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HOST RESPONSE AND INFLAMMATION

Silencing of Ac45 Simultaneously Inhibits Osteoclast-Mediated Bone Resorption and Attenuates Dendritic Cell-Mediated Inflammation through Impairing Acidification and Cathepsin K Secretion

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ABSTRACT Endodontic disease is characterized by inflammation and destruction of periapical tissues, leading to severe bone resorption and tooth loss. ATP6AP1 (Ac45) has been implicated in human immune diseases, yet the mechanism underlying how Ac45 regulates immune response and reaction in inflammatory diseases remains unknown. We generated endodontic disease mice through bacterial infection as an inflammatory disease model and used adeno-associated virus (AAV)-mediated *Ac45* RNA interference knockdown to study the function of Ac45 in periapical inflammation and bone resorption. We demonstrated that the AAV small hairpin RNA targeting *Ac45* (AAV-sh-*Ac45*) impaired cellular acidification, extracellular acidification, and bone resorption. Our results showed that local delivery of AAV-sh-*Ac45* in periapical tissues in bacterium-induced inflammatory lesions largely reduced bone destruction, inhibited inflammation, and dramatically reduced mononuclear immune cells. T-cell, macrophage, and dendritic cell infiltration in the periapical lesion was dramatically reduced, and the periodontal ligament was protected from inflammation-induced destruction. Furthermore, AAV-sh-*Ac45* significantly reduced osteoclast formation and the expression of proinflammatory cytokines, such as tumor necrosis factor alpha, interleukin-10 (IL-10), IL-12, IL-1α, IL-6, and IL-17. Interestingly, AAV-sh-*Ac45* impaired mature cathepsin K secretion more significantly than that by AAV-sh-*C1* and AAV-sh-*CtsK*. Unbiased genome-wide transcriptome sequencing analysis of *Ctsk*$^{-/-}$ dendritic cells stimulated with lipopolysaccharide demonstrated that the ablation of Ctsk dramatically reduced dendritic cell-mediated inflammatory signaling. Taken together, our results indicated that AAV-sh-*Ac45* simultaneously inhibits osteoclast-mediated bone resorption and attenuates dendritic cell-mediated inflammation through impairing acidification and cathepsin K secretion. Thus, Ac45 may be a novel target for therapeutic approaches to attenuate inflammation and bone erosion in endodontic disease and other inflammation-related osteolytic diseases.

KEYWORDS Ac45, adeno-associated virus, bone resorption, inflammation, RNAi silencing, endodontic disease

Dental caries is one of the most prevalent infectious diseases in the world, affecting approximately 80% of children and the majority of adults. Dental plaque bacteria increase B-cell and T-cell activation through, in part, the activation of TLR signaling, which promotes both inflammation and osteoclast differentiation, and activity that

Citation Yang W, Zhu Z, Li L, McVicar A, Gao N, Wang L, Li Y-P, Chen W. 2021. Silencing of Ac45 simultaneously inhibits osteoclast-mediated bone resorption and attenuates dendritic cell-mediated inflammation through impairing acidification and cathepsin K secretion. Infect Immun 89:e00436-20. https://doi.org/10.1128/IAI.00436-20.

Editor Marvin Whiteley, Georgia Institute of Technology School of Biological Sciences

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Received 26 July 2020
Returned for modification 20 August 2020
Accepted 29 September 2020
Accepted manuscript posted online 19 October 2020
Published 15 December 2020

ultimately results in periapical tissue inflammation and bone degradation around teeth (1). Infection-induced dental caries may result in periapical disease, leading to dental pulp necrosis, periapical inflammation and bone resorption, and subsequent loss of teeth. It may also increase inflammation incidence in other parts of the body and may lead to systemic manifestations, such as infective endocarditis. The pulp tissue infection spreads throughout the root canal system toward the apical foramen and into the periodontal ligament (PDL), leading to endodontic disease and periapical bone resorption (2). Endodontic disease therapy consists of removing necrotic pulp tissue and preventing local inflammation. Although this treatment has a high degree of success, endodontic failures still happen, and complete bone healing or reduction of the apical lesion may not occur after treatment (3). Thus, there is an urgent need for novel treatments that can target both inflammation and bone resorption in endodontic disease.

Vacuolar-type H^+-ATPases (V-ATPases) are responsible for proton secretion and intracellular vesicle acidification. V-ATPases have been implicated in many physiological processes, including exocytosis, endocytosis, intracellular membrane trafficking, cell–cell fusion, and membrane fusion (4). The V-ATPases are composed of two multiprotein domains, V1 and V0. The V1 domain, consisting of eight subunits (A to H), is located in the cytoplasm and hydrolyses ATP, whereas the V0 domain, consisting of five subunits (a, d, e, c, and c′′), is embedded in the organelle membrane. Ac45 is an accessory subunit of the V-ATPase complex encoded by *ATP6AP1*, and it is a type I transmembrane protein associated with the V-ATPase membrane domain (V0) (5). V-ATPase subunit ATP6AP1 (Ac45) is essential for osteoclast-mediated extracellular acidification and protease exocytosis. Previous studies have shown that targeted suppression of Ac45 impairs intracellular acidification and endocytosis, processes that are requirements for osteoclastic bone resorption (6, 7). A recent study investigating the expression, distribution, and activity of V-ATPase isoforms in invasive prostate adenocarcinoma (PC-3) cells indicated that Ac45 plays a central role in navigating the V-ATPase to the plasma membrane and, thus, is an important factor in expression of the phenotype in invasive prostate adenocarcinoma (8). Furthermore, different Ac45 protein isoforms were discovered in human brain, liver, and B cells, indicating the presence of tissue-specific regulation of organelle acidification (9). Notably, the clinical phenotype of Ac45 deficiency in humans includes hepatopathy and immune abnormalities, suggesting an important role of Ac45 in human immune diseases (9). Moreover, we reported that the knockdown of *Ac45* in the mouse model of periodontal disease prevents alveolar bone loss and periodontal tissue inflammation (7). However, although Ac45 has been implicated in immune and inflammatory diseases, the mechanism underlying the roles of Ac45 in inflammatory diseases such as endodontic disease remains unknown.

To investigate the role and underlying mechanisms of *Ac45* in endodontic disease, we characterized the therapeutic potential of recombinant adeno-associated virus (AAV)-mediated *Ac45* silencing to simultaneously target inflammation and periapical bone resorption. Ablation of *Ac45* in the periapical lesions of periapical disease mice largely decreased bone destruction, impaired osteoclast activation, significantly reduced the infiltration of T cells, macrophages, and dendritic cells (DC) in the periapical lesion, and protected the PDL from destruction caused by inflammation due to the significant decrease in mononuclear immune cell infiltration. In addition, AAV-mediated *Ac45* knockdown also reduced the expression of bacterial infection-stimulated proinflammatory cytokines. Further, we demonstrated that extracellular acidification and cellular acidification were impaired due to *Ac45* silencing. We further showed that *Ac45* silencing in periapical tissues can slow periapical disease progression, alleviate inflammation, and prevent bone erosion. Mechanistically, we demonstrated that loss of extracellular and cellular acidification by AAV-sh-*Ac45* reduced inflammation and the secretion of mature Ctsk, an activator of TLR signaling, indicating that AAV-sh-*Ac45* attenuated inflammation in the periapical tissues and periodontal ligament through inhibiting TLR signaling pathway activation related to extracellular acidification, cellular acidification, and lysosomal trafficking. Thus, our results indicate that targeting *Ac45*

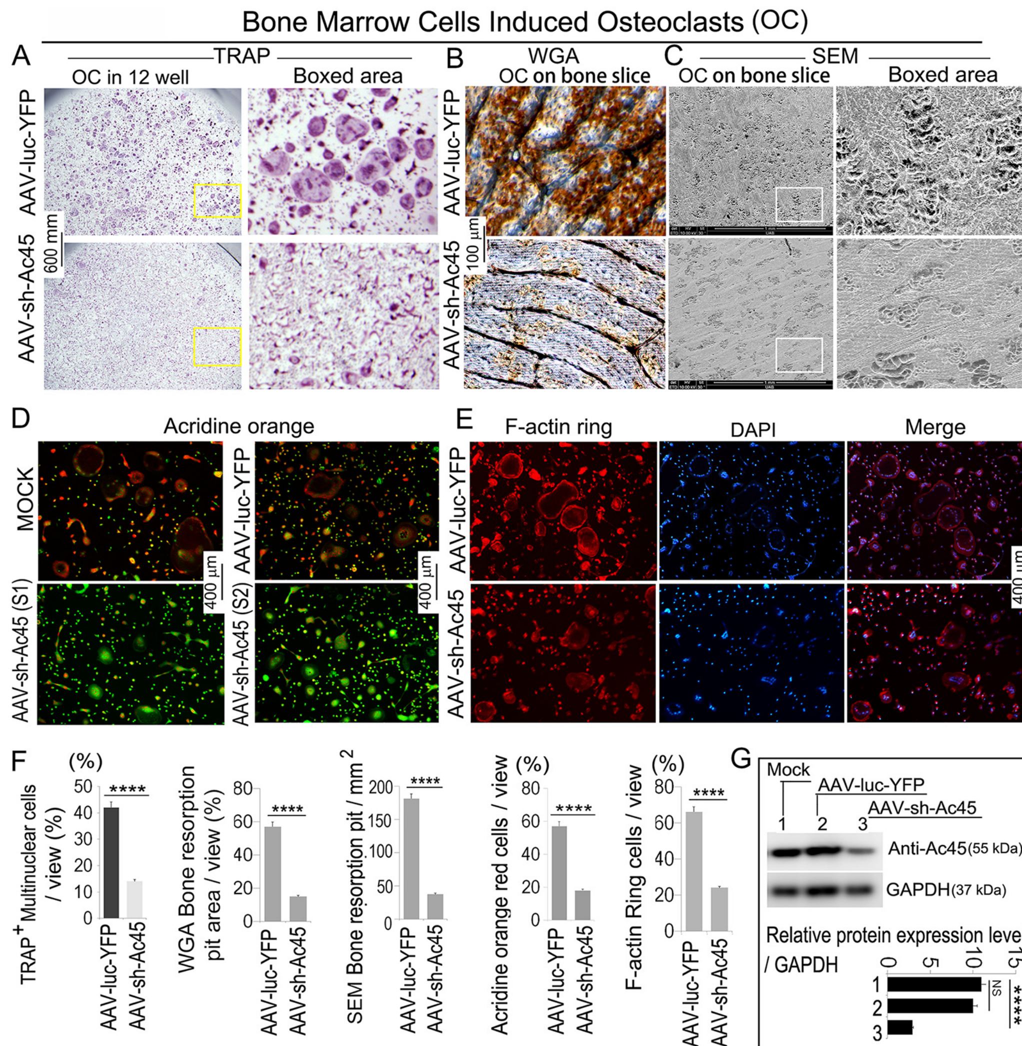

FIG 1 AAV-sh-*Ac45* efficiently knocked down expression of *Ac45* and impaired cellular acidification and bone resorption *in vitro*. (A) TRAP staining of MBM treated with AAV-luc-YFP or AAV-sh-Ac45. (B) Wheat germ agglutinin (WGA) stain of bone resorption pit of the AAV-sh-*Ac45* and AAV-luc-YFP groups. (C) Resorption lacunae were visualized by scanning electron microscopy (SEM). (D) Acridine orange staining of osteoclasts, including cells without fusion (<3 nuclei). (E) F-actin ring formation was detected in MBM treated with AAV-luc-YFP or AAV-sh-*Ac45*. (F) Quantification of TRAP-positive cells, WGA bone resorption pits, scanning electron microscopy, percentage of red multinucleated cells of acridine orange staining, and F-actin ring cells shown in panels A to D. (G) Western blot and quantification analysis of *Ac45* expression in MBM stimulated with macrophage-colony-stimulating facotr/RANKL for 3 days and transduced with AAV-luc-YFP or AAV-sh-*Ac45* or left untreated (mock).

results in novel therapeutic approaches for diseases of osteoclast overactivation, such as periapical disease, and is a target in other inflammatory diseases in humans.

RESULTS

AAV-sh-*Ac45* efficiently knocked down expression of *Ac45* and impaired cellular acidification and bone resorption *in vitro*. To enable knockdown of *Ac45*, we generated short hairpin RNA (shRNA) that targeted the expression of *Ac45* to evaluate the effect of inhibition of Ac45 on osteoclasts. We performed tartrate-resistant acid phosphatase (TRAP) staining of mouse bone marrow (MBM) isolated from wild-type BALB/cJ mice, cultured with *M-CSF* and *RANKL* to generate osteoclasts (10), and then transduced with either AAV-luc-yellow fluorescent protein (YFP) or AAV-sh-*Ac45* (Fig. 1). Our results show that AAV-mediated *Ac45* silencing reduced the number of osteoclasts *in vitro* by 70%, suggesting impaired osteoclast differentiation following *Ac45* silencing (Fig. 1A and F). Acridine orange staining and F-actin ring staining were conducted to

evaluate extracellular and cellular acidification and osteoclast function, respectively (Fig. 1D and E). The results demonstrate that lentivirus-sh-*Ac45* severely impaired both osteoclast extracellular acidification and function compared to the control (Fig. 1D to F). Interestingly, besides a reduction in osteoclast-mediated cellular acidification, we found that mononuclear cell acidification was also inhibited by *Ac45* silencing (Fig. 1D to F), which suggests that *Ac45* silencing inhibits lysosomal acidification. We also found that *Ac45* knockdown severely affected osteoclast-mediated bone resorption, as shown by wheat germ agglutinin and scanning electron microscopy analysis, which demonstrated that bone resorption was decreased by 70% following *Ac45* silencing (Fig. 1B, C, and F). To confirm the effect of *Ac45* silencing, we examined *Ac45* expression in MBM isolated from wild-type BALB/cJ mice, cultured with *M-CSF* and *RANKL* to generate osteoclasts (10) and transduced with AAV-sh-*Ac45* or AAV-luc-YFP. The analysis of protein levels in MBM through Western blot analysis revealed that osteoclasts transduced with AAV-sh-*Ac45* had a 70% reduction in *Ac45* expression compared to untreated osteoclasts (mock) or osteoclasts transduced with AAV-luc-YFP (Fig. 1G). Our results demonstrated that *Ac45* silencing results in impaired extracellular and cellular acidification, osteoclast differentiation, and bone resorption.

AAV-sh-*Ac45* effectively transduced periapical tissue and knocked down the expression of *Ac45 in vivo*. We used a mouse model of periapical lesion induction to determine the efficacy of AAV-sh-*Ac45* in reducing the severity of endodontic disease (1, 11). Mandibular first-molar dental pulp was exposed and infected with a mixture of four common endodontic pathogens: *Peptostreptococcus micros*, *Streptococcus intermedius*, *Prevotella intermedia*, and *Fusobacterium nucleatum*. We conducted fluorescence analysis of the infected mice treated with AAV-sh-*Ac45*. Compared to the uninfected controls, AAV vectors successfully infiltrated the periapical tissues of the infected mice treated with AAV-sh-*Ac45* (Fig. 2A). As revealed by immunohistochemistry staining, Ac45-positive cells were reduced in the AAV-sh-*Ac45* group compared to the AAV-luc-YFP group by 78%, indicating that AAV-sh-*Ac45* efficiently inhibited the expression of *Ac45* in the transduced periapical tissue (Fig. 2B and C). These results demonstrate that *Ac45* silencing protects against periapical disease-induced bone destruction in the mouse model of periapical disease.

AAV-mediated *Ac45* knockdown reduced alveolar bone resorption in periapical area. To determine the efficacy of AAV-sh-*Ac45* in protecting oral tissues against inflammation and bone resorption due to endodontic disease, we utilized the mouse model of periapical lesion induction (1, 11). Radiographic imaging of the distal root of the first mandibular molar was performed to compare the periapical bone resorption in uninfected mice and infected mice treated with either AAV-luc-YFP or AAV-sh-*Ac45* (Fig. 3A). The X-ray of the crown and distal root of the first mandibular molar revealed that the disease control mice had significantly increased periapical bone resorption surrounding the mandibular first molar root compared to the uninfected control group. Notably, the AAV-sh-*Ac45*-treated group displayed minimal bone resorption, which was similar to that of the normal control group (Fig. 3A). These results were further confirmed by microcomputed tomography two-dimensional (2D) images and 3D analysis of bone volume/total volume (BV/TV) ratios of the periapical area surrounding the distal root of the first molar (Fig. 3B and C), which showed that infected mice treated with AAV-sh-*Ac45* had a 57% reduction in infection-induced bone resorption compared to infected mice with AAV-luc-YFP treatment (Fig. 3D). Collectively, these data demonstrated that AAV-mediated *Ac45* knockdown protected against bone resorption in periapical disease *in vivo*.

AAV-sh-*Ac45* attenuates inflammation in the periodontal ligament and periapical lesions through inhibiting immune cell infiltration. To examine how *Ac45* knockdown attenuates bone destruction *in vivo*, tooth sections from normal and infected mice treated with AAV-sh-*Ac45* or AAV-luc-YFP were stained with hematoxylin and eosin (H&E) (Fig. 4A). H&E staining demonstrated that periapical tissue sections from infected mice treated with AAV-sh-*Ac45* had significantly less bone resorption, as shown by images of the furcation, disto-root, and mesio-root areas (Fig. 4A). The

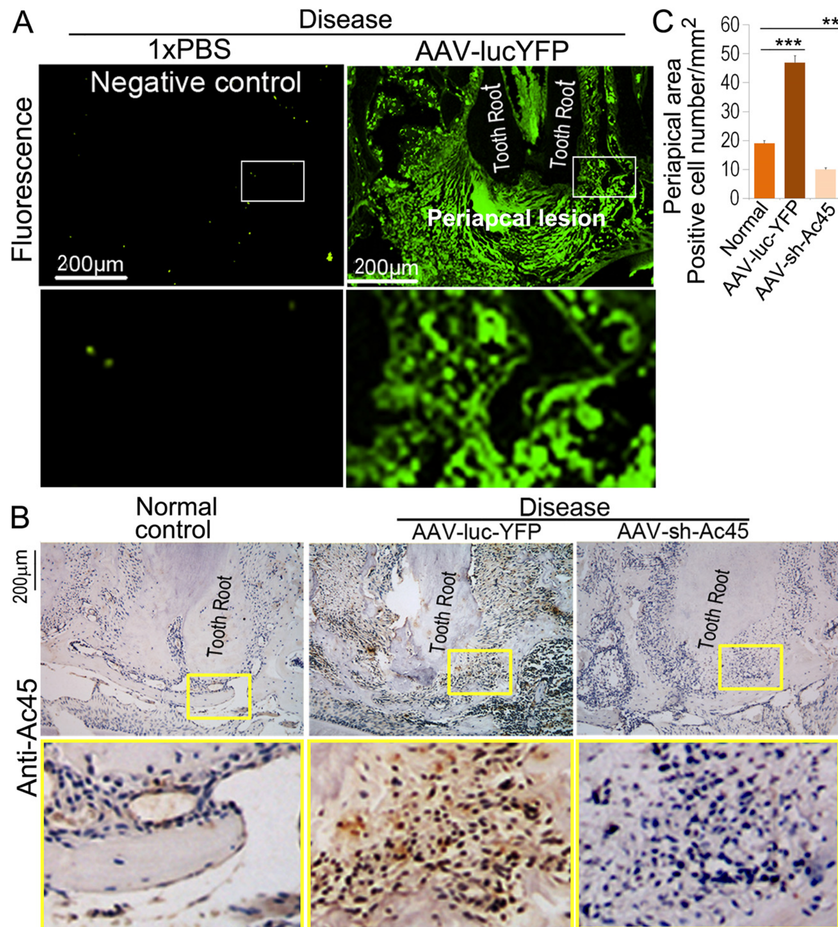

FIG 2 AAV-sh-*Ac45* effectively transduced endodontic tissue *in vivo*. (A) Enhanced green fluorescence expression by the AAV-infected cells in groups treated with phosphate-buffered saline (PBS) or AAV-luc-YFP. Corresponding immunohistochemistry (IHC) images are shown in panel B. (B) Representative images from immunochemistry staining of anti-Ac45 reveal that AAV-sh-*Ac45* treatment reduces expression of Ac45 *in vivo*. (C) Quantification of Ac45-positive cell numbers in the periapical area in panel B. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; NS, not significant.

quantification of bone resorption in the furcation area showed a 60% reduction in bone resorption of the alveolar bone in the AAV-sh-*Ac45* group compared to the AAV-luc-YFP group (Fig. 4C). Furthermore, we found that immune cell infiltration in the periapical lesion was dramatically increased in infected mice with AAV-luc-YFP treatment, as shown by H&E stain, whereas immune cell infiltration was dramatically reduced in the periapical lesions of the AAV-sh-*Ac45* treatment group (Fig. 4A). Interestingly, as shown by high-magnification images of the mesio-root area, there were significantly fewer mononuclear leukocytes in the AAV-sh-*Ac45* group than the AAV-luc-YFP group, as indicated by nuclear morphology, compared to bacterium-infected mice treated with AAV-luc-YFP (32 + 12 versus 118 + 6 per section, respectively, $P < 0.001$) (Fig. 4B). Furthermore, the width of the periodontal ligament (PDL) seen in the AAV-sh-*Ac45* treatment group was similar to that of the uninfected normal group and significantly shorter in width than that of the AAV-luc-YFP group (Fig. 4B, red arrows show the width of the PDL). Inhibition of mononuclear cell infiltration into the periapical tissues and periodontal ligament after *Ac45* silencing indicates a critical role of Ac45 in the immune response and may be the cause of the immunodeficiency seen in humans with hemizygous missense mutations in *Ac45* (9). In conclusion, these data demonstrate that AAV-sh-*Ac45* treatment attenuates inflammatory responses in the periapical lesions through inhibiting immune cell infiltration.

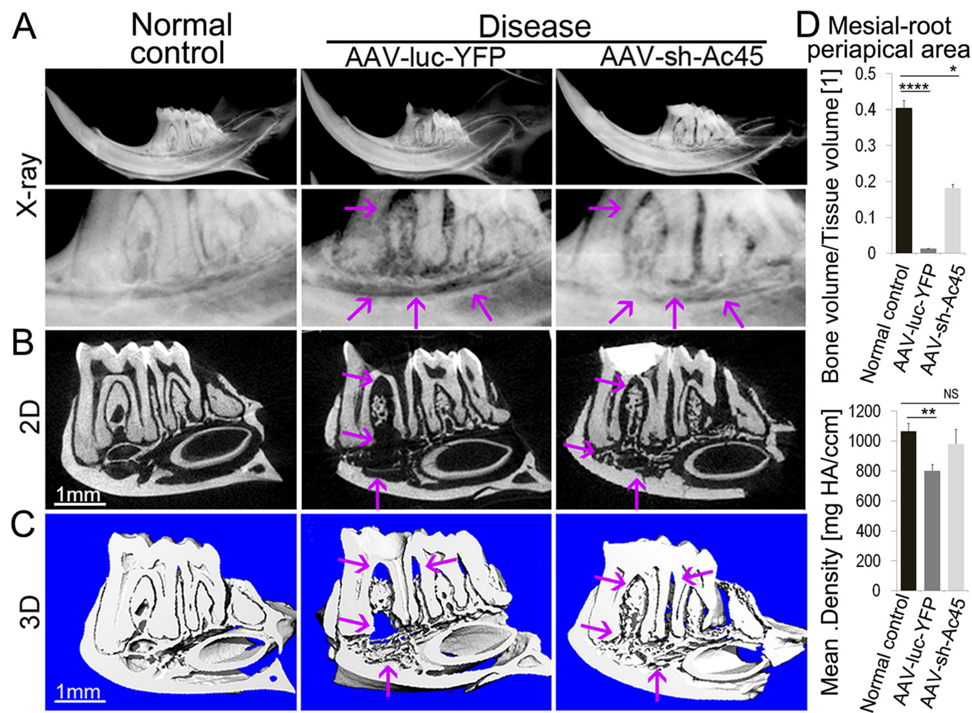

FIG 3 AAV-mediated *Ac45* knockdown reduced alveolar bone resorption in periapical area. (A and B) X-ray imaging (A) and microcomputed tomography (μCT) analysis (B) of the crown and distal root of the mandibular first molar and patent apical foramen, extracted from WT BALB/cJ mice that did not receive bacterial infection or any form of treatment (normal) and infected mice treated with AAV-luc-YFP or AAV-sh-*Ac45* (disease). (C) Three-dimensional (3D) reconstruction of two-dimensional (2D) μCT analysis shown in panel B. (D) Quantification of bone volume/tissue volume and bone density measured for periapical lesions in panel B. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. $n = 9$. Purple arrows indicate severe apical bone loss.

AAV-sh-*Ac45* largely decreased osteoclast numbers in the periapical lesion area. To further examine how AAV-sh-*Ac45* treatment attenuates bone destruction *in vivo*, tooth sections from normal and infected mice treated with AAV-sh-*Ac45* or AAV-luc-YFP were stained with TRAP, which indicated that AAV-sh-*Ac45* treatment reduced the number of activated osteoclasts *in vivo* by 67% (Fig. 5A and B). Consistent with our *in vitro* results, which showed significant changes in osteoclast differentiation following *Ac45* silencing, we found that osteoclast differentiation was significantly reduced following *Ac45* silencing in the endodontic disease model due to attenuated inflammation compared to the infected AAV-luc-YFP-treated mice. Notably, under inflammatory conditions, activated T cells can induce osteoclastogenesis via RANKL-dependent and RANKL-independent mechanisms (12). Thus, upon *Ac45* silencing, the T-cell-mediated immune response was inhibited, which in turn impaired RANKL-stimulated osteoclast differentiation. Furthermore, anti-Ctsk immunochemistry staining revealed that AAV-sh-*Ac45* treatment reduced expression of Ctsk *in vivo* by 66%, while Ctsk expression was not detected in the normal control group (Fig. 5C and D). Collectively, these data demonstrated that AAV-mediated *Ac45* knockdown prevented periapical bone resorption *in vivo* by impairing osteoclast differentiation.

AAV-sh-*Ac45* significantly decreased the number of macrophages and dendritic cells in the periapical lesion area. Immunofluorescence staining of alveolar sections indicates that uninfected mice (normal) and bacterium-infected mice treated with AAV-sh-*Ac45* have fewer CD11c-positive mature dendritic cells than bacterium-infected mice treated with AAV-luc-YFP (Fig. 6A and B). Quantification analysis showed the percentage of CD11c-positive dendritic cells in periapical lesions in the AAV-sh-*Ac45* group decreased by 70% compared to that of the AAV-luc-YFP group (Fig. 6D). Immunochemistry staining of alveolar sections indicates that uninfected mice (normal) and bacterium-infected mice treated with AAV-sh-*Ac45* have fewer F4/80-positive

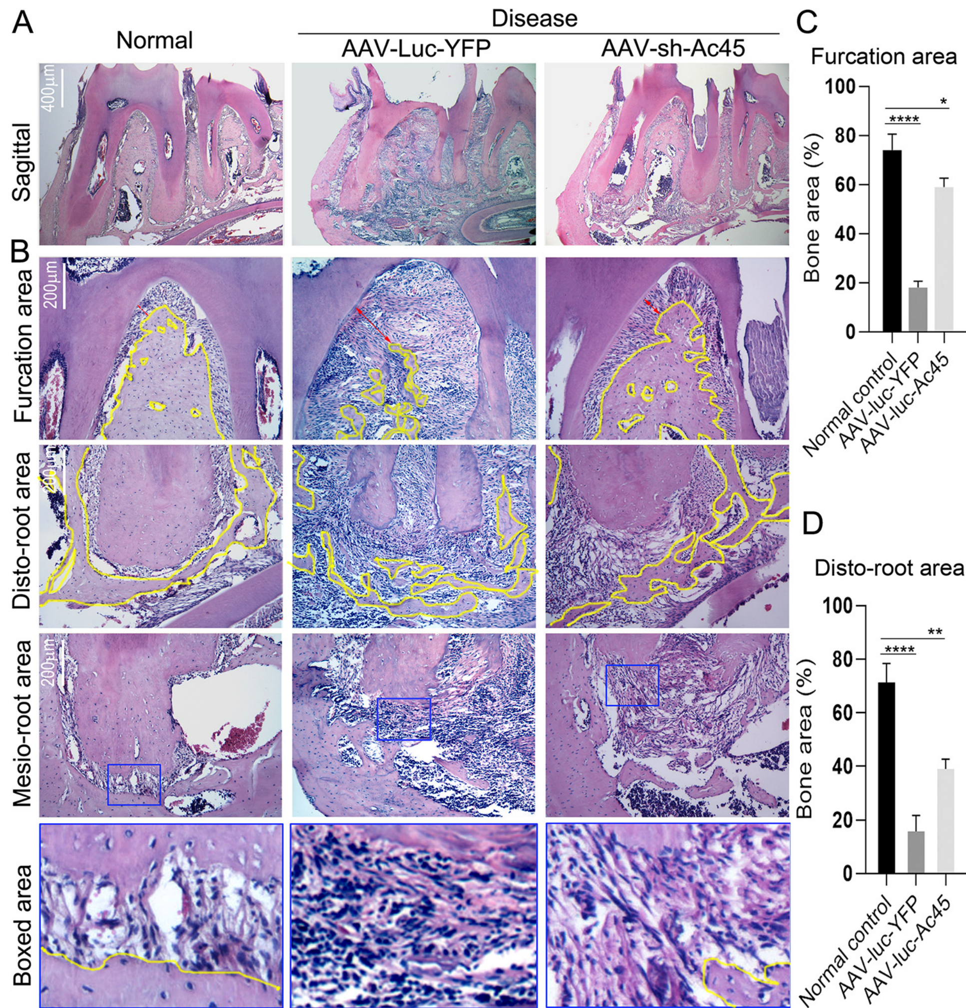

FIG 4 *Ac45* knockdown decreased mononuclear cell infiltration in the periapical area and periodontal ligament. (A and B) Hematoxylin and eosin (H&E) staining of sections from uninfected mice (normal), bacterium-infected mice treated with AAV-luc-YFP (control), and bacterium-infected mice treated with AAV-sh-*Ac45*. Mononuclear cell infiltration was significantly decreased in the AAV-sh-*Ac45* treatment group compared to the control AAV-luc-YFP group in the furcation area and the mesial- and disto-root areas ($n = 21$). Yellow outlined areas indicate bone. The periodontal ligament (PDL) is shown outside the yellow outlined region. The width of the PDL is indicated by the red arrows. (C) Quantification of bone resorption in the furcation area in panel B. (D) Quantification of bone resorption in the disto-root area in panel B. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. $n = 9$.

macrophages than bacterium-infected mice treated with AAV-luc-YFP (Fig. 6B to D). Notably, AAV-sh-*Ac45*-treated mice showed a 75% reduction in F4/80-positive macrophages compared to bacterium-infected mice treated with AAV-luc-YFP (Fig. 6D). Dendritic cells are critical in immune responses for antigen presentation and Ctsk secretion, which leads to TLR9 signaling activation (13). Similarly, macrophages are also antigen-presenting cells that have a phenotype similar to that of dendritic cells. Consistent with this finding, F4/80-positive macrophages were also decreased in the periapical area after *Ac45* silencing (Fig. 6C and D). These data indicate that *Ac45* silencing reduces dendritic cell and macrophage infiltration, leading to the attenuated inflammation seen in AAV-sh-*Ac45*-treated mice.

AAV-sh-*Ac45* significantly decreased the number of T cells in the periapical lesion area. Through immunofluorescence staining, we revealed that the number of $CD3^+$ T cells in the periapical tissues displayed a reduction in the AAV-sh-*Ac45* treatment group similar to that of the AAV-luc-YFP control group (36 + 8 versus 92 + 7, $P < 0.001$), indicating that AAV-sh-*Ac45* also reduced T cells *in vivo* (Fig. 7A to C). Under inflammatory conditions, activated T cells can induce osteoclastogenesis via RANKL-

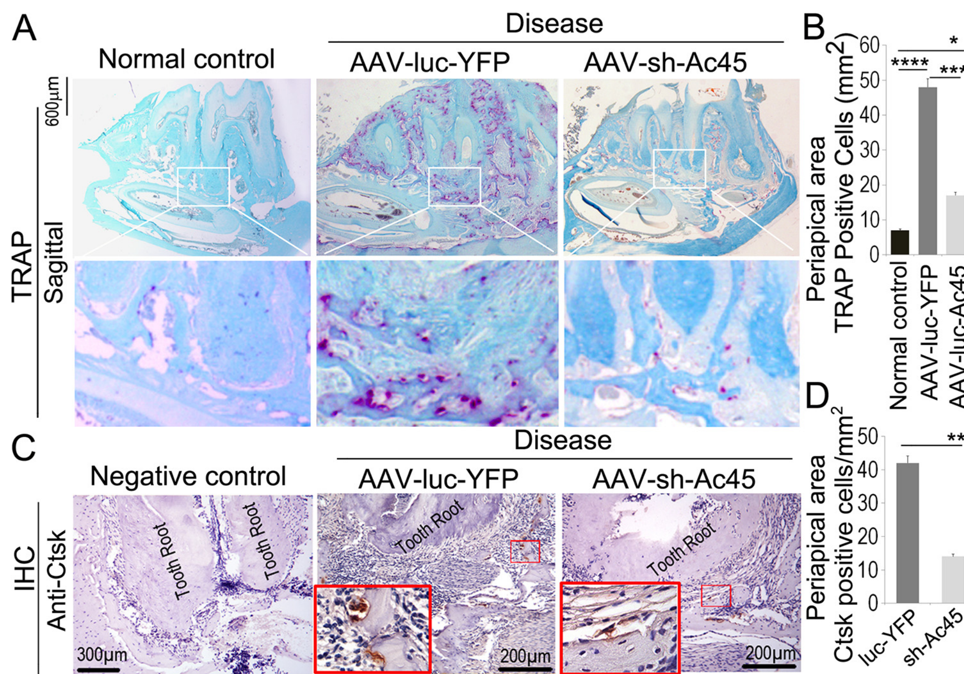

FIG 5 AAV-sh-*Ac45* reduced osteoclast-mediated alveolar bone resorption. (A) Representative figures from TRAP staining of sections from normal and infected mice treated with AAV-luc-YFP or AAV-sh-*Ac45*. (B) Quantification of the number of TRAP-positive cells in the periapical area in panel A. (C) Representative figures from immunochemistry staining of anti-Ctsk reveals that AAV-sh-*Ac45* treatment reduces expression of Ctsk *in vivo*. (D) Quantification of the number of Ctsk-positive cells in the periapical area in panel C. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. $n = 9$.

dependent and RANKL-independent mechanisms (12). Thus, upon *Ac45* knockdown, the T-cell-mediated immune response was inhibited, impairing RANKL-stimulated osteoclast differentiation. These results indicated that in addition to inhibiting bone resorption, AAV-sh-*Ac45* also reduces inflammatory cell infiltration.

AAV-sh-*Ac45* reduced expression levels of osteoclast marker genes and cytokines in periapical tissues. To investigate potential mechanisms underlying how *Ac45* knockdown attenuates bone resorption and inflammation, we examined the protein levels of mature cathepsin K (Ctsk) in the supernatant from MBM-induced osteoclasts (Fig. 8A). Interestingly, *Ac45* silencing can dramatically reduce the protein levels of mature Ctsk secreted from MBM-induced osteoclasts, suggesting that Ac45 regulates lysosomal secretion of Ctsk (Fig. 8A). Ctsk plays an important role in the activation of TLR signaling by cleaving TLR receptors. To examine Ctsk-mediated activation of TLRs and downstream signaling, we performed unbiased genome-wide transcriptome sequencing (RNA-seq) analysis of CTSK$^{-/-}$ dendritic cells stimulated with LPS (see Fig. S1 in the supplemental material). Cathepsin K knockout was confirmed in Ctsk$^{-/-}$ dendritic cells, and beta-catenin was used as a positive control. mRNA expression levels of various TLRs were examined in Ctsk$^{-/-}$ dendritic cells with notably reduced expression of TLR6/1/3 and, to a lesser extent, TLR9 (Fig. S1). However, TLR4 expression was slightly upregulated in mutant DC. It is unclear whether the expression at the receptor level is of transcriptional significance. Therefore, downstream molecules in TLR4 and TLR9 signaling were further explored. We found significantly reduced expression of TLR downstream targets (Fig. S1). Furthermore, the expression of inflammatory molecules in NF-κB and tumor necrosis factor α (TNF-α)-related signaling were also downregulated in *Ctsk*$^{-/-}$ dendritic cells (Fig. S1). To investigate the effect of *Ac45* silencing on inflammatory cytokines at the protein level, enzyme-linked immunosorbent assays (ELISA) were performed. We found that both infected groups had elevated levels of interleukin-12 (IL-12), IL-6, and IL-17, which are TLR signaling pathways targeting downstream genes, compared to those of uninfected controls. However, the levels of

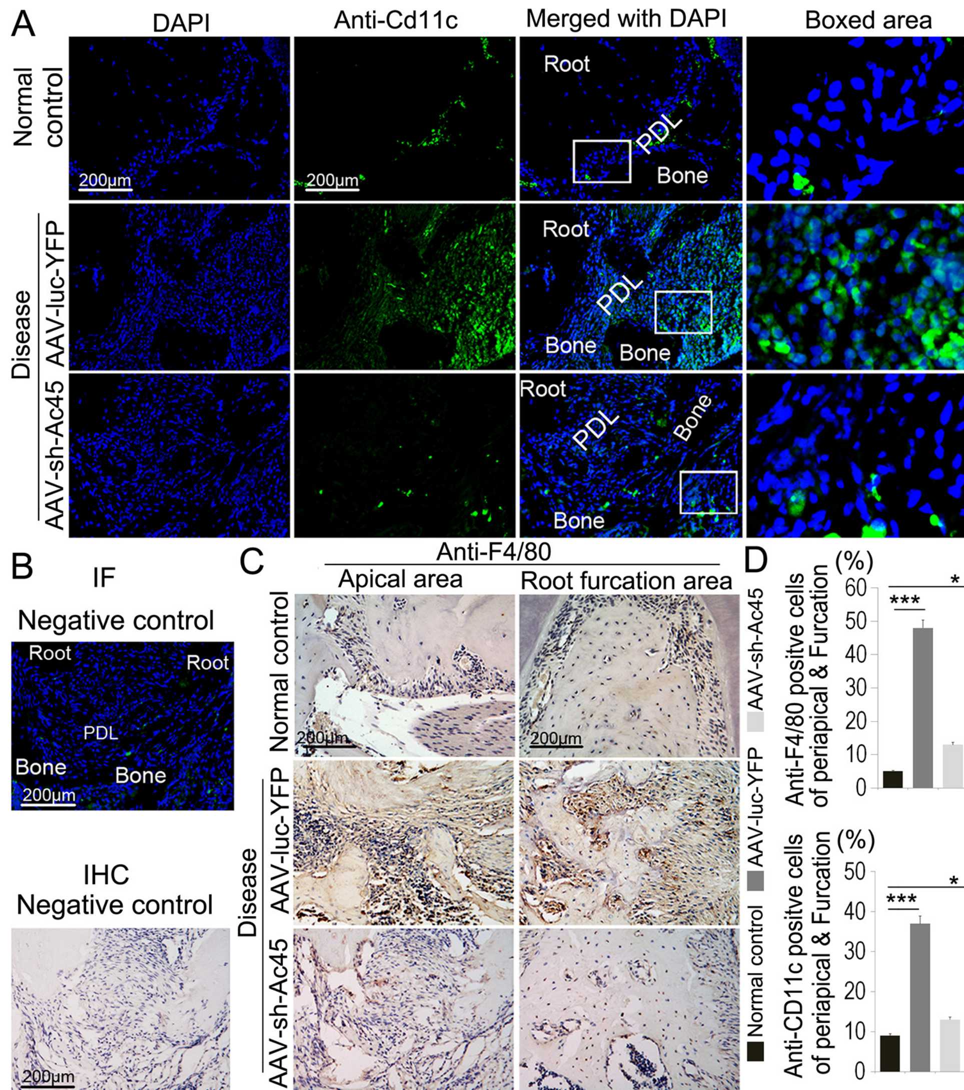

FIG 6 AAV-mediated *Ac45* knockdown decreased the number of dendritic cells and macrophages in the periapical area. (A) Representative figures from immunofluorescence staining of alveolar sections indicated that uninfected mice (normal) ($n = 12$) and bacterium-infected mice treated with AAV-sh-*Ac45* ($n = 12$) have fewer CD11c-positive (green) dendritic cells than bacterium-infected mice treated with AAV-luc-YFP ($n = 12$). Cell nuclei were labeled using 4′,6-diamidino-2-phenylindole (DAPI) DNA stain (blue). (B) Negative control for anti-CD11c immunofluorescence staining (without primary antibody) and negative control for anti-F4/80 immunohistochemistry staining (without primary antibody). (C) Representative immunohistochemistry staining from alveolar sections indicated that uninfected mice (normal) ($n = 12$) and bacterium-infected mice treated with AAV-sh-*Ac45* ($n = 12$) have fewer macrophages than bacterium-infected mice treated with AAV-luc-YFP ($n = 12$). (D) Quantification analysis of CD11c-positive dendritic cell percentages in periapical lesions in the AAV-sh-*Ac45* group compared to the normal group and AAV-luc-YFP group, and quantification analysis of F4/80 positive macrophages percentage in periapical lesions in the AAV-sh-*Ac45* group compared to the normal group and AAV-luc-YFP group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; NS, not significant.

these mediators were largely reduced in the AAV-sh-*Ac45* treatment group compared to the group treated with AAV-luc-YFP (Fig. 8B). Furthermore, we found that the protein levels of TNF-α, IL-10, and IL-1α were elevated in the infected group treated with AAV-luc-YFP, but the protein levels of these inflammatory cytokines remained similar to those of uninfected controls in the infected group treated with AAV-sh-Ac45 (Fig. 8B). IL-10 has been shown to contribute to the anti-inflammatory or immunosuppressive effects under inflammatory conditions (14). The examination of inflammatory markers in periapical tissues by quantitative reverse transcription-PCR (qRT-PCR) analysis revealed that the AAV-sh-*Ac45* treatment group had significantly lower expression of

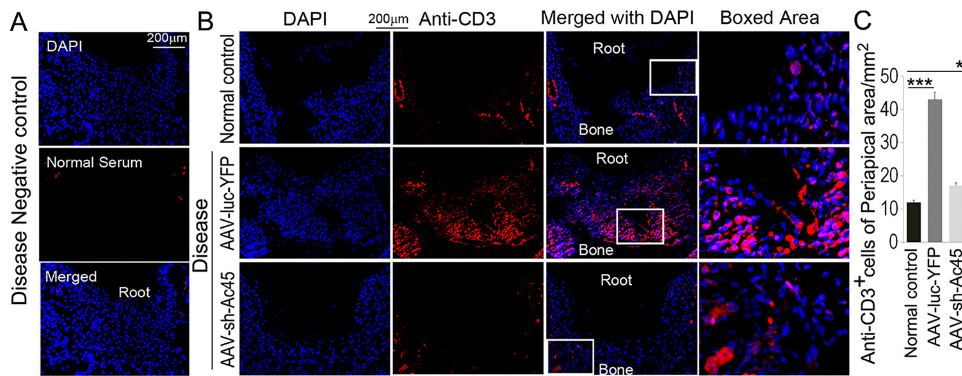

FIG 7 AAV-sh-*Ac45* significantly decreased the number of T cells in the periapical area. (A) Disease negative-control group (without primary antibody). (B) Representative images from immunofluorescence staining of alveolar sections indicated that uninfected mice (normal) ($n = 12$) and bacterium-infected mice treated with AAV-sh-*Ac45* ($n = 12$) have significantly fewer CD3-positive (red) T cells than infected mice treated with AAV-luc-YFP ($n = 12$). Cell nuclei were labeled using DAPI DNA stain (blue). (C) Quantification analysis of CD3-positive cell percentages in periapical lesions in the AAV-sh-*Ac45* group compared to the normal group and AAV-luc-YFP group. *, $P < 0.05$; ***, $P < 0.005$. NS, not significant.

inflammatory mediators TNF-α, IL-6, and IL-17, as well as the osteoclast gene encoding Ctsk, than the AAV-luc-YFP group (Fig. 8C), while bone formation markers osterix (OSX), osteopontin (OPN), and osteocalcin (OCN) were significantly increased in the AAV-sh-*Ac45* treatment group compared to both the uninfected control and AAV-luc-YFP treatment groups (Fig. 8C). Mature Ctsk is involved in cleaving TLR9, which results in the activation of TLR9 signaling responsible for inflammatory responses (13). In low-pH microenvironments, mature Ctsk is secreted from lysosomes of immune cells or following osteoclast extracellular acidification. Due to osteoclast malfunction after *Ac45* silencing, the acidic environment required for bone resorption is disrupted, which inhibits the maturation of Ctsk. The data show that *Ac45* knockdown disrupts cellular and extracellular acidification and may block Ctsk maturation in dendritic cells or osteoclasts and TLR9 signaling activation. IL-6 is secreted by osteoblasts in response to bone resorption and is important for osteoclast differentiation (15). In conclusion, we found that *Ac45* knockdown significantly reduced proinflammatory cytokine expression and Ctsk maturation, indicating that *Ac45* regulates TLR signaling in endodontic disease.

DISCUSSION

In this study, we investigated the mechanism underlying how Ac45 regulates the immune response and inhibits inflammation in endodontic disease. We used the AAV2 vector to silence *Ac45* gene expression to reduce bone resorption and inflammation induced by bacterial infection in the dental pulp. The AAV vector efficiently silenced the expression of *Ac45*, reduced osteoclast bone resorption *in vitro*, and blocked cellular and extracellular acidification. Remarkably, transduction of periapical lesions with AAV-sh-*Ac45* largely reduced infection-stimulated bone resorption *in vivo* and resulted in significantly fewer infiltrating mononuclear cells, including T cells and dendritic cells. Furthermore, AAV-sh-*Ac45* reduced bacterial infection-stimulated proinflammatory cytokine expression and dendritic cell maturation by regulation of TLR signaling through to disrupted cellular acidification, lysosomal trafficking, and protease exocytosis, indicating that targeting *Ac45* facilitates the design of novel therapeutic approaches for osteoclast overactivation-related diseases, such as periapical disease.

We previously reported that silencing of *Ac45* in the mouse model of periodontal disease prevents alveolar bone loss and periodontal tissue inflammation (7); however, the mechanisms by which Ac45 regulates immune cell activation, and which immune cells target different TLR signaling pathways in endodontic disease, have not been explored. In this study, we investigated the mechanism by which Ac45 regulates the

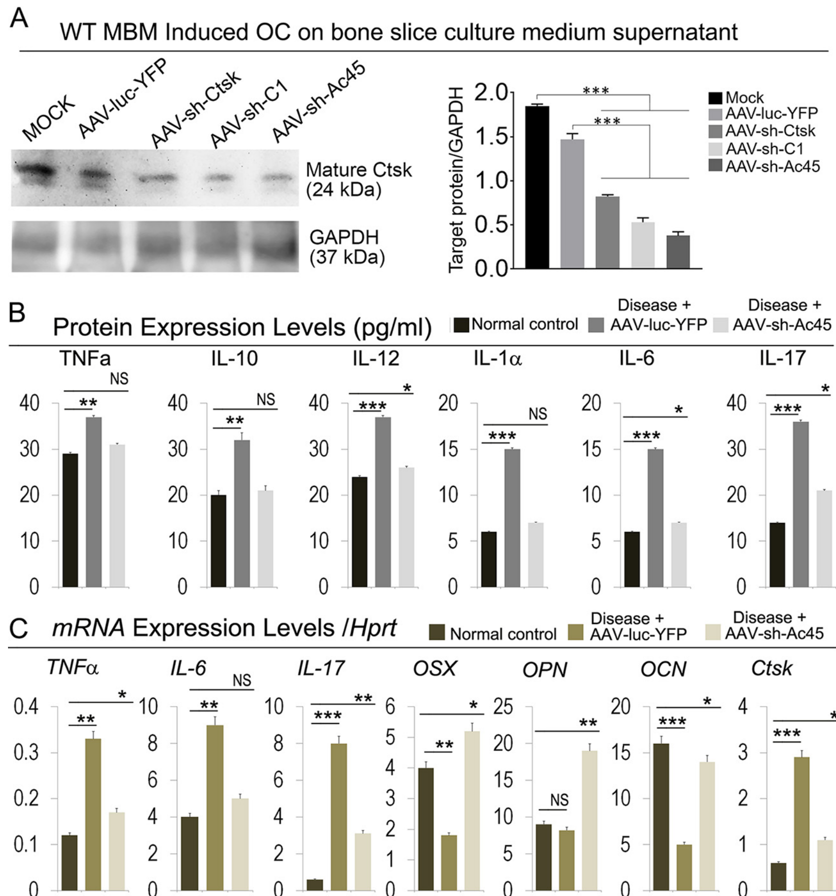

FIG 8 AAV-sh-*Ac45* reduced the expression of osteoclast marker genes and cytokines in the periapical tissues, potentially through regulating TLR signaling. (A) Western blot to detect the protein levels of Ctsk in the supernatant from MBM-induced osteoclasts cultured on bone slices. Mock-infected and AAV-luc-YFP groups served as positive controls, while AAV-sh-Ctsk and AAV-sh-C1 groups served as negative controls. (B) Cytokines of TNF-α, IL-10, IL-12, IL-1α, IL-6, and IL-17 in the periapical tissues were detected by ELISA (pool of 3 samples each time in each group for three independent experiments). (C) Quantitative PCR of *Ac45* in uninfected mice (normal), bacterium-infected mice treated with AAV-luc-YFP, or infected mice treated with AAV-sh-*Ac45*. *hprt* was used as an endogenous control. qRT-PCR was performed on TNF-α, IL-6, IL-17, Osx, OPN, OCN, and Ctsk in the periapical tissues of uninfected mice (normal) or bacterium-infected mice treated with AAV-luc-YFP or with AAV-sh-*Ac45*. Expression levels were normalized to the housekeeping gene encoding hypoxanthine-guanine phosphoribosyl transferase (*hprt*) (pool of 3 samples each time in each group for three independent experiments). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. NS, not significant.

activation of immune cells and bone resorption under pathological conditions in the mouse model of endodontic disease. Notably, the infiltration of mononuclear cells (dendritic cells, T cells, and macrophages) into the periapical tissues and PDL in the AAV-sh-*Ac45* treatment group was largely reduced in the AAV-luc-YFP-treated group, indicating potential effects of *Ac45* silencing to attenuate the inflammatory and immune response in periapical disease. These findings are consistent with previous reports that deficiency of Ac45 leads to immunodeficiency (9). Our data demonstrate that Ac45 may regulate inflammatory responses through inhibiting TLR signaling pathway activation related to both extracellular and cellular acidification and lysosomal trafficking. Mediation of inflammatory signals by immune cells and cytokines in periapical disease significantly influenced osteoclast differentiation and function through either direct or indirect effects on osteoclast precursors in the bony microenvironment (16). Dendritic cells are critical in immune responses for antigen presentation and Ctsk secretion, which leads to TLR9 signaling activation (13). Similarly, osteoclasts can express numerous immune receptors (17, 18). Our previous study demonstrated that

Ac45 regulates extracellular acidification, lysosomal trafficking, and protease exocytosis, and that osteoclasts deficient in Ac45 have a lack of Ctsk exocytosis into the resorption lacuna (5). Mature Ctsk is involved in cleaving TLR9, which results in the activation of TLR9 signaling, responsible for inflammatory responses (13). In low-pH microenvironments, mature Ctsk is secreted from lysosomes of immune cells or from osteoclasts following cellular and extracellular acidification. Due to osteoclast malfunction after *Ac45* silencing, the acidic environment is disrupted, which inhibits the maturation of Ctsk. This disruption in acidification largely contributes to inhibited dendritic cell maturation seen in AAV-sh-*Ac45*-treated mice due to loss of TLR signaling activation. We demonstrated that AAV-sh-*Ac45* impaired mature cathepsin K secretion more significantly than that by AAV-sh-*C1* and AAV-sh-*CtsK*, which we have previously shown to be involved in regulating the immune response (19–23). Interestingly, Ewald et al. were unable to block TLR9 processing using cathepsin inhibitors, and they did not observe defects in TLR9 signaling in macrophages and dendritic cells from Ctsk-deficient mice (24). However, through unbiased genome-wide RNA-seq studies, we showed that ablation of Ctsk in dendritic cells resulted in significantly reduced TLR downstream target expression. Our data demonstrate that *Ac45* knockdown disrupts extracellular and cellular acidification and may block Ctsk maturation in dendritic cells and TLR signaling activation through inhibiting extracellular and cellular acidification and lysosomal trafficking. Although other cathepsins may also play key roles in mediating TLR signaling activation in acidic microenvironments, TLR signaling is critical for the T-cell-mediated immune response as well as cytokine secretion. Our data show that $CD3^+$ T-cell numbers were significantly decreased in periapical lesions in the AAV-sh-*Ac45* treatment group, indicating that *Ac45* knockdown inhibits the inflammatory response in periapical disease through modulating TLR signaling.

Osteoclasts function as the primary cells to mediate periapical bone resorption. Receptor activator of nuclear factor-$\kappa\beta$ ligand (RANKL) stimulates osteoclast differentiation and was found to be expressed in human dental pulp cells (25). T and B cells may also express RANKL (26, 27); however, the contribution of these cell types to stimulating osteoclastogenesis and activating osteoclasts in periapical bone resorption is unclear. Osteoclasts with *Ac45* silencing have impaired exocytosis and lysosomal trafficking, indicated by a lack of lysosomal trafficking to the ruffled border and a lack of Ctsk exocytosis into the resorption lacuna (5). Studies indicate that osteoclasts and their precursors regulate immune responses and osteoblast formation and function by means of direct cell-cell contact through ligands and receptors and through the expression of clastokines. Osteoclasts have been implicated as playing important roles in immune responses beyond mediating bone resorption (28). Dendritic cells are critical in immune responses for antigen presentation and Ctsk secretion, leading to TLR9 signaling activation (13). Similarly, macrophages are also antigen-presenting cells that have a phenotype similar to that of dendritic cells. In this study, we observed the potential of AAV-sh-*Ac45* treatment to attenuate bone resorption and inflammation in the periapical disease mice. Although further studies are needed, the effect of AAV-sh-*Ac45* on the number of osteoclasts may be an indication of a direct effect on osteoclast precursor proliferation and fusion or an indirect effect from the inhibition of dendritic cell maturation and T-cell activation.

TLRs are important components of the innate immune response through recognition of different microbe-associated molecular patterns, and TLR signaling activation results in specific cellular transcriptional programs and the expression of immune mediators such as proinflammatory cytokines (29). TLRs act as primary detectors that sense a multitude of microbial components, elicit innate immune responses, and subsequently activate the transcription factor NF-κB, which regulates the gene expression of numerous inflammatory cytokines, including IL-1, IL-6, TNF-α, and IL-12 (30). Myeloid dendritic cells express several TLRs, such as TLR2 and TLR4, which trigger dendritic cell maturation in response to bacterial peptidoglycan and lipopolysaccharides (31). TLR4 recognizes LPS in Gram-negative bacteria, while TLR2 plays a major role in the recognition of various bacterial components (32). TLR2 and TLR4 upregulation

has been shown in bacterium-infected dental pulp, which suggests innate immune responses involving the TLRs as signaling receptors contribute to the pathogenesis of pulp inflammation (32). TLR9 specifically recognizes CpG DNA of bacteria and viruses (33). TLR signaling is crucial for the secretion of cytokines and the T-cell-mediated immune response. Activated T cells can induce osteoclastogenesis via RANKL-dependent and RANKL-independent mechanisms under inflammatory conditions (12). TLR2 signaling leads to the production of interleukin 17 (IL-17) by immune cells, which is an important effector cytokine produced by cells of the immune system (34). We found that AAV-sh-*Ac45* treatment significantly reduced proinflammatory cytokines TNF-α, IL-10, IL-12, IL-6, and IL-17 at both the protein and mRNA levels. These findings were consistent with the reduced CD3-positive T cells and decreased inflammation by knockdown of Ac45. It has been shown that TLR2 signaling promotes IL-17A production during oropharyngeal candidiasis (34); interestingly, in our study we found that protein and mRNA levels of IL-17 were decreased in the infection group following *Ac45* knockdown, suggesting a role of Ac45 in the TLR2 signaling pathway. Furthermore, other studies have shown that IL-1, IL-6, and TNF-α regulate mononuclear preosteoclast proliferation and differentiation into osteoclast progenitors and preosteoclast fusion (35, 36). We also found reduced protein levels of IL-10 following *Ac45* knockdown. IL-10 is an important anti-inflammatory cytokine that suppresses immunoproliferative and inflammatory responses, and it downregulates proinflammatory cytokine and chemokine synthesis (37, 38). IL-10 also regulates osteoblastic bone formation and inhibits osteoclastic bone resorption (39–41). Thus, under inflammatory conditions, *Ac45* silencing may play a key role in inhibiting TLR signaling pathway activation in immune cells.

V-ATPases have been implicated in numerous physiological processes, including exocytosis, endocytosis, membrane fusion, cell-cell fusion, and intracellular membrane trafficking (4). A recent study by Smith et al. investigated the expression, distribution, and activity of V-ATPase isoforms in invasive prostate adenocarcinoma (PC-3) cells and revealed that isoforms of membrane subunit *a* associate with the accessory protein Ac45 (8). Knockdown of *Ac45* stalled the transit of isoform *a*1 and transferrin-transferrin receptor, decreased proton efflux, and reduced cell growth and invasiveness, indicating that Ac45 plays a central role in navigating the V-ATPase to the plasma membrane and, thus, is an important factor in the expression of the phenotype in invasive prostate adenocarcinoma (8). Jansen et al. showed that different Ac45 protein isoforms were discovered in human brain, liver, and B cells, indicating the presence of tissue-specific regulation of organelle acidification (9), while previous studies have also shown that the clinical phenotype of Ac45 deficiency in humans causes an immunodeficiency with hepatopathy, cognitive impairment, and abnormal protein glycosylation (9), suggesting an important role of Ac45 in immune diseases. Notably, in our study we found that *Ac45* silencing plays a key role in attenuating inflammation and bone resorption in periapical disease by significantly reducing inflammatory cytokine expression of TLR signaling pathway-targeted downstream genes, as well as decreasing macrophages, dendritic cells, and T cells in the periapical lesion. Our results demonstrate that silencing of *Ac45* in the periapical lesion dramatically inhibited infection-induced inflammation, providing new insights into the role of Ac45 in modulating the immune response through inhibiting TLR signaling.

In conclusion, we investigated the therapeutic effect of AAV-sh-*Ac45* in periapical disease of inhibiting inflammation and bone resorption and demonstrated that AAV-sh-*Ac45* protected the periodontal ligament from inflammation-inducted destruction by impairing cellular and extracellular acidification, cathepsin K secretion, TLR signaling activation, and dendritic cell maturation. Our study provides important insights into the mechanism underlying the role of Ac45 in inflammatory diseases and osteolytic diseases, such as endodontic disease, and how Ac45 modulates inflammation and osteoclast-mediated bone resorption. Endodontic disease results in both bone erosion and soft-tissue damage caused by inflammation; thus, a gene therapy against a single target that can significantly inhibit inflammation and bone loss simultaneously may have tremendous potential as a therapeutic approach in humans. The insights resulting

from this study may assist in the design of novel treatments for endodontic disease and other osteolytic and inflammatory diseases.

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 Institutional Animal Care and Use Committee and followed all recommendations of ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines.

Design and construction of shRNA. Using the Dharmacon siDESIGN Centre (http://www.dharmacon.com) as described in our recent publication (42), we generated shRNAs that would simultaneously target Ac45. As a control vector, we used AAV-H1-luc-YFP (gift from Sonoko Ogawa), which contains a luciferase yellow fluorescent protein (YFP) cassette (43).

Pulp exposure, bacterial infection, and transduction of AAV vectors. The periapical disease mouse model was produced as we previously described (1, 11). Bacterial culture, infection, and viral vector transduction in a site-specific manner was performed as described previously (1, 11).

Data quantification and statistical analyses. Experimental data are reported as means ± standard deviations from triplicate independent samples. The figures are representative of the data ($n = 21$). Data were analyzed with the two-tailed Student's t test. P values of <0.05 were considered significant. Data quantification analyses were performed using the NIH ImageJ program as described previously (1, 11, 44).

Data availability. The RNA-seq data are available upon request. All other data are contained within the manuscript.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

ACKNOWLEDGMENTS

We thank Diep Edwards for her bioinformatics assistance. We thank Liang Hao for his technical assistance. We thank Sergei Musatov and Sonoko Ogawa for kindly providing the AAV-H1-luc-YFP and AAV-H1 vectors and for helpful suggestions. We are grateful for assistance from the Small Animal Phenotyping Core and Metabolism Core Laboratory at the University of Alabama at Birmingham.

This work was supported by the National Institutes of Health (DE023813, DE028264, AR074954, and AR075735 to Y.P.L.; AR070135 and AG056438 to W.C.). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Study design: W.C. and Y.P.L. Study conduct: W.Y. and Z.Z. Data collection and analysis: W.Y., Z.Z., N.G., A.M., L.W., Y.P.L., L.L., and W.C. Drafting manuscript: W.C., Y.P.L., L.L., W.Y., and A.M. Revising manuscript: W.C., Y.P.L., W.Y., A.M., and L.L. All authors approved the final version of the manuscript for submission. W.C. and Y.P.L. take responsibility for the integrity of data analysis.

We have no conflicts of interest to declare.

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