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C1 silencing attenuates inflammation and alveolar bone resorption in endodontic disease

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Abstract

Introduction—Endodontic disease, one of the most prevalent chronic infectious diseases worldwide, occurs when the dental pulp becomes infected and inflamed, leading to bone destruction around the tooth root, severe pain, and tooth loss. Though many studies have tried to develop therapies to alleviate the bone erosion and inflammation associated with endodontic disease, there is an urgent need for an effective treatment.

Methods—In this study, we used a gene-based therapy approach by administering recombinant adeno-associated virus (AAV)-mediated *Atp6v1c1* knockdown to target both periapical bone resorption and inflammation in the mouse model of endodontic disease.

Results—The results showed that *Atp6v1c1* knockdown is simultaneously capable of reducing bone resorption by 70% through impaired osteoclast activation, inhibiting inflammation by decreasing T-cell infiltration in the periapical lesion by 75%, and protected the periodontal ligament (PDL) from destruction caused by inflammation. Notably, AAV-mediated gene therapy of *Atp6v1c1* knockdown significantly reduced proinflammatory cytokine expression, including tumor necrosis factor α, interleukin-1α, interleukin-17, interleukin-12, and interleukin-6 levels in periapical tissues caused by bacterial infection. Quantitative real-time polymerase chain reaction revealed that *Atp6v1c1* knockdown reduced osteoclast-specific functional genes (ie, *Ctsk*) in periapical tissues.

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The authors deny any conflicts of interest related to this study

Conclusions—Our results demonstrated that AAV-mediated *Atp6v1c1* knockdown in periapical tissues slowed endodontic disease progression, prevented bone erosion, and alleviated inflammation in the periapical tissues and periodontal ligament potentially through regulation of TLR signaling, indicating that targeting *Atp6v1c1* may facilitate the design of novel therapeutic approaches to reduce inflammation and bone erosion in endodontic disease.

Keywords

RNAi knockdown; Gene therapy; Atp6v1c1; periapical disease; gingival inflammation; oral bone resorption

Introduction

Dental plaque bacteria is the main cause of dental caries. If left untreated, the bacteria progresses to the dental pulp in the root canal, leading to pulp inflammation and necrosis and further bone destruction around the tooth root, severe pain, and tooth loss. The periodontal ligament (PDL) is comprised of specialized connective tissue fibers that attach the tooth to the alveolar bone. During endodontic disease progression, inflammation in the periapical area and PDL leads to tooth loss. Periapical bone destruction is mainly induced by enhanced osteoclast formation and function, initiated by extracellular acidification and following the degradation of the organic constituent of bone. The bacteria increases T-cell and B-cell activation, which promotes both inflammation and osteoclast differentiation (1). Currently, endodontic disease is treated by mechanical removal of the infected pulp tissue, followed by obturation of the root canal space with an inert filling material (2), however periapical bone regeneration may take as long as two years, and in some cases complete healing is never achieved. Thus, there is urgent need for a novel osteoclast-specific inhibitor which can simultaneously target bone loss and inflammation in periapical disease.

Atp6v1c1, a subunit of the V-ATPase, is highly expressed in osteoclasts, is mainly localized on the ruffled border of activated osteoclasts (3). Functioning as a subunit of osteoclast proton pump Atp6i, *Atp6v1c1* can be induced by receptor activator for NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) during osteoclast differentiation (3). The mature osteoclasts attach to the bone surface to deliver the proton pump V-ATPase and then release extracellular matrix-digesting acid proteases, such as Cathepsin K (Ctsk), for the degradation of bone matrices (4–6). Our previous research demonstrated that osteoclast acidification activity and bone resorption is impaired through silencing of *Atp6v1c1* (7), suggesting its therapeutic potential for diseases of excessive osteoclastic bone resorption. Furthermore, our previous studies have revealed that silencing of *Atp6v1c1* can prevent cancer progression and metastasis, indicating its potential function in immune responses (8). As a subunit of Atp6i, *Atp6v1c1* is expressed in immune cells such as macrophages and dendritic cells, as well as osteoclasts (7, 9), and may play an osteoimmune role during endodontic disease pathogenesis. The regulation of both osteoclast differentiation and the immune response is crucial for the maintenance of alveolar bone volume, as disruptions may result in pathologic osteoclastic diseases such as endodontic disease.

We hypothesized that local Atp6v1c1 silencing in the periapical lesion could inhibit osteoclastic activity and inflammation simultaneously. To investigate the potential role of C1 in periapical disease, we used a poly-microbial induced periapical mouse model in conjunction with the AAV knockdown system to investigate the effect of Atp6v1c1 silencing in periapical disease. We propose AAV mediated knockdown of Atp6v1c1 as a novel therapeutic target for the treatment of endodontic disease.

Materials and Methods

Study approval.

All animal experimentation was carried out according to the legal requirements of the Association for Assessment and Accreditation of the Laboratory Animal Care International and the University of Alabama at Birmingham IACUC, and followed all recommendations of ARRIVE guidelines.

AAV RNAi viral production and purification.

We purchased the AAV Helper-Free System (AAV Helper-Free System Catalog #240071, Stratagene). Viral production was accomplished using a triple-transfection, helper-free method, and purified with a modified version of a published protocol (10).

Pulp exposure, bacterial infection, and transduction of AAV vectors.

The periapical disease mouse model was produced as we previously described (11, 12). Bacterial culture, infection, and viral vectors transduction in a site-specific manner was performed as described (11, 12).

Statistical analysis.

Experimental data are reported as mean ± SD of triplicate independent samples. The figures are representative of the data (n=15). Data quantification analyses were performed using the NIH Image J Program as described (11–13).

Results

Atp6v1c1 knockdown impaired osteoclast function including osteoclast-mediated bone resorption and extracellular acidification *in vitro*.

To enable knockdown of *Atp6v1c1*, we generated shRNA that targeted *Atp6v1c1*. We used the AAV2 serotype and subcloned shRNA targeting *Atp6v1c1* into the AAV.H1 vector (gift from Dr. Sergei Musatov) (Fig. 1A). To confirm the effect of *Atp6v1c1* silencing, we examined the expression of *Atp6v1c1* in mouse bone marrow (MBM) isolated from wild-type BALB/cJ mice, cultured with *M-CSF* and *RANKL* to generate osteoclasts (14), and transduced with AAV-sh-*Atp6v1c1* or AAV-luc-YFP. Western blot analysis revealed that osteoclasts transduced with AAV-sh-*Atp6v1c1* have an 80% reduction in *Atp6v1c1* expression compared to untreated osteoclasts (mock) or osteoclasts transduced with AAV-luc-YFP (Fig. 1B). Overall, our results indicate that AAV-sh-*Atp6v1c1* efficiently knocked down *Atp6v1c1* expression. To investigate how *Atp6v1c1* knockdown can affect osteoclast differentiation and function, TRAP staining, Wheat germ agglutinin staining, and scanning

electron microscopy were performed. Notably, osteoclast number was not significantly changed after transduction with AAV-sh-*Atp6v1c1,* as shown by TRAP staining (Fig. 1C, 1F). We further sought to investigate the effects of AAV-sh-*Atp6v1c1* on osteoclasts function by examining bone resorption. Wheat germ agglutinin staining demonstrated that compared to AAV-luc-YFP treatment, AAV-mediated *Atp6v1c1* knockdown reduced osteoclast-mediated bone resorption by 80% (Fig. 1D, G). Consistently, visualization of the resorption lacunae through scanning electron microscopy demonstrated that AAV-sh-*Atp6v1c1* completely impaired bone resorption *in vitro* (Figs. 1E, H). To further investigate how *Atp6v1c1* knockdown can affect osteoclast function, we examined osteoclast extracellular acidification. Osteoclasts were induced from wild-type mouse bone marrow (MBM) stimulated by M-CSF and RANKL for 3 days. After transduction by lentivirus mediated *Atp6v1c1* knockdown, Acridine Orange staining was conducted to evaluate osteoclast extracellular acidification (Fig. 1I). We found that lentivirus-sh-*Atp6v1c1* severely impaired extracellular acidification compared to the scrambled control (Fig. 1I, J). The actin ring is formed during osteoclast maturation, and is a key structure for osteoclast extracellular acidification. Thus, to further investigate the cause of impaired acidification ability, we detect the actin ring formation in mock, C1 knockdown and AAV-*luc*-YFP osteoclasts (Fig. 1K). Results showed that C1 depleted osteoclasts fail to form normal actin ring. Our results demonstrated that *Atp6v1c1* silencing results in impaired osteoclast extracellular acidification and bone resorption, but not differentiation.

Atp6v1c1 depletion reduced infection-stimulated periapical bone resorption through reduced osteoclast differentiation.

In order to determine the efficacy of AAV-sh-*Atp6v1c1* in improving the health of oral tissues affected by endodontic disease, we used a model of periapical lesion induction (11, 12). Radiographic imaging of the distal root of mandibular first molar was performed to compare the periapical bone resorption in uninfected normal mice and infected mice treated with either AAV-luc-YFP or AAV-sh-*Atp6v1c1* (Fig. 2A). We found that the infected group treated with AAV-luc-YFP had significantly more bone resorption compared to the normal control, while AAV-sh-*Atp6v1c1* treatment in infected mice protected periapical bone against resorption as shown by X-ray (Fig. 2A, red arrows) and micro computed tomography (μCT) analysis (Fig. 2B, red arrows). The percentage of bone volume/total volume (BV/TV) was increased by 25% in the AAV-sh-*Atp6v1c1* treated mice compared to the AAV-luc-YFP treatment group (Fig. 2C). In order to further examine how AAV-sh-*Atp6v1c1* treatment attenuates bone destruction *in vivo,* tooth sections from normal and infected mice treated with AAV-sh-*Atp6v1c1* or AAV-luc-YFP were stained with tartrate-resistant acid phosphatase (TRAP), which indicated that AAV-sh-*Atp6v1c1* treatment impaired the number of activated osteoclasts *in vivo* by 70% (Fig. 2D, E). While our *in vitro* results did not show any significant changes in osteoclast differentiation following *Atp6v1c1* silencing, we found that osteoclast differentiation was significantly reduced following *Atp6v1c1* silencing in endodontic disease model due to attenuated inflammation, compared to the infected AAV-luc-YFP treated mice. Under inflammatory conditions, activated T-cells can induce osteoclastogenesis via RANKL-dependent and RANKL-independent mechanisms (15). Thus, upon *Atp6v1c1* knockdown, T-cell mediated immune response was inhibited, impairing RANKL–stimulated osteoclast differentiation. Collectively, these data

demonstrated that AAV-mediated *Atp6v1c1* knockdown prevent periapical bone resorption *in vivo* by impairing osteoclast differentiation.

Atp6v1c1 knockdown attenuates inflammation in the periodontal ligament and periapical lesions through inhibiting immune cell infiltration.

To further examine how *Atp6v1c1* knockdown attenuates bone destruction *in vivo,* tooth sections from normal and infected mice treated with AAV-sh-*Atp6v1c1* or AAV-luc-YFP were stained with hematoxylin and eosin (H&E) (Fig. 3A). We found that immune cells (monocytes, macrophages and granulocytes) infiltration in the periapical lesion was significantly increased in the infected group treated with AAV-luc-YFP as shown by H&E stain, whereas immune cells infiltration was dramatically reduced in the periapical lesions of the AAV-sh-*Atp6v1c1* group (Fig. 3A). Notably, treatment with AAV-sh-*Atp6v1c1* reduced the width of the periodontal ligament (PDL) by 50% compared to the AAV-luc-YFP treated disease group, and was similar to the normal control (Fig. 3A). Consistent with the μCT results, quantification analysis of the bone resorption area showed that the AAV-sh-*Atp6v1c1* treated mice had a 50% increase in bone area compared to the disease group treated with AAV-luc-YFP (Fig. 3B). To further investigate the role of *Atp6v1c1* in inflammation, immunofluorescence (IF) and IHC staining for CD3 positive T-cells was performed in uninfected normal mice and infected mice treated with AAV-luc-YFP or AAV-sh-*Atp6v1c1* (Fig. 3C, E). The data demonstrated that infiltration of CD3 positive T-cells in the AAV-sh-*Atp6v1c1* group was reduced by 75% compared with that of the AAV-luc-YFP group (Fig. 3D, F), indicating that AAV-sh-*Atp6v1c1* knockdown attenuates inflammatory responses in the periapical lesions through inhibiting immune cell infiltration.

AAV-mediated *Atp6v1c1* knockdown reduced the expression of inflammatory cytokines in the periapical lesions and periodontal ligament (PDL).

We confirmed *Atp6v1c1* knockdown *in vivo* by immunohistochemical (IHC) stain in the periapical lesion area, and found that the expression of *Atp6v1c1* had been efficiently knocked down in the AAV-sh-*Atp6v1c1* treated group (Fig. 4A, B). Notably, the width of the PDL was reduced by 50% in the *AAV-sh-Atp6v1c1* group compared to the AAV-luc-YFP treated mice, indicating attenuated inflammation in *AAV-sh-Atp6v1c1* treated mice (Fig. 4A). We performed ELISA to evaluate the effect of *Atp6v1c1* silencing on the protein levels of the pro-inflammatory cytokines IL-1α, IL-6, IL-17, IL-10, IL-12, and TNFα (Fig. 4C). Interestingly, our data showed that the protein levels of IL-1α, IL-6, IL-17, IL-12 and TNFα were significantly decreased in the AAV-sh-*Atp6v1c1* treated group compared to the AAV-luc-YFP group, while IL-10 protein levels were increased in the AAV-sh-*Atp6v1c1* treated group (Fig. 4C). Produced by mast cells, IL-10 has been shown to contribute to the anti-inflammatory or immunosuppressive effects in inflammatory conditions (16). IL-6 is secreted by osteoblasts in response to bone resorption and is important for osteoclast differentiation (17). The relative mRNA expression level of the osteoclast marker gene *Cathepsin K* was decreased by 60% in the AAV-sh-*Atp6v1c1* treated group compared to the AAV-luc-YFP treatment group (Fig. 4D). In addition, the expression levels of pro-inflammatory markers *TNF*α and *IL-17* were decreased by 50% and 75%, in the AAV-sh-*Atp6v1c1* treated group respectively, AAV-sh-*Atp6v1c1* also reduced the expression of *IL-6,* which is important for osteoclast differentiation, by 50% (Fig. 4D). Previous studies have

demonstrated that V-ATPases are required for mTORC1 activation stimulated by amino acids (18), thus we tested co-localization of Atp6v1c1 and mTOR in 4T1 epithelial cells (SFig. 1). Our results showed co-expression of Atp6v1c1 and mTOR in epithelial cells, indicating its relationship with mTOR signaling (Fig. S1). Thus, Atp6v1c1 may regulate inflammation though the mTOR and/or TLR pathways. Furthermore, our data demonstrate that Atp6v1c1 is involved, not only in osteoclasts, but also in dendritic cells, T cells, and macrophages. It is also possible that mTOR signaling is mediated by Atp6v1c1 in both osteoclasts and immune cells. TNF-α and IL-10 are downstream cytokines in TLR2/4 signaling, which is involved in the periapical disease pathogenesis (19). Our results showed that the expression of TNF-α is down-regulated in Atp6v1c1 depletion group, while IL-10 is upregulated. Furthermore, knockdown of Atp6v1c1 inhibited the levels of IL-12 and IL-6, which are regulated by TLR9. These results suggested that Atp6v1c1 may regulate TLRs signaling in endodontic disease. In conclusion, we found that *Atp6v1c1* knockdown significantly reduced proinflammatory cytokine expression, indicating that *Atp6v1c1* may regulate TLRs signaling in endodontic disease.

Discussion

To investigate the role of *Atp6v1c1* in periapical disease, we characterized the efficacy of a gene therapy using recombinant adeno-associated virus (AAV)-mediated *Atp6v1c1* knockdown to target periapical bone resorption and inflammation simultaneously. Depletion of *Atp6v1c1* in the mouse model of periapical disease reduced bone destruction by 70%, impaired osteoclast activation, decreased T-cell infiltration in the periapical lesion by 75%, and protected the PDL from destruction caused by inflammation. Furthermore, AAV-mediated *Atp6v1c1* knockdown also reduced bacterial infection-stimulated pro-inflammatory cytokines expression. Notably, our data also showed that osteoclast extracellular acidification was impaired due to *Atp6v1c1* silencing. Our results demonstrated that AAV-mediated *Atp6v1c1* knockdown in periapical tissues can slow endodontic disease progression, prevent bone erosion, and alleviate inflammation, indicating that targeting *Atp6v1c1* may result in novel therapeutic approaches for diseases of osteoclast overactivation such as endodontic disease.

In this study, we found that depletion of *Atp6v1c1* in the mouse model of endodontic disease reduced bone destruction. We previously demonstrated that *Atp6i* is an osteoclast-specific proton pump which is essential for osteoclast-mediated extracellular acidification during bone resorption (20). As a subunit of *Atp6i*, *Atp6v1c1* is located in the V1 domain of V-ATPase and is considered to be directly responsible for regulating the dissociative mechanism of the V-ATPase (21, 22). Moreover, *Atp6v1c1* regulates intracellular and intraorganellar pH together with other subunits (23, 24). Our results demonstrated that local administration of AAV-sh-*Atp6v1c1* in the apical area inhibited bone resorption, potentially resulting from osteoclast malfunction due to *Atp6v1c1* knockdown. Biochemical analysis revealed that *Atp6v1c1* stabilizes the V-ATPase complex assembly and increases proton pump activity, which promotes bone resorption (25–27). AAV-sh-*Atp6v1c1* impairs the proton exchange, thus the acidic microenvironment that favors bone resorption cannot be maintained. AAV-sh-*Atp6v1c1* rescued bone resorption and restored the bone surrounding

the tooth root; therefore, the reduced bone resorption might be related to the malfunction of osteoclasts after *Atp6v1c1* knockdown.

Notably, *Atp6v1c1* not only protects against bone resorption in endodontic disease, but also protected the periapical tissues and PDL from destruction caused by inflammation, yet the mechanism underlying how *Atp6v1c1* regulates inflammation is still unknown. Inflammatory signals mediated by immune cells and cytokines in endodontic disease have a significant influence over osteoclast differentiation and function through direct or indirect effects on osteoclast precursors in the bony microenvironment (28). Similarly, osteoclasts can express numerous immune receptors (29, 30). Our data show that AAV-sh-*Atp6v1c1* decreased CD3 positive T-cells and inflammatory cytokines, indicating that *Atp6v1c1* knockdown not only impairs osteoclast function, but also the immune response. TLR signaling is critical for cytokine secretion and the T-cell mediated immune response. Activated T-cells can induce osteoclastogenesis via RANKL-dependent and RANKL-independent mechanisms under inflammatory conditions (15). Thus, upon *Atp6v1c1* knockdown, T-cell mediated immune response was inhibited, impairing RANKL–stimulated osteoclast differentiation. A previous study reported that activated Ctsk is responsible for the cleavage of TLR9, and the activation of TLR9 signaling responsible for inflammatory responses (31). Under a low pH microenvironment, activated Ctsk is secreted either from immune cell lysosomes or following osteoclast extracellular acidification. Due to osteoclasts malfunction after *Atp6v1c1* silencing, the acidic environment for bone resorption is interrupted, thus inhibiting Ctsk activation. Our data demonstrate that *Atp6v1c1* knockdown impairs osteoclast extracellular acidification and may block Ctsk activation thus inhibiting TLR9 signaling.

We further evaluated the effects of *Atp6v1c1* silencing on the levels of pro-inflammatory cytokines in periapical tissues, and found that IL-6 expression was reduced after *Atp6v1c1* knockdown. IL-6 is secreted by osteoblasts in response to bone resorption and is important for osteoclast differentiation (17). We found similar decreases in the expression of the classic pro-inflammatory mediators TNF-α and IL-12 following *Atp6v1c1* knockdown. Although further studies are needed determine the exact mechanism underlying how C1 depletion can inhibit inflammation, TLR signaling may be involved. TLRs can recognize the microorganisms and their components, which stimulate the production of inflammatory cytokines. TLR2/TLR4 has been demonstrated to be expressed in endodontic disease (32), and while TLR4 knockout mice showed reduced bone destruction in periapical lesions, TLR2 deficient mice showed increased periapical lesion size and increased osteoclast numbers. TLR9 signaling has been demonstrated to stimulate IL-6 and IL-12 production (31). We found that IL-6 and IL-12 protein levels were decreased in the Atp6v1c1 knockdown group, suggesting that *Atp6v1c1* may mediate the immune response through TLR9 signaling. Cintra et al. demonstrated that apical periodontitis increased serum levels of IL-17 (33). AAV-sh-*Atp6v1c1* significantly decreased the levels of IL-1α and IL-17 in the mouse model of periapical disease. Furthermore, IL-10, which can suppress proinflammatory responses in inflammatory conditions (16), was increased following *Atp6v1c1* silencing. Thus, loss of extracellular acidification by AAV-sh-*Atp6v1c1* may inhibit Ctsk activation and inhibit TLR9 signaling in periapical disease, suggesting a critical role of *Atp6v1c1* in mediating immune responses. The periodontal ligament (PDL) is

comprised of specialized connective tissue fibers that attach the tooth to the alveolar bone, and in endodontic disease, inflammation of the PDL leads to tooth loss. Interestingly, our data show that AAV-sh-*Atp6v1c1* protected the PDL from destruction caused by inflammation, although the mechanism underlying how *Atp6v1c1* regulates inflammation warrants further study.

In conclusion, we investigated the therapeutic effect of AAV-sh-*Atp6v1c1* in periapical disease to inhibit osteoclastic bone resorption and inflammation, and revealed that AAV-sh-*Apt6v1c1* protected the PDL from destruction caused by inflammation by impairing of osteoclast extracellular acidification and potentially through regulating TLRs signaling. This work provides important insights into the role of *Apt6v1c1* in osteolytic and inflammatory diseases such as periapical disease, and the mechanisms underlying how *Apt6v1c1* modulates osteoclast-mediated bone resorption and inflammation. Insights resulting from this study may assist in developing novel treatments for periapical disease and other osteolytic and inflammatory diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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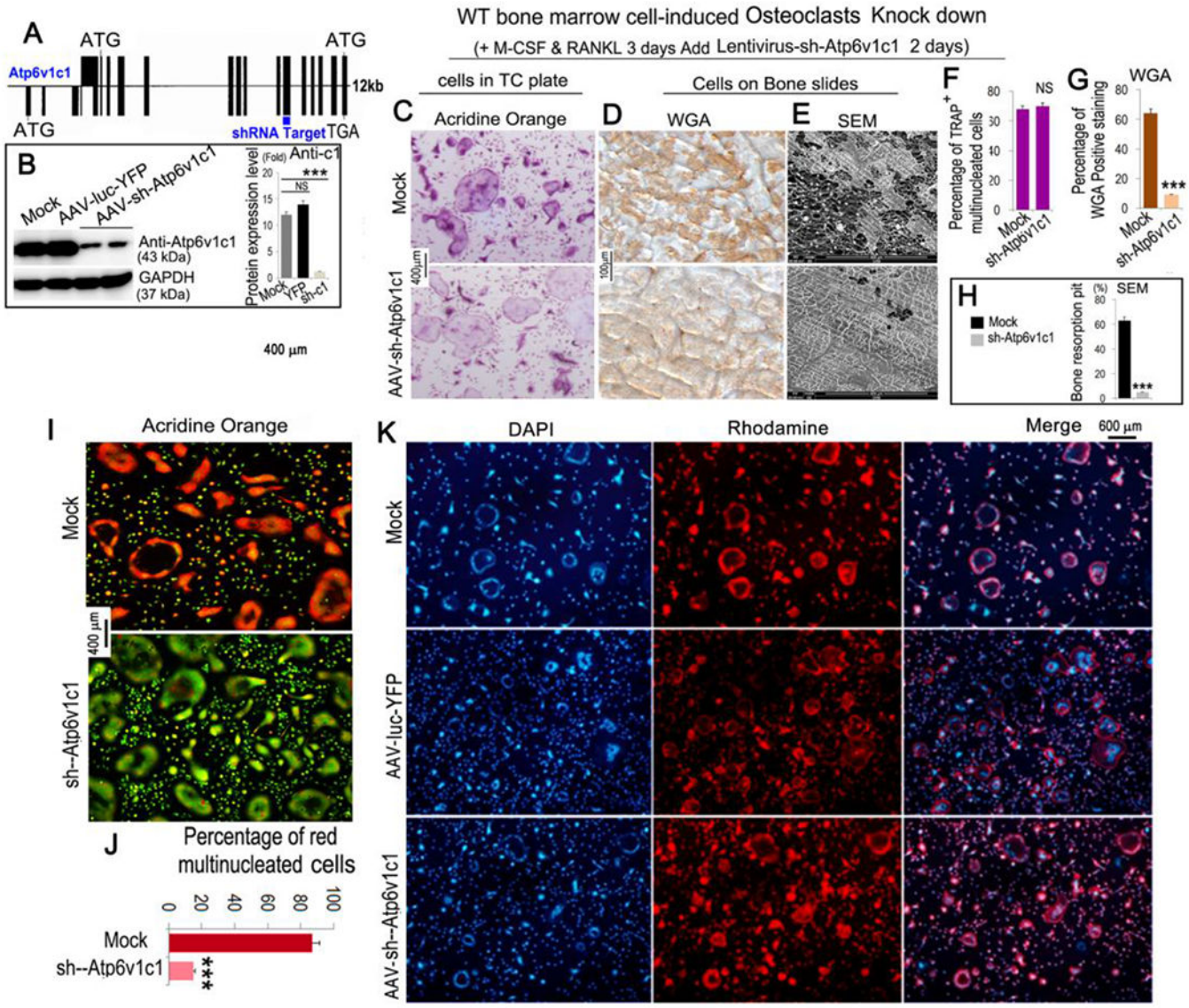

Figure 1. AAV-sh-*Atp6v1c1* simultaneously targeted and efficiently knocked down the expression of *Atp6v1c1*.

(A) Diagram of loci illustrating *Atp6v1c1* zone of homology and shRNA specific for *Atp6v1c1* mRNA. **(B)** Western blot and quantification analysis of *Atp6v1c1* expression in MBM stimulated with M-CSF/RANKL for 3 days and transduced with AAV-luc-YFP or AAV-sh-*Atp6v1c1* or left un-treated (mock). **(C)** TRAP Staining of the mock and AAV-sh-*Atp6v1c1* treated group. **(D)** Wheat germ agglutinin (WGA) stain of the bone resorption pit of the un-treated and AAV-sh-*Atp6v1c1* groups. **(E)** Resorption lacunae visualized by scanning electron microscopy (SEM). **(F)** Quantification of the percentage of TRAP positive multinucleated cells in C. **(G)** Quantification of WGA staining in D. **(H)** Quantification of bone resorption pits in E. **(I)** Acridine orange staining of wild-type MBM induced osteoclasts treated with or without AAV-sh-*Atp6v1c1 in vitro.* **(J)** Quantification of the percentage of red multinucleated cells in I. **(K)** F-actin ring formation assay shows disrupted

ringed structures of F-actin dots (actin rings) in M-CSF/RANKL-induced AAV-sh-*Atp6v1c1* OCs compared to Mock and AAV-luc-YFP control. ***p<0.001; NS, not significant. n=9.

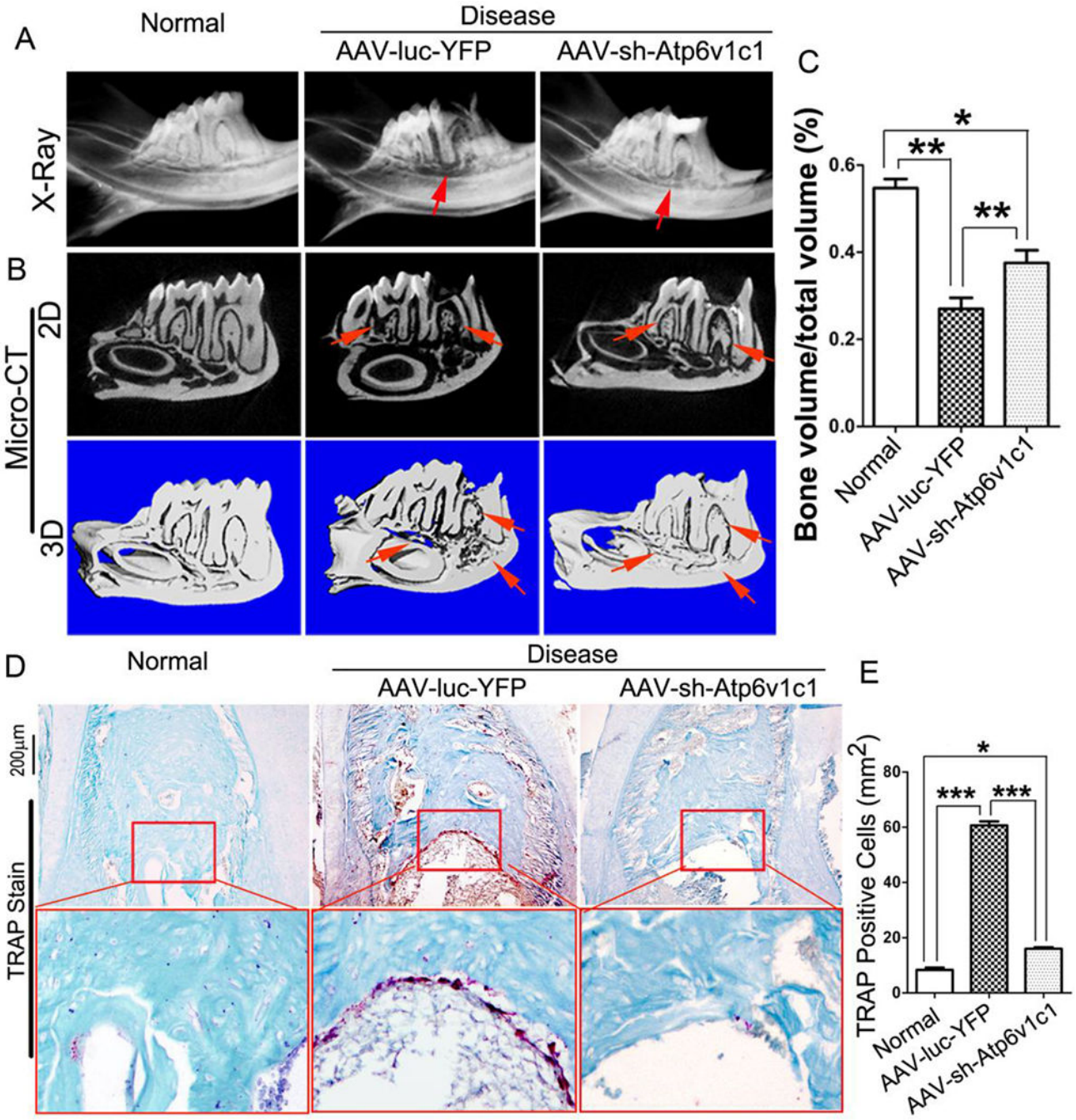

Figure 2. AAV-sh-*Atp6v1c1* impaired osteoclast-mediated bone resorption in the periapical lesion area *in vivo* through reduced osteoclast differentiation.

(A) Representative figures of X-ray from the normal, AAV-luc-YFP and AAV-sh-*Atp6v1c1* group. Red arrows indicate the bone resorption area in the x-ray images. **(B)** 2-D and 3-D μCT analysis of the periapical lesion area. Red arrows indicate the bone defect area in μCT imaging. **(C)** Quantification of BV/TV from the un-treated, AAV-luc-YFP treated, and AAV-sh-*Atp6v1c1* treated mice in B. **(D)** TRAP staining of sections from normal mice and infected mice treated with AAV-luc-YFP or AAV-sh-*Atp6v1c1*. **(E)** Quantification of TRAP

positive cells in the periapical lesion area in D. * $p<0.05$. ** $p<0.01$. *** $p<0.001$; NS, not significant. n=9.

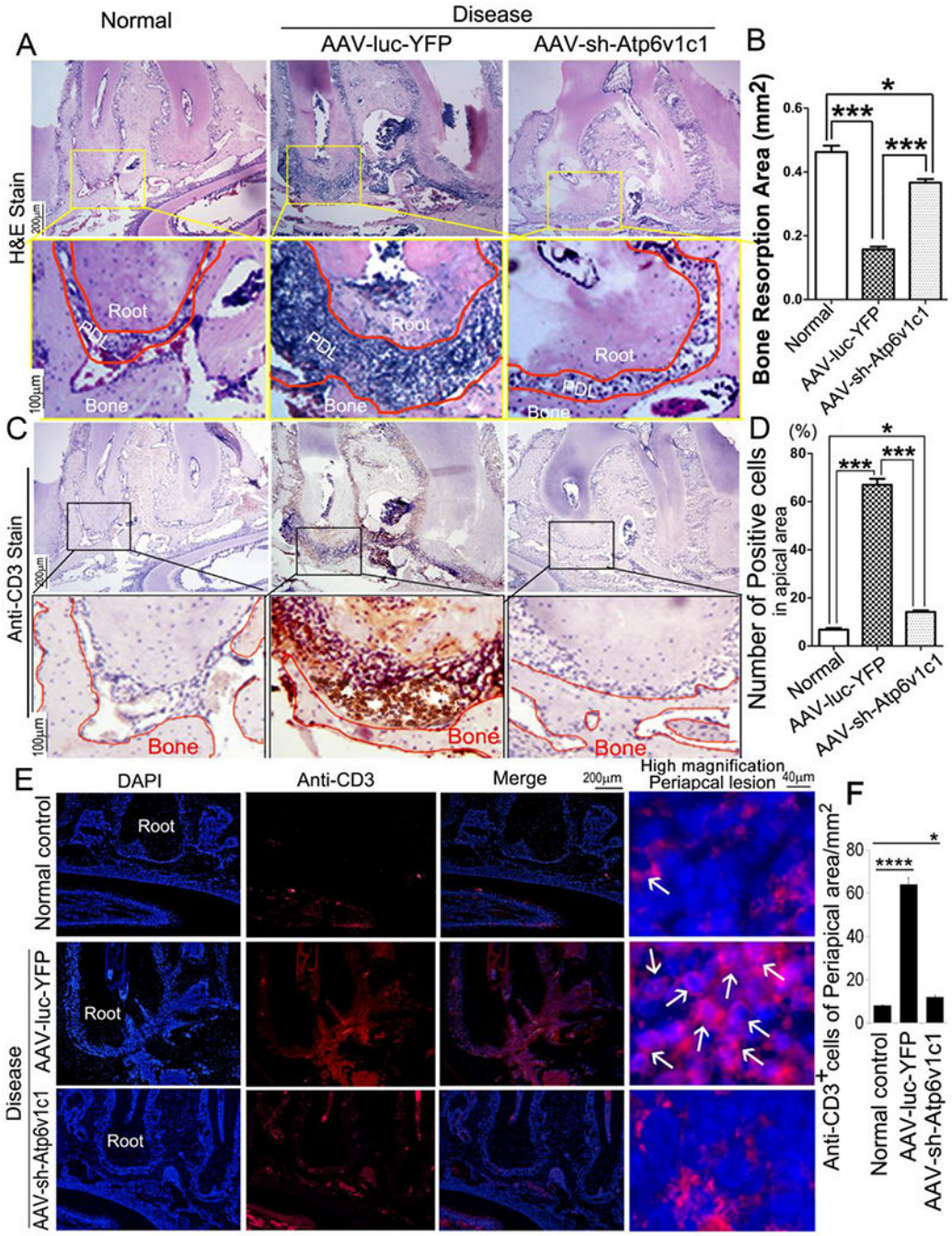

Figure 3. Silencing of *Atp6v1c1* attenuates inflammation in the periapical area and periodontal ligament (PDL), bone resorption, and CD3 positive T cells in the periapical lesion area.

(A) Representative images of hematoxylin and eosin (H&E) stain of normal or bacteria-infected mice treated with AAV-sh-*Atp6v1c1* or AAV-luc-YFP. The red outline indicates the periodontal ligament area. **(B)** Quantification of bone resorption area of the tooth root section in A. **(C)** Immunohistochemical staining was used to detect CD3 positive T cells in periapical lesions. The red outline indicates the alveolar bone area. **(D)** Quantification of CD3 positive cells in the apical area in C in normal control, AAV-luc-YFP treated, and AAV-

sh-*Atp6v1c1* treated mice. **(E)** Immunofluorescence staining for CD3 antibody to detect CD3 positive T cells in periapical lesions. **(F)** Quantification of CD3 positive T cells in the periapical area in E. * $p<0.05$. ***$p<0.001$. ****$p<0.0001$. n=9.

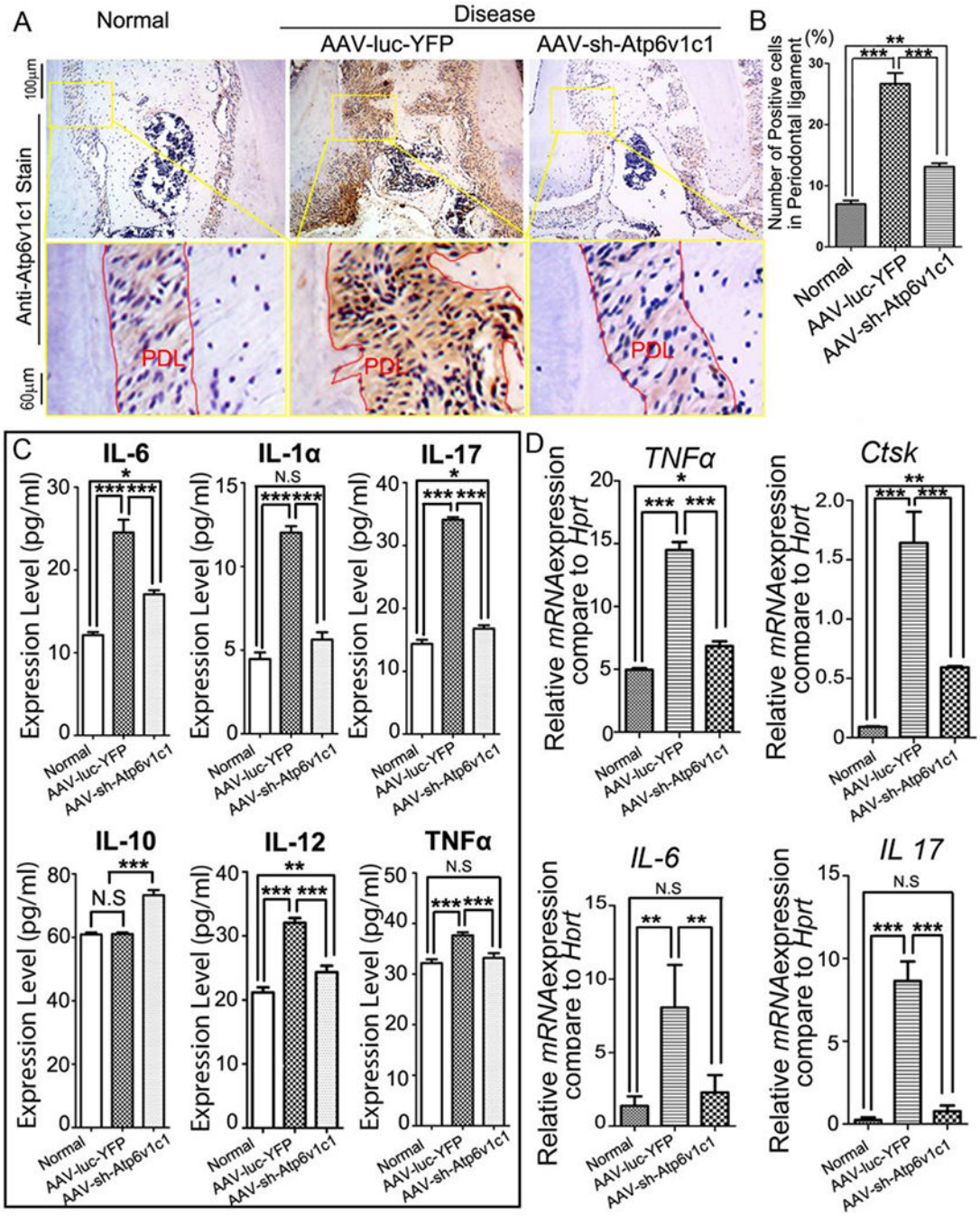

Figure 4. AAV-mediated *Atp6v1c1* knockdown reduced the expression of inflammatory cytokines in the periapical lesion.

(A) Immunohistochemical staining was used to verify the effectiveness of AAV-sh-*Atp6v1c1* knockdown *Atp6v1c1* in periapical tissues. The red outline indicates the periodontal ligament area. **(B)** Quantification of *Atp6v1c1* positive cells in the periodontal ligament area in normal control, AAV-luc-YFP treated, and AAV-sh-*Atp6v1c1* treated mice in A. **(C)** IL-6, IL-1α, IL-17, IL-10, IL-12, and TNFα levels in the periapical tissues were detected by ELISA. **(D)** Quantitative real-time polymerase chain reaction of OC marker gene (i.e. Ctsk)

and pro-inflammatory cytokines (i.e. TNFα, IL-6, and IL-17) in periapical tissues of uninfected mice (normal) or bacteria-infected mice treated with AAV-luc-YFP or with AAV-sh-Atp6v1c1. Expression levels were normalized to Hprt. * $p<0.05$. ** $p<0.01$. *** $p<0.001$; N.S, not significant. n=9.