C1 Silencing Attenuates Inflammation and Alveolar Bone Resorption in Endodontic Disease

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SIGNIFICANCE

Atp6v1c1 silencing attenuates alveolar bone resorption and protects the periodontal ligament from destruction caused by inflammation in endodontic disease. Insights resulting from this study may assist in developing novel treatments for endodontic disease and other osteolytic and inflammatory diseases.

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ABSTRACT

Introduction: Endodontic disease, 1 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. Although 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 protecting the periodontal ligament 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. **Conclusions:** Our results showed 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 toll-like receptor signaling, indicating that targeting *Atp6v1c1* may facilitate the design of novel therapeutic approaches to reduce inflammation and bone erosion in endodontic disease. *(J Endod 2019;45:898–906.)*

KEY WORDS

Atp6v1c1; RNA interference knockdown; gene therapy; gingival inflammation; oral bone resorption; periapical disease

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- 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 2 years, and in some cases complete healing is never achieved. Thus, there is urgent need for a novel osteoclast-specific inhibitor that can simultaneously target bone loss and inflammation in periapical disease.

Atp6v1c1, a subunit of vacuolar-type H^+-ATPase (V-ATPase), is highly expressed in osteoclasts and 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 of nuclear factor kappa-B 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 showed that osteoclast acidification activity and bone resorption are impaired through silencing of *Atp6v1c1*[3], 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[7]. As a subunit of Atp6i, *Atp6v1c1* is expressed in immune cells such as macrophages and dendritic cells, as well as osteoclasts[3,8], 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 because 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 polymicrobial-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 performed 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 Institutional Animal Care and Use Committee and followed all recommendations of Animal Research: Reporting of In Vivo Experiments guidelines.

AAV RNA Interferences Viral Production and Purification

We purchased the AAV Helper-Free System (AAV Helper-Free System Catalog #240071; Stratagene, San Diego, CA). Viral production was accomplished using a triple-transfection, helper-free method and purified with a modified version of a published protocol[9].

Pulp Exposure, Bacterial Infection, and Transduction of AAV Vectors

The periapical disease mouse model was produced as we previously described[1,10]. Bacterial culture, infection, and viral vector transduction in a site-specific manner was performed as described[1,10].

Statistical Analysis

Experimental data are reported as the mean ± standard deviation of triplicate independent samples. The figures are representative of the data ($n = 15$). Data quantification analyses were performed using ImageJ (National Institutes of Health, Bethesda, MD) as described[1,10,11].

RESULTS

Atp6v1c1 Knockdown Impaired Osteoclast Function Including Osteoclast-mediated Bone Resorption and Extracellular Acidification *In Vitro*

To enable knockdown of *Atp6v1c1*, we generated short hairpin RNA that targeted *Atp6v1c1*. We used the AAV2 serotype and subcloned short hairpin RNA targeting *Atp6v1c1* into the AAV.H1 vector (courtesy of Dr. Sergei Musatov, Weill Cornell Medical College, New York City, New York) (Fig. 1*A*). 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[12], 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 with untreated osteoclasts (mock) or osteoclasts transduced with AAV-luc-YFP (Fig. 1*B*). Overall, our results indicate that AAV-sh-*Atp6v1c1* efficiently knocked down *Atp6v1c1* expression. To investigate how *Atp6v1c1* knockdown can affect osteoclast differentiation and function, tartrate-resistant acid phosphatase (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. 1*C* and *F*). We further sought to investigate the effects of AAV-sh-*Atp6v1c1* on osteoclast function by examining bone resorption. Wheat germ agglutinin staining showed that compared with AAV-luc-YFP treatment, AAV-mediated *Atp6v1c1* knockdown reduced osteoclast-mediated bone resorption by 80% (Fig. 1*D* and *G*). Consistently, visualization of the resorption lacunae through scanning electron microscopy showed that AAV-sh-*Atp6v1c1* completely impaired bone resorption *in vitro* (Fig. 1*E* and *H*). To further investigate how *Atp6v1c1* knockdown can affect osteoclast function, we examined osteoclast extracellular acidification. Osteoclasts were induced from wild-type 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. 1*I*). We found that lentivirus-sh-*Atp6v1c1* severely impaired extracellular acidification compared with the scrambled control (Fig. 1*I* and *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 detected actin ring formation in mock C1 knockdown and AAV-*luc*-YFP osteoclasts (Fig. 1*K*). The results showed that C1-depleted osteoclasts fail to form a 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[1,10]. Radiographic imaging of the distal root of the 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. 2*A*). We found that the infected group treated with AAV-luc-YFP had significantly more bone resorption compared with the normal control, whereas AAV-sh-*Atp6v1c1* treatment in infected mice protected periapical bone against resorption as shown by X-ray (Fig. 2*A*, *red arrows*) and micro–computed tomographic (μCT) analysis (Fig. 2*B*, *red arrows*). The percentage of bone volume/total volume was increased by 25% in the AAV-sh-*Atp6v1c1*–treated mice compared with the AAV-luc-YFP treatment group (Fig. 2*C*). In order to further examine how AAV-sh-*Atp6v1c1* treatment attenuates bone destruction *in vivo*, tooth sections from normal and infected mice treated with

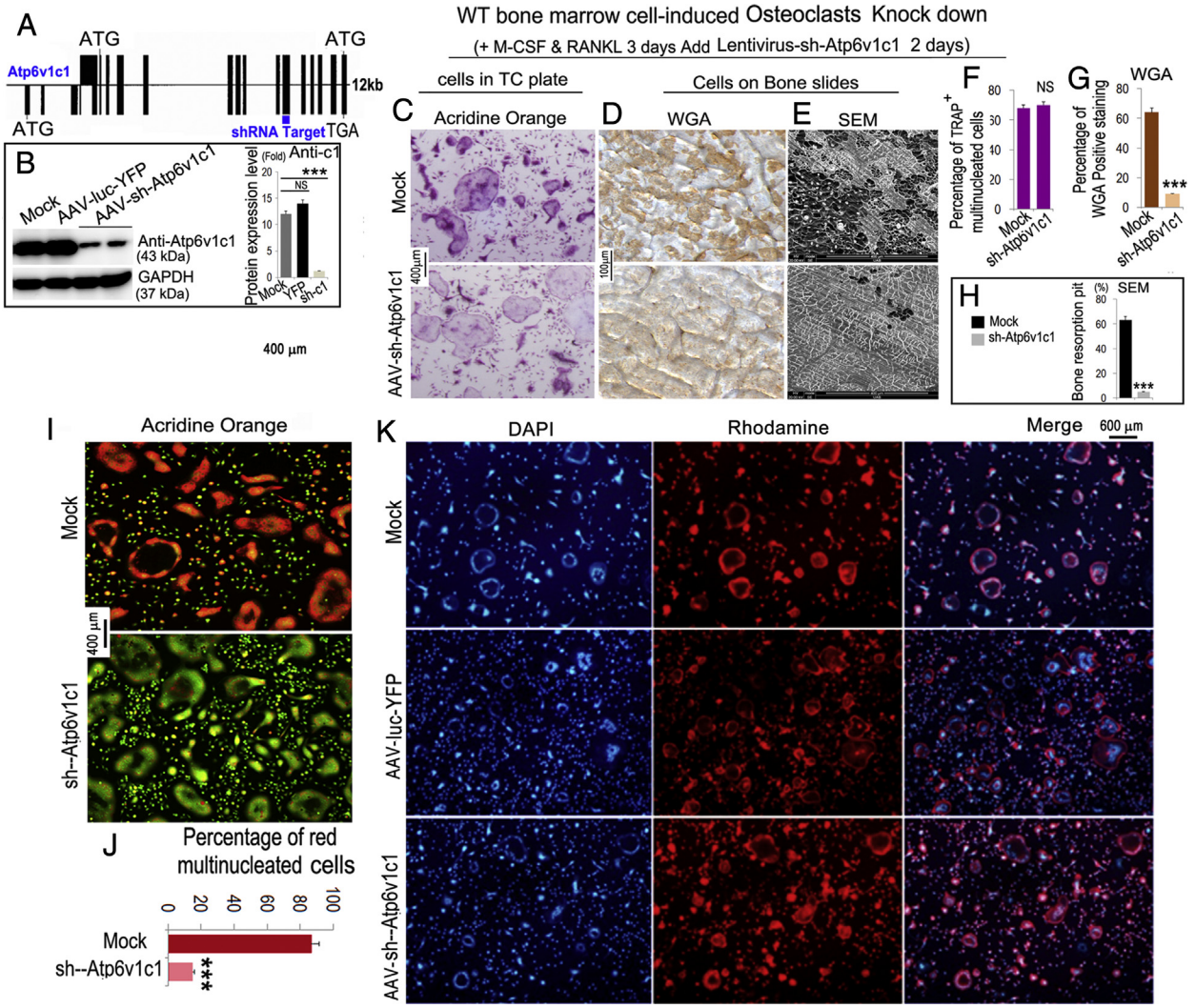

FIGURE 1 – AAV-sh-*Atp6v1c1* simultaneously targeted and efficiently knocked down the expression of *Atp6v1c1*. (*A*) A diagram of loci illustrating the *Atp6v1c1* zone of homology and short hairpin RNA specific for *Atp6v1c1* messenger RNA. (*B*) Western blot and quantification analysis of *Atp6v1c1* expression in MBM stimulated with M-CSF/RANKL for 3 days and transduced with AAV-luc-YFP or AAV-sh-*Atp6v1c1* or left untreated (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 untreated 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* osteoclasts compared with mock and AAV-luc-YFP control. ***$P < .001$. NS, not significant. $n = 9$.

AAV-sh-*Atp6v1c1* or AAV-luc-YFP were stained with TRAP, which indicated that AAV-sh-*Atp6v1c1* treatment impaired the number of activated osteoclasts *in vivo* by 70% (Fig. 2*D* and *E*). Although our *in vitro* results did not show any significant changes in osteoclast differentiation after *Atp6v1c1* silencing, we found that osteoclast differentiation was significantly reduced after *Atp6v1c1* silencing in an endodontic disease model because of attenuated inflammation compared with the infected AAV-luc-YFP–treated mice. Under inflammatory conditions, activated T cells can induce osteoclastogenesis via RANKL-dependent and RANKL-independent mechanisms[13]. Thus, upon *Atp6v1c1* knockdown, the T-cell–mediated immune response was inhibited, impairing RANKL-stimulated osteoclast differentiation. Collectively, these data showed that AAV-mediated *Atp6v1c1* knockdown prevented 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-eosin (H&E) (Fig. 3*A*). We found that immune cell (monocyte, macrophage, and granulocyte) infiltration in the periapical lesion was significantly increased in the infected group treated with AAV-luc-YFP as shown by H&E staining, whereas immune cell infiltration was dramatically reduced in the periapical lesions of the AAV-sh-*Atp6v1c1* group (Fig. 3*A*). Notably, treatment with AAV-sh-*Atp6v1c1* reduced the width of the PDL by 50% compared with the AAV-luc-YFP–treated disease group and was similar to the normal control (Fig. 3*A*). Consistent with the μCT results, quantification analysis of the bone resorption area showed

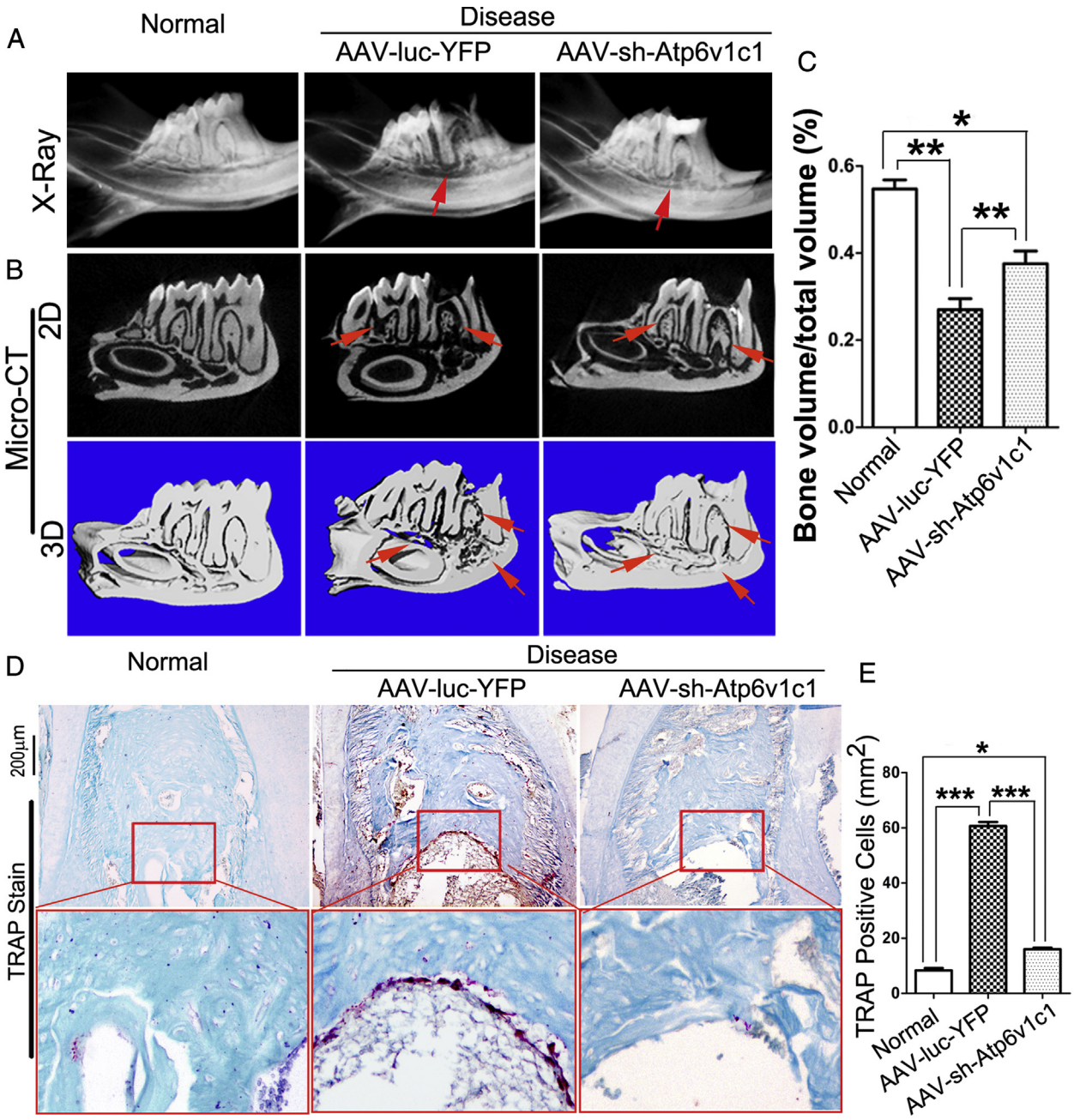

FIGURE 2 – AAV-sh-*Atp6v1c1*–impaired osteoclast-mediated bone resorption in the periapical lesion area *in vivo* through reduced osteoclast differentiation. (*A*) Representative figures of X-ray from the normal, AAV-luc-YFP, and AAV-sh-*Atp6v1c1* groups. *Red arrows* indicate the bone resorption area in the X-ray images. (*B*) Two-dimensional and 3-dimensional μCT analysis of the periapical lesion area. *Red arrows* indicate the bone defect area in μCT imaging. (*C*) Quantification of bone volume/total volume from the untreated, 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*. $^*P < .05$. $^{**}P < .01$. $^{***}P < .001$. NS, not significant. $n = 9$.

that the AAV-sh-*Atp6v1c1*–treated mice had a 50% increase in bone area compared with the disease group treated with AAV-luc-YFP (Fig. 3*B*). To further investigate the role of *Atp6v1c1* in inflammation, immunofluorescence and immunohistochemical 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. 3*C* and *E*). The data showed 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. 3*D* and *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 PDL

We confirmed *Atp6v1c1* knockdown *in vivo* by immunohistochemical stain in the periapical lesion area and found that the expression of *Atp6v1c1* had been efficiently knocked down

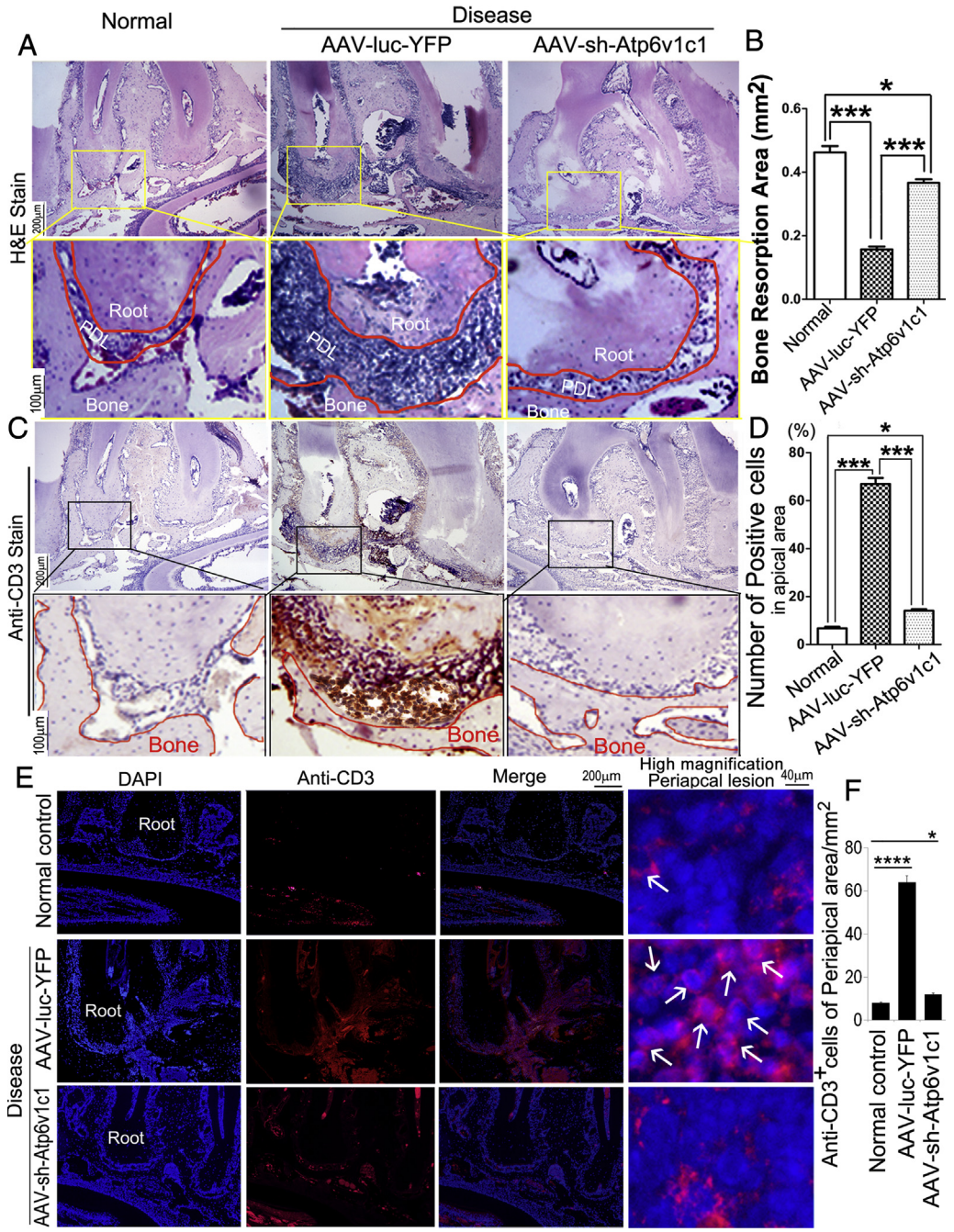

FIGURE 3 – Silencing of *Atp6v1c1* attenuates inflammation in the periapical area and PDL, bone resorption, and CD3-positive T cells in the periapical lesion area. (*A*) Representative images of hematoxylin-eosin stain of normal or bacteria-infected mice treated with AAV-sh-*Atp6v1c1* or AAV-luc-YFP. 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*. $^{*}P < .05$. $^{***}P < .001$. $^{****}P < .0001$. $n = 9$.

in the AAV-sh-*Atp6v1c1*–treated group (Fig. 4*A* and *B*). Notably, the width of the PDL was reduced by 50% in the *AAV-sh-Atp6v1c1* group compared with the AAV-luc-YFP–treated mice, indicating attenuated inflammation in *AAV-sh-Atp6v1c1*–treated mice (Fig. 4*A*). We performed enzyme-linked immunosorbent assay to evaluate the effect of *Atp6v1c1* silencing on the protein levels of the proinflammatory cytokines interleukin (IL)-1α, IL-6, IL-17, IL-10, IL-12, and tumor necrosis factor alpha (TNF-α) (Fig. 4*C*). Interestingly, our data showed that the protein levels of IL-1α, IL-6, IL-17, IL-12, and TNF-α were significantly decreased in the AAV-sh-*Atp6v1c1*–treated group compared with the AAV-luc-YFP group, whereas IL-10 protein levels were increased in the AAV-sh-*Atp6v1c1*–treated group (Fig. 4*C*). Produced by mast cells, IL-10 has been shown to contribute to the anti-inflammatory or immunosuppressive effects in inflammatory

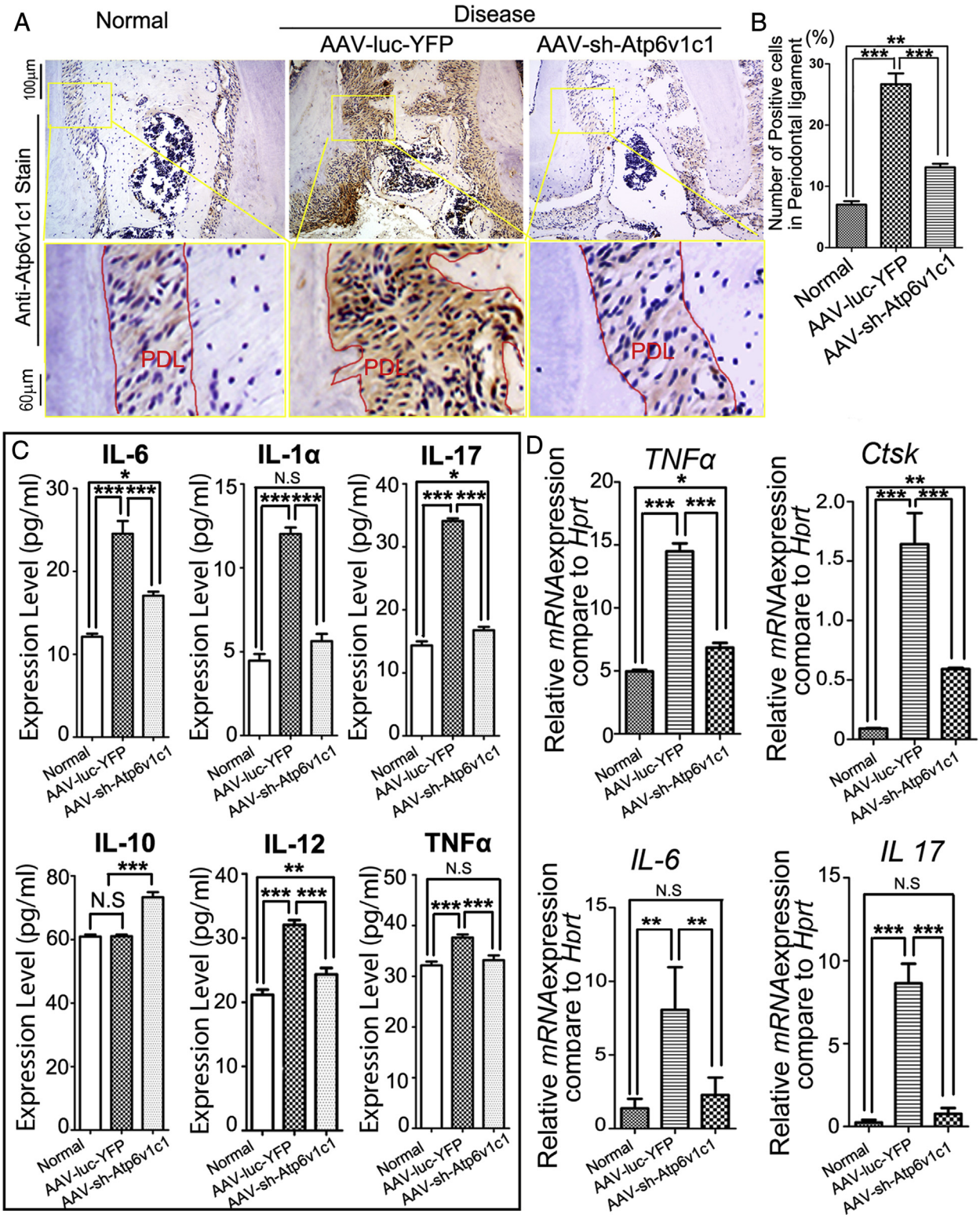

FIGURE 4 – AAV-mediated *Atp6v1c1* knockdown reduced the expression of inflammatory cytokines in the periapical lesion. (*A*) Immunohistochemical staining was used to verify the effectiveness of AAV-sh-*Atp6v1c1* knockdown *Atp6v1c1* in periapical tissues. The *red outline* indicates the PDL area. (*B*) Quantification of *Atp6v1c1*-positive cells in the PDL 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 TNF-α levels in the periapical tissues were detected by enzyme-linked immunosorbent assay. (*D*) Quantitative real-time polymerase chain reaction of osteoclast marker gene (ie, Ctsk) and proinflammatory cytokines (ie, TNF-α, 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 hypoxanthine guanine phosphoribosyl transferase. $^{*}P < .05$. $^{**}P < .01$. $^{***}P < .001$. NS, not significant. $n = 9$.

conditions[14]. IL-6 is secreted by osteoblasts in response to bone resorption and is important for osteoclast differentiation[15]. The relative messenger RNA expression level of the osteoclast marker gene *Ctsk* was decreased by 60% in the AAV-sh-*Atp6v1c1*–treated group compared with the AAV-luc-YFP treatment group (Fig. 4*D*). In addition, the expression levels of proinflammatory 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. 4*D*). Previous studies have shown that V-ATPases are required for mammalian target of rapamycin complex 1 activation stimulated by amino acids[16]; thus, we tested colocalization of Atp6v1c1 and mammalian target of rapamycin (mTOR) in 4T1 epithelial cells (Supplemental Fig. S1 is available online at www.jendodon.com). Our results showed coexpression of Atp6v1c1 and mTOR in epithelial cells, indicating its relationship with mTOR signaling (Supplemental Fig. S1 is available online at www.jendodon.com). Thus, Atp6v1c1 may regulate inflammation though the mTOR and/or toll-like receptor (TLR) pathways. Furthermore, our data show 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[17]. Our results showed that the expression of TNF-α was down-regulated in the Atp6v1c1 depletion group, whereas IL-10 was up-regulated. 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 TLR signaling in endodontic disease. In conclusion, we found that *Atp6v1c1* knockdown significantly reduced proinflammatory cytokine expression, indicating that *Atp6v1c1* may regulate TLR signaling in endodontic disease.

DISCUSSION

To investigate the role of *Atp6v1c1* in periapical disease, we characterized the efficacy of a gene therapy using recombinant 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 proinflammatory cytokine expression. Notably, our data also showed that osteoclast extracellular acidification was impaired because of *Atp6v1c1* silencing. Our results showed 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 showed that *Atp6i* is an osteoclast-specific proton pump that is essential for osteoclast-mediated extracellular acidification during bone resorption[18]. 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[19,20]. Moreover, *Atp6v1c1* regulates intracellular and intraorganellar pH together with other subunits[3,21]. Our results demonstrated that local administration of AAV-sh-*Atp6v1c1* in the apical area inhibited bone resorption, potentially resulting from osteoclast malfunction caused by *Atp6v1c1* knockdown. Biochemical analysis revealed that *Atp6v1c1* stabilizes the V-ATPase complex assembly and increases proton pump activity, which promotes bone resorption[22-24]. 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[25]. Similarly, osteoclasts can express numerous immune receptors[26,27]. 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[13]. Thus, upon *Atp6v1c1* knockdown, the 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[28]. Under a low pH microenvironment, activated Ctsk is secreted either from immune cell lysosomes or after osteoclast extracellular acidification. Because of osteoclast malfunction after *Atp6v1c1* silencing, the acidic environment for bone resorption is interrupted, thus inhibiting Ctsk activation. Our data show 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 proinflammatory 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[15]. We found similar decreases in the expression of the classic proinflammatory mediators TNF-α and IL-12 after *Atp6v1c1* knockdown. Although further studies are needed to 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 shown to be expressed in endodontic disease[29], and although 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 shown to stimulate IL-6 and IL-12 production[28]. 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[30] showed that apical periodontitis increased serum levels of IL-17. 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[14], was increased after *Atp6v1c1* silencing. Thus, the 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 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 osteoclast extracellular acidification and potentially through regulating TLR 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.

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Yi-Ping Li and Yuehua Liu contributed equally to this study.

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The authors deny any conflicts of interest related to this study.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found in the online version at www.jendodon.com (https://doi.org/10.1016/j.joen.2019.02.024).

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