1544. [PubMed: 14634645]
 14. Tu Q, Zhang J, Fix A, et al. Targeted overexpression of BSP in osteoclasts promotes bone metastasis of breast cancer cells. *J Cell Physiol.* 2009; 218:135–145. [PubMed: 18756497]
 15. Jiang H, Chen W, Zhu G, et al. RNAi-Mediated Silencing of Atp6i and Atp6i Haploinsufficiency Prevents Both Bone Loss and Inflammation in a Mouse Model of Periodontal Disease. *PLoS ONE.* 2013; 8:e58599. [PubMed: 23577057]
 16. Yang S, Hao L, McConnell M, et al. Inhibition of Rgs10 Expression Prevents Immune Cell Infiltration in Bacteria-induced Inflammatory Lesions and Osteoclast-mediated Bone Destruction. *Bone research.* 2013; 1:267–281. [PubMed: 24761229]
 17. Yang SY, Li YP. RGS10-null mutation impairs osteoclast differentiation resulting from the loss of [Ca²⁺]_i oscillation regulation. *Gene Dev.* 2007; 21:1803–1816. [PubMed: 17626792]
 18. Yang S, Wei D, Wang D, Phimphilai M, Krebsbach PH, Franceschi RT. In vitro and in vivo synergistic interactions between the Runx2/Cbfa1 transcription factor and bone morphogenetic protein-2 in stimulating osteoblast differentiation. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research.* 2003; 18:705–715.
 19. Yang S, Li YP. RGS10-null mutation impairs osteoclast differentiation resulting from the loss of [Ca²⁺]_i oscillation regulation. *Genes Dev.* 2007; 21:1803–1816. [PubMed: 17626792]
 20. Chen W, Yang SY, Abe Y, et al. Novel pycnodysostosis mouse model uncovers cathepsin K function as a potential regulator of osteoclast apoptosis and senescence. *Hum Mol Genet.* 2007; 16:410–423. [PubMed: 17210673]
 21. Chen W, Ma J, Zhu G, et al. Cbfb deletion in mice recapitulates cleidocranial dysplasia and reveals multiple functions of Cbfb required for skeletal development. *Proceedings of the National Academy of Sciences of the United States of America.* 2014; 111:8482–8487. [PubMed: 24850862]
 22. Goodson JM, Haffajee AD, Socransky SS, et al. Control of periodontal infections: a randomized controlled trial I. The primary outcome attachment gain and pocket depth reduction at treated sites. *Journal of clinical periodontology.* 2012; 39:526–536. [PubMed: 22512461]
 23. Evans C. Arthritis gene therapy trials reach phase II. *The Journal of rheumatology.* 2010; 37:683–685. [PubMed: 20360201]
 24. Mease PJ, Wei N, Fudman EJ, et al. Safety, Tolerability, and Clinical Outcomes after Intraarticular Injection of a Recombinant Adeno-associated Vector Containing a Tumor Necrosis Factor Antagonist Gene: Results of a Phase 1/2 Study. *Journal of Rheumatology.* 2010; 37:692–703. [PubMed: 20032102]
 25. Zhou Q, Guo RL, Wood R, et al. Vascular Endothelial Growth Factor C Attenuates Joint Damage in Chronic Inflammatory Arthritis by Accelerating Local Lymphatic Drainage in Mice. *Arthritis Rheum-U.S.* 2011; 63:2318–2328.
 26. Carter BJ. Adeno-associated virus vectors in clinical trials. *Hum Gene Ther.* 2005; 16:541–550. [PubMed: 15916479]
 27. Jönsson D, Nebel D, Bratthall G, Nilsson BO. The human periodontal ligament cell: a fibroblast-like cell acting as an immune cell. *J Periodontal Res.* 2011; 46:153–157. [PubMed: 21118418]
 28. Chen W, Zhu G, Hao L, Wu M, Ci H, Li Y-P. C/EBP α regulates osteoclast lineage commitment. *Proceedings of the National Academy of Sciences.* 2013; 110:7294–7299.
 29. Li YP, Chen W, Liang Y, Li E, Stashenko P. Atp6i-deficient mice exhibit severe osteopetrosis due to loss of osteoclast-mediated extracellular acidification. *Nat Genet.* 1999; 23:447–451. [PubMed: 10581033]
 30. O'Gradaigh D, Compston JE. T-cell involvement in osteoclast biology: implications for rheumatoid bone erosion. *Rheumatology.* 2004; 43:122–130. [PubMed: 12867576]
 31. Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat Rev Immunol.* 2007; 7:292–304. [PubMed: 17380158]
 32. Nakashima T, Takayanagi H. Osteoclasts and the immune system. *J Bone Miner Metab.* 2009; 27:519–529. [PubMed: 19455385]

33. Saftig P, Hunziker E, Wehmeyer O, et al. Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*. 1998; 95:13453–13458. [PubMed: 9811821]
34. Lappin DF, Macleod CP, Kerr A, Mitchell T, Kinane DF. Anti-inflammatory cytokine IL-10 and T cell cytokine profile in periodontitis granulation tissue. *Clin Exp Immunol*. 2001; 123:294–300. [PubMed: 11207661]
35. Manhart SS, Reinhardt RA, Payne JB, et al. Gingival cell IL-2 and IL-4 in early-onset periodontitis. *Journal of periodontology*. 1994; 65:807–813. [PubMed: 7990015]
36. Li Y-P, Chen W. Characterization of Mouse Cathepsin K Gene, the Gene Promoter, and the Gene Expression. *Journal of Bone and Mineral Research*. 1999; 14:487–499. [PubMed: 10234569]
37. Asagiri M, Hirai T, Kunigami T, et al. Cathepsin K-dependent toll-like receptor 9 signaling revealed in experimental arthritis. *Science*. 2008; 319:624–627. [PubMed: 18239127]
38. Kamolmatyakul S, Chen W, Li YP. Interferon-gamma down-regulates gene expression of cathepsin K in osteoclasts and inhibits osteoclast formation. *Journal of dental research*. 2001; 80:351–355. [PubMed: 11269728]
39. Kamolmatyakul S, Chen W, Yang S, et al. IL-1 α stimulates cathepsin K expression in osteoclasts via the tyrosine kinase-NF- κ B pathway. *Journal of dental research*. 2004; 83:791–796. [PubMed: 15381721]
40. Gao B, Chen W, Hao L, et al. Inhibiting periapical lesions through AAV-RNAi silencing of cathepsin K. *Journal of dental research*. 2013; 92:180–186. [PubMed: 23166044]
41. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. *Periodontology 2000*. 1994; 5:78–111. [PubMed: 9673164]
42. Seymour GJ, Ford PJ, Cullinan MP, Leishman S, Yamazaki K. Relationship between periodontal infections and systemic disease. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases*. 2007; 13(Suppl 4):3–10.
43. Irwin CR, Myrillas TT. The role of IL-6 in the pathogenesis of periodontal disease. *Oral diseases*. 1998; 4:43–47. [PubMed: 9655045]
44. Liu D, Xu JK, Figliomeni L, et al. Expression of RANKL and OPG mRNA in periodontal disease: possible involvement in bone destruction. *International journal of molecular medicine*. 2003; 11:17–21. [PubMed: 12469211]
45. Shirodaria S, Smith J, McKay IJ, Kennett CN, Hughes FJ. Polymorphisms in the IL-1A Gene are Correlated with Levels of Interleukin-1 α Protein in Gingival Crevicular Fluid of Teeth with Severe Periodontal Disease. *Journal of dental research*. 2000; 79:1864–1869. [PubMed: 11145356]
46. Delima AJ, Oates T, Assuma R, et al. Soluble antagonists to interleukin-1 (IL-1) and tumor necrosis factor (TNF) inhibits loss of tissue attachment in experimental periodontitis. *Journal of clinical periodontology*. 2001; 28:233–240. [PubMed: 11284536]
47. Miller CS, King CP Jr, Langub MC, Kryscio RJ, Thomas MV. Salivary biomarkers of existing periodontal disease: A cross-sectional study. *The Journal of the American Dental Association*. 2006; 137:322–329. [PubMed: 16570465]
48. Azuma MM, Samuel RO, Gomes-Filho JE, Dezan-Junior E, Cintra LTA. The role of IL-6 on apical periodontitis: a systematic review. *International Endodontic Journal*. 2014; 47:615–621. [PubMed: 24224782]
49. Orozco A, Gemmell E, Bickel M, Seymour GJ. Interleukin-1 β , interleukin-12 and interleukin-18 levels in gingival fluid and serum of patients with gingivitis and periodontitis. *Oral Microbiol Immunol*. 2006; 21:256–260. [PubMed: 16842511]
50. Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annual review of immunology*. 2011; 29:71–109.

Figure 1. AAV efficiently transduced osteoclasts and ctsk knockdown by AAV impairs osteoclast function *in vitro*

(A) Immunofluorescent photomicrograph of AAV-sh-luc-YFP control and AAV-sh-Ctsk treatment groups seven days after transduction. (B) Western blot analysis of Ctsk expression from the mock, AAV-sh-Luc-YFP, and AAV-sh-Ctsk treated groups. (C) Quantification of Western blot analysis demonstrates that the AAV-sh-Ctsk treated group showed significant reduction in Ctsk expression as compared to the AAV-sh-luc-YFP control group. (D) Untransduced osteoclasts (mock) and osteoclasts transduced with AAV-sh-Ctsk or AAV-sh-luc-YFP (control) stained with acridine orange to show extracellular acidification. (E) Bone resorption pits were visualized by WGA and SEM analyses. (F) Quantification of bone pits from SEM analysis shows that bone resorption is significantly lower in the AAV-sh-Ctsk treated group as compared to the Mock and AAV-sh-luc-YFP groups (n=3). *: $P < 0.05$, **: $P < 0.01$, N.S: $P > 0.05$.

Figure 2. AAV can effectively transduce periodontal tissue, display a local distribution pattern, and inhibit osteoclast differentiation *in vivo*

(A) Image of the mouse maxillary molars showing the sites of AAV administration (black arrows). (B) Image analysis at 2 and 5 weeks after a single administration of AAV-sh-luc-YFP into the lower right jaw. Luciferase expression was sustained and limited to the local injection sites (N=15, n=5 samples in each group; experiments were repeated 3 times). (C) Fluorescent micrographs of AAV-infected cells in uninfected mice and infected mice treated with AAV-sh-Ctsk and AAV-sh-luc-YFP. (G, gingival; P, pulp; PDL, periodontal ligament) (N=15, n=5 samples in each group; experiments were repeated 3 times). (D) TRAP staining reveals the presence of osteoclasts surrounding the tooth root structures in both AAV-sh-luc-YFP and AAV-sh-Ctsk treated groups (n=3). (E) Localization of Ctsk expression (Red, anti-Ctsk; Blue, nuclei counterstaining with Hoechst 33342) in the murine periodontal tissue transduced with AAV-sh-luc-YFP or AAV-sh-Ctsk (P, pulp; PDL, periodontal ligament) (n=3). (F) Quantification of TRAP positive osteoclasts in lesion areas. (G) Quantification of Ctsk positive cells in periodontal tissue area. *: P<0.05, **: P<0.01, ***: P<0.001, N.S. indicates P>0.05.

Figure 3. Ctsk silencing by AAV protects mice from bone loss in *P. gingivalis*-induced periodontitis

(A) Periodontitis as indicated by alveolar bone loss and root exposure was examined in normal group versus *P. gingivalis*-infected mice treated with AAV-sh-Ctsk, AAV-sh-luc-YFP or PBS. (B) Quantification of bone resorption in the normal group, AAV-sh-luc-YFP, PBS treated, and AAV-sh-Ctsk treated groups (N=21, n=7, repeated 3 times). (C) H&E staining of sections from uninfected mice (Normal) and *P. gingivalis*-infected mice treated with AAV-sh-Ctsk, AAV-sh-luc-YFP or PBS. (D) The width of periodontal ligament in the uninfected mice and *P. gingivalis*-infected mice treated with AAV-sh-Ctsk, AAV-sh-luc-YFP or PBS (N=15, n=5, repeated 3 times). (E) IFC staining of alveolar sections from different groups. Cell nuclei were labeled using DAPI staining (blue). Blue and green fluorescence merged with bright DIC (differential interference contrast) and DAPI shows the shape of the tooth (N=15, n=5, repeated 3 times). (F) Quantification of CD3-positive T cells shows a

significant reduction in T cell number in the AAV-sh-Ctsk group as compared to the AAV-sh-luc-YFP and PBS group. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$, N.S: $P>0.05$.

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Figure 4. Ctsk Knockdown decreases the number of CD11c and Ctsk double positive cells in periodontal lesion areas

(A) IFC staining of CD11c (red) and Ctsk (yellow) double positive DCs in the periodontal lesion area in the uninfected mice (Normal) and *P. gingivalis*-infected mice treated with AAV-sh-Ctsk, AAV-sh-luc-YFP and PBS (N=15, n=5, repeated 3 times) at 56 days. Blue and green fluorescence merged with bright DIC (differential interference contrast) and DAPI shows the shape of the periodontal tissue. (B) Normal serum served as a negative control of the same area (Without primary antibody). (C) Quantification of double positive DCs analysis demonstrates that Ctsk has significantly reduced expression of CD11c (red) and Ctsk (yellow) double positive DCs in different groups. **: P<0.01. N.S: No Significance.

Figure 5. AAV-sh-Ctsk reduces the expression of inflammatory factors in the periodontal tissues (A) qRT-PCR of osteoclast-specific genes (i.e. Acp5, RANKL and OPG) and inflammatory cytokines (IL-1 α , IL-1 β , and IL-6) in the periodontal tissues of uninfected mice (normal) or *P. gingivalis*-infected mice treated with AAVsh- luc-YFP or with AAV-sh-Ctsk. Expression levels were normalized to Hprt (N=15, n=5, repeated 3 times). (B) The level of inflammatory factors IL-1 α , IL-6, IL-12, IL-17A, TNF- α , IFN- γ and IL-10 in the periodontal tissues as detected by ELISA (pooled 3 samples each time in each group on three independent experiments). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, N.S: $P > 0.05$.