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Original Article

Quercetin through miR-147−5p/Clip3 axis reducing Th17 cell differentiation to alleviate periodontitis

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ABSTRACT

Background: Quercetin (QU) plays an important role in treating periodontitis; however, the mechanism through which microRNAs regulate Th17 cell differentiation has not been determined.
Methods: QU was administered intragastrically to periodontitis rats once a day for one month. The morphology of alveolar bone was observed by micro-CT, gingival tissue structure was observed by HE staining, IL-6, TNF-α, IL-17A, RORγt, FOXP3 and IL-10 were detected by immunohistochemical staining, and Th17 and Treg cells in the peripheral blood were detected by flow cytometry. CD4$^+$T cells were induced to differentiate into Th17 cells in vitro. Cell viability was determined by CCK8, and IL-17A and RORγt were detected by qPCR. Th17 cells were detected by flow cytometry, microRNA sequencing and bioinformatics analysis were used to screen key microRNAs, the phenotypic changes of Th17 cells were observed after overexpressed microRNAs via mimics. TargetScan database, in situ hybridization, and dual-luciferase reporter experiment were used to predict and prove target genes of microRNAs. The phenotype of Th17 cells was observed after overexpression of microRNA and target gene.
Results: Compared with periodontitis group, the distance from cementoenamel junction(CEJ) to alveolar bone(AB) was decreased, the structure of gingival papilla was improved, IL-6, TNF-α, IL-17, and RORγt were downregulated, FOXP3 and IL-10 were upregulated, the proportion of Th17 decreased and Treg increased in peripheral blood after QU treatment. Compared with Th17 cell group, mRNA levels of IL-17A and RORγt were decreased, and proportion of Th17 cells was significantly lower in the coculture group. MiR-147−5p was low in control group, upregulated in Th17 cell group, and downregulated after QU intervention, it's eight bases were inversely related to 3′UTR of Clip3, miR-147−5p with Clip3 were co-located in cells of periodontal tissue. Compared with those in Th17-mimicsNC + QU cells, the mRNA levels of RORγt and IL-17A upregulated, and proportion of Th17 cells increased in Th17-miR-147−5p + QU cells. The miR-147−5p mimics inhibited the luciferase activity of the WT Clip3 3′UTR but had no effect on the Mut Clip3 3′UTR. Clip3 was significantly downregulated after the overexpression of miR-147−5p. Mimics transfected with miR-147−5p reversed the decrease in the proportion of Th17 cells induced by QU, while the overexpression of Clip3 antagonized the effect of miR-147−5p and further reduced the proportion of Th17 cells. Moreover, the overexpression of miR-147−5p reversed the decreases in the mRNA levels of IL-17 and RORγt induced by QU treatment, while pcDNA3.1 Clip3 treatment further decreased the mRNA levels of IL-17 and RORγt.
Conclusion: QU reducing inflammatory response and promoting alveolar bone injury and repair, which closely relative to inhibit the differentiation of CD4$^+$T cells into Th17 cells by downregulating miR-147−5p to promote the activation of Clip3.

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1. Introduction

Periodontitis is triggered by pathogens and long-term inflammation and is associated with cardiovascular, neurodegenerative and autoimmune diseases [1,2]. As the sixth most common human disease [3], there are many patients with this disease, but the problems associated with periodontal tissue regeneration and repair have not been resolved. Th17 cells play an important role in driving and regulating the occurrence and development of periodontal inflammation [4,5]; these cells are key media in the destruction of alveolar bone [6] and maintain the inflammatory response in periodontitis through secreting a variety of cytokines and recruiting and activating neutrophils, resulting in excessive activation of osteoclasts and disruption of the dynamic balance between bone formation and absorption [7,8].

QU is a flavonoid that has significant anti-inflammatory, antioxidant and immunomodulatory effects. It has been found that QU inhibits Th17 differentiation, reduces IL-17 release and increases the expression of anti-inflammatory factors by downregulating MAPK-TLR4 signalling pathway [9]. Moreover, QU significantly reduces the content of inflammatory mediators, regulates the Th17/Treg balance and has an anti-inflammatory effect [10]. QU inhibits differentiation of Th17 cells through inhibiting the activity of STAT3 and activating peroxisome proliferator receptor γ [11]. QU activates Toll3 signalling pathway and promotes immunomodulatory effect of mesenchymal stem cells [12]. In brief, an increasing number of studies have shown that QU plays an important role in inhibiting Th17 cell differentiation to reduce inflammation, but the underlying mechanism in periodontitis needs to be further clarified.

Th17 cell differentiation is regulated by microRNAs [13], which mainly affect downstream pathways by negatively regulating the expression of target genes [14]. MicroRNAs are important for the differentiation of immune cells and osteoclasts [15]. Upregulation of miR-590−3p inhibits the differentiation of Th17 cells and improves clinical symptoms of systemic lupus erythematosus [16]. MiR-124−3p binds to the transcription factor YY1 to enhance the differentiation of Th17 cells and promote rheumatoid arthritis [17]. Upregulation of miR-302C-5p can downregulate STAT3, inhibit the differentiation of Th17 cells and alleviate ulcerative colitis [18]. However, there is still a lack of miRNAs induced by QU to inhibit the differentiation of Th17 cells to interfere with periodontitis.

Therefore, rats with periodontitis were treated with QU by intragastric administration, and the efficacy of QU in the treatment of periodontitis was evaluated by imaging, histopathology, and serological techniques. Then flow cytometry, microRNA sequencing and bioinformatics analysis, and gene transfection were subsequently performed to determine the effect of QU on the differentiation of CD4$^+$T cells into Th17 cells and clarify the mechanism through which microRNA regulates periodontitis, so as to provide a new scientific basis for the use of QU in the treatment of periodontitis.

2. Materials and methods

2.1. Animals

Sprague–Dawley (SD) rats (n = 30, 180–220 g) were purchased from Kunming Medical University experimental animal center (SCXK (Dian) K2018-0002). The animal experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals approved by the animal ethics committee of Kunming Medical University (permission number: kmmu20231531).

2.2. Intragastric administration of QU to periodontitis model rats

Healthy SD rats were used as the control group (n = 10). Periodontitis was induced by ligating the maxillary second molar of SD rats with silk thread, and the rats were randomly divided into periodontitis (n = 10) and treatment (n = 10) groups. The plants in the treatment group were administered QU (75 mg/kg) suspended in 0.5% CMC-Na every day for one month, while the control and periodontitis groups were administered 0.5% CMC-Na.

2.3. Micro-CT

SD rats were euthanized by excessive 3% pentobarbital sodium, the maxilla fixed with 4% paraformaldehyde for 24 h was placed in a NEMO Micro-CT (NMC-100) and scanned by a Cruiserr with a resolution of 12 μm. The distance from the CEJ to the AB of the maxillary second molar was analysed via three-dimensional reconstruction with an Avatar 1.5.0.

2.4. Hematoxylin-eosin (HE) staining

Periodontal tissues were separated and fixed in 4% paraformaldehyde, and decalcification was carried out with 10% EDTA calcium carbonate solution. Periodontal tissue was dehydrated, embedded in paraffin wax and cut into 5 μm thick slices from the cheek side. The slices were baked in a constant temperature oven at 60 °C for 12 min. After xylene was dewaxed, the sections were stained with hematoxylin-eosin, sealed with gum, and observed.

2.5. Immunohistochemical (IHC) staining

After the maxillary sections were deparaffinized, antigen retrieval was performed. BSA was added to cover the padlocks for blocking, and primary antibodies against IL6, TNFα, IL-1β, IL-17, RORγt, FOXP3, and IL-10 (Servicebio) were added and incubated overnight. The slides were then incubated with biotinylated secondary antibodies followed by incubation with the streptavidin-peroxidase complex.

2.6. Isolation and purification of naïve CD4$^+$T cells

C57BL/6 mice at 6−8 weeks of age were euthanized to separate the spleens, the cell suspension was resuspended in Mojosort™ Buffer (Biolegend), filtered through a 70 μm filter (Biosharp), and centrifuged at 300×g for 5 min. The cells were adjusted to 1 × 10^8/ml with Mojosort™ Buffer, Biotin-Antibody Cocktail were added according to the instructions of Mojosort™ Mouse CD4 Naïve T Cell Isolation Kit (BioLegend) for magnetic bead separation, then incubated on ice for 15 min, Mojosort™ Buffer was added, and the unlabelled cells were placed at the magnetic pole for 5 min after being blown.

2.7. Induction of Th17 cell differentiation and intervention with QU

The plates were coated with anti-mouse CD3 (5 μg/mL) (BioLegend) overnight at 4 °C, and the concentration of the purified naïve CD4$^+$T cells was adjusted to 2 × 10^6/mL. Anti-mouse CD28 (5 μg/mL) (BioLegend), IL-6 (100 ng/mL) (Peprotech), TGF-β1 (1 ng/mL) (Peprotech), IL-23 (5 ng/mL) (Novoprotein), anti-mouse IL-4 (10 μg/mL) (BioLegend), and anti-mouse IFN-γ (10 μg/mL) (BioLegend) were added, mixed with QU (0, 1, 3, 10 μM) (sigma) and incubated for 3 days.

2.8. MicroRNA sequencing and bioinformatics analysis

Total RNA was extracted from the cells in each group through TRIzol(Invitrogen), purity and integrity of the RNA were detected via a NanoDrop (Nanodrop 2000, USA) and an Agilent 2100 (Agilent Technologies). After the cDNA library was constructed, an Agilent 2100 and ABI StepOnePlus Real-Time PCR System (Life Technologies) were used to test its quality and yield. Sequencing was performed via an Illumina HiSeq2000 (Illumina, San Diego, CA, USA). Volcano and heatmaps were drawn by conventional methods.

2.9. Dual-luciferase reporter assay

The psiCHECK-2 vector containing the wild-type (wt) or mutant (mut) Clip3 binding site was constructed. Mmu-miR-147—5p mimics or miRNA-NC were cotransfected into cells with wild-type Clip3 or mut-Clip3, respectively. A luciferase assay was performed after 48 h.

2.10. Cell transfection and differentiation

The cell concentration was adjusted to 5×10^5 cells/mL with basal medium, the cells were seeded into six-well plates, and a mixture of the miR-147—5p mimics or pcDNA3.1 Clip3 and Lipo3000 were added. The medium was replaced with complete medium after 6 h of transfection, after which the cells were induced for 24 h, and incubated for 3 days in the presence or absence of QU.

2.11. In situ hybridization

The paraffin sections of periodontal tissue were incubated with pre-hybridization solution, the probe (miR-147—5p , 5'-TAGCAGAAGCATTTCCGCACAC-3' , 500 nM) hybrid solution (G3045) was used to hybridize overnight, added preheated branch probe hybridization solution (Servicebio), washed with preheated SSC solution. The corresponding signal probe was added, and sealed with 3%BSA, the primary antibody of clip3 (Rab, 1:100) was incubated, after washed with PBS the fluorescein-labeled secondary antibody (488 labeled goat anti-rabbit) was added. The nuclei and seals were re-dyed by DAPI, and images were collected.

2.12. Flow cytometry

The cells were stimulated with eBioscience Cell Stimulation Cocktail (2 μg/mL) (eBioscience) for 5 h, collected, centrifuged, resuspended in FACS buffer, stained with PE-labeled CD4 (Biolegend), and incubated at 4 °C in the dark for 20 min. In $1 \times$ Cytofix/Cytoperm Buffer (BD) suspension cells, 4 °C avoid light incubation for 30 min, $1 \times$ Permeabilization Wash Buffer (BD) fixed and $500 \times g$ centrifugal 5 min. $1 \times$ Permeabilization Wash Buffer after heavy rupture in suspension cells, according to the instruction manual to join APC IL-17A (Biolegend) dyeing was carried out on the intracellular cytokines, 4 °C avoid light incubation for 30 min, flow cytometry was used for detection.

2.13. qPCR

Total RNA was extracted via a Takara MiniBEST Universal RNA Extraction Kit (Takara, Japan), and microRNAs were extracted via a miRcute miRNA Extraction Kit (Tiangen, China). Reverse transcription into cDNA was performed using PrimeScriptTM RT Master Mix (Perfect Real Time) or a Mir-X miRNA First-Strand Synthesis Kit (Takara, Japan); the QuantStudioTM Real-Time

PCR system was used to perform q-PCR with TB Green Premix Ex TaqTM II (Tli RNaseH Plus) (Takara, Japan). The sequences of primers used were as follows.

gene	Primer sequences
RORγt	F:5'-GACTGGAGGACCTTCTACGG -3'
	R:5'-CTCCATGAAGCCTGAAAGCC -3'
IL-17A	F:5'-GGAATCACAATCCACGAAATCC-3'
	R:5'-GGGATATCTCTCAGGGTCCTCAT-3'
GAPDH	F: 5'-GGTTGTCTCCTGCGACTTCA-3'
	R: 5'-TGGTCCAGGGTTTCTTACTCC-3'
Clip3	F: 5'-CCGAAGAGAACACAAAGGGAAGA -3'
	R: 5'-GTAACCTGAGCAAAGTCAGTCT-3'
miR-147—5p	GTGTGCGGAAATGCTTCTGCT
miR-181a-1-3p	ACCATCGACCGTTGATTGTACC
miR-10b-5p	TACCCTGTAGAACCGAATTTGTG
miR-181b-5p	AACATTCATTGCTGTCGGTGGGTT
U6	GGAACGATACAGAGAAGATTAGC

2.14. Statistical analysis

All the experiments were repeated at least three times, and the data are presented as the means ± SD. GraphPad Prism 9 software was used for statistical analysis, and Student's *t*-test or one-way analysis of variance (ANOVA) was used for statistical analysis. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. QU relieves ligature-induced periodontitis symptoms

QU was intragastrically administered to periodontitis rats once a day for one month. Micro-CT revealed that the distance from the CEJ to the AB and the extent of alveolar bone resorption were significantly lower in the QU treatment group than in the periodontitis group (Fig. 1A and B). HE staining revealed that inflammatory cell infiltration, collagen fibre breakage and attachment loss were decreased in the QU treatment group than in the periodontitis group (Fig. 1C). Compared with those in the periodontitis group, the expression of TNFα, IL-1β and IL-6 in the QU treatment group was decreased (Fig. 1D and E). The above experimental results showed that QU can promote the repair of alveolar bone injury and reduce inflammatory reactions in periodontitis rats.

3.2. QU diminishes Th17 cell proportion in periodontitis rats

Th17 cells drive and regulate the occurrence and development of periodontitis. Therefore, we observed the effect of QU on Th17 cells in the periodontal tissue and peripheral blood of periodontitis rats. The immunohistochemistry results (Fig. 2A and B) showed that the expression of IL-17 and RORγt increased and that of FOXP3 and IL-10 decreased in the periodontitis group, while the expression of IL-17 and RORγt significantly decreased, and that of FOXP3 and IL-10 increased after QU treatment. The flow cytometry results (Fig. 2C and D) showed that, compared with that in the normal group, the proportion of Th17 cells in the periodontitis group was greater, but there was no significant change in the Treg cell population; moreover, the proportion of Th17 cells was lower, while the Treg cell population was greater in the treatment group. The above results suggest that QU downregulates inflammatory factors, upregulates anti-inflammatory factors, and inhibits Th17 cell differentiation in periodontitis.
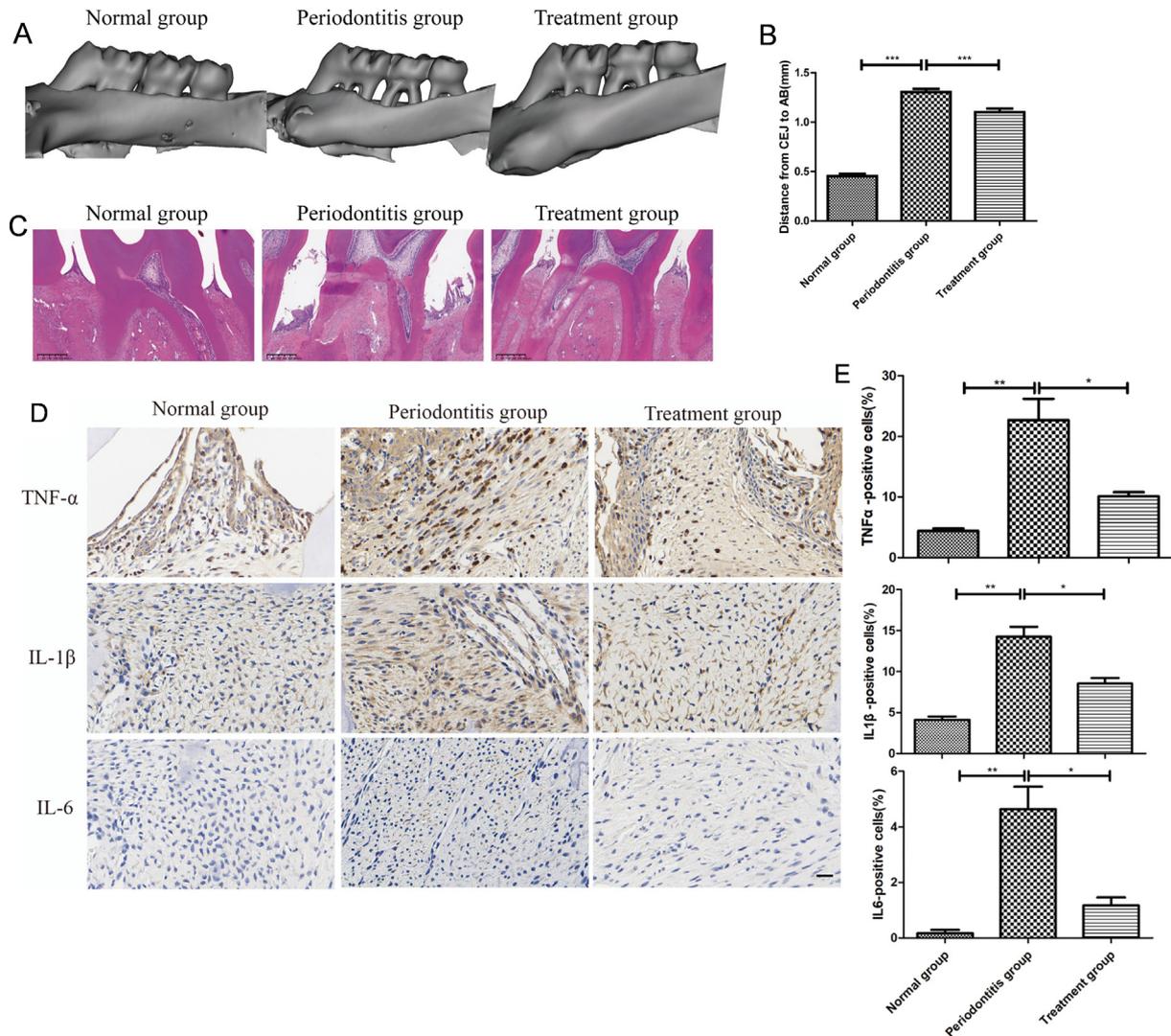
Fig. 1. QU alleviated symptoms of experimental periodontitis in rats. (A, B) The alveolar bone morphology and the distance from CEJ to AB were analysed via micro-CT. (C) Pathological changes of periodontal tissues were evaluated via HE staining (400 μm). (D) Immunohistochemistry staining were performed to detect the expression of the TNFα, IL-1β and IL-6 (20 μm). (E) Statistical analysis of the changes of TNFα, IL-1β and IL-6 among groups. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.3. QU represses Th17 cell differentiation in vitro

To further clarify the effect of QU on the differentiation of Th17 cells, we isolated and purified CD4$^+$T cells from the mouse spleen and induced them to differentiate into Th17 cells. Moreover, QU was added to observe the phenotypic changes in Th17 cells. The flow cytometry results showed that the proportion of CD4$^+$IL17A$^+$ cells in the Th17 group increased and decreased significantly after QU intervention in a concentration gradient-dependent manner within the 1−10 μM treatment (Fig. 3A and B). Furthermore, the same change trend was observed for the mRNA level. qPCR results showed that the upregulation of RORγt and IL-17A in the Th17 group, while RORγt and IL-17A decreased significantly after QU treatment (Fig. 3C and D). The above results showed that QU markedly inhibited the differentiation of Th17 cells.

3.4. Differential microRNAs

The differentiation of Th17 cells is regulated by microRNAs [19], to elucidate how QU regulates microRNAs to decrease Th17 cells. We performed microRNA sequencing and bioinformatics analysis,

results shown that top5 microRNAs of mmu-miR-181a-1-3p, mmu-miR-147−5p, mmu-miR-181b-5p, mmu-miR-181c-3p, mmu-miR-10b-5p with low level in control, upregulated in Th17, and down-regulated in Th17+QU group (Fig. 4A and B). qPCR further examined the changes in the top 5 microRNAs among the groups (Fig. 4C), mmu-miR-147−5p was consistent with of the results of the sequencing analysis, while the other miRNAs were different from those of the sequencing analysis. At present, whether mmu-miR-147−5p can regulate Th17 cell differentiation has not been reported in periodontitis. Therefore, from an innovative point of view, we selected mmu-miR-147−5p as the key molecule for follow-up research.

3.5. QU downregulates miR-147−5p to inhibit Th17 cell differentiation

To explore the role of miR-147−5p in the inhibition of Th17 cell differentiation by QU, miR-147−5p mimics were transfected into CD4$^+$T cells, which were then induced to differentiate into Th17 cells in the presence of QU. The flow cytometry results showed that the proportion of CD4$^+$IL17A$^+$ T cells decreased after QU
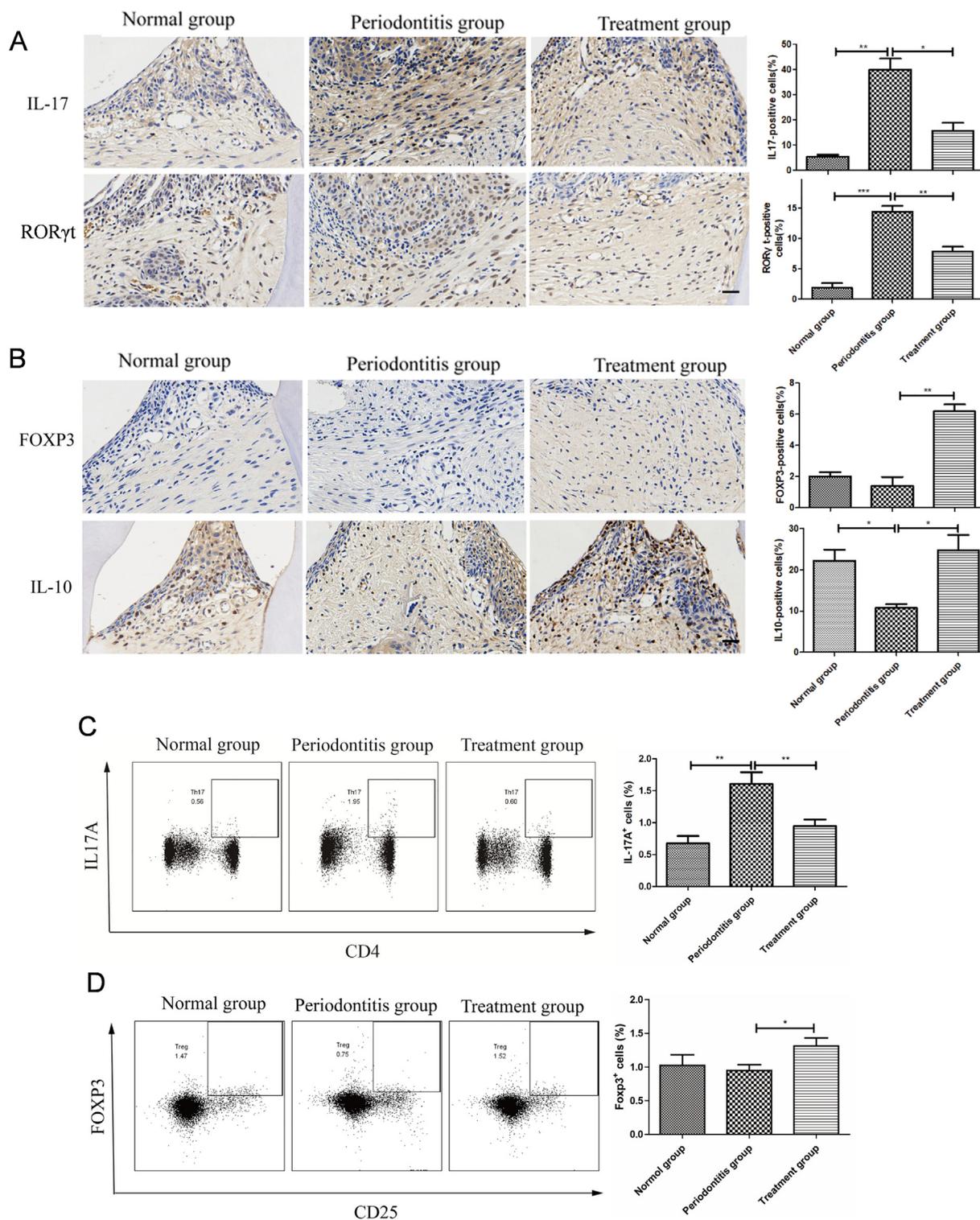
Fig. 2. QU inhibits Th17 cell differentiation in vivo. (A–B) The expression of IL-17, RORγt, FOXP3 and IL-10 were detected via immunohistochemistry (20 μm). (C–D) CD4$^+$IL17A$^+$ T cells and CD4$^+$CD25$^+$FOXP3$^+$ T cells in peripheral blood were measured by flow cytometry. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

intervention, but this decrease was partially reversed after the overexpression of miR-147–5p (Fig. 5A and B). In addition, the qPCR results revealed the same trend; that is, QU intervention decreased the relative expression of IL-17A and RORγt, while these decreases were reversed after the overexpression of miR-147–5p (Fig. 5C and D). These results suggest that QU inhibits the differentiation of Th17 cells through reducing miR-147–5p.

3.6. QU inhibits Th17 cell differentiation via the miR-147–5p/Clip3 axis

In order to explore the downstream molecular mechanism of miR-147–5p regulation through QU, we used the TargetScan database to predict the target gene of miR-147–5p. The results showed that eight bases of miR-147–5p with the 3′UTR sequence of
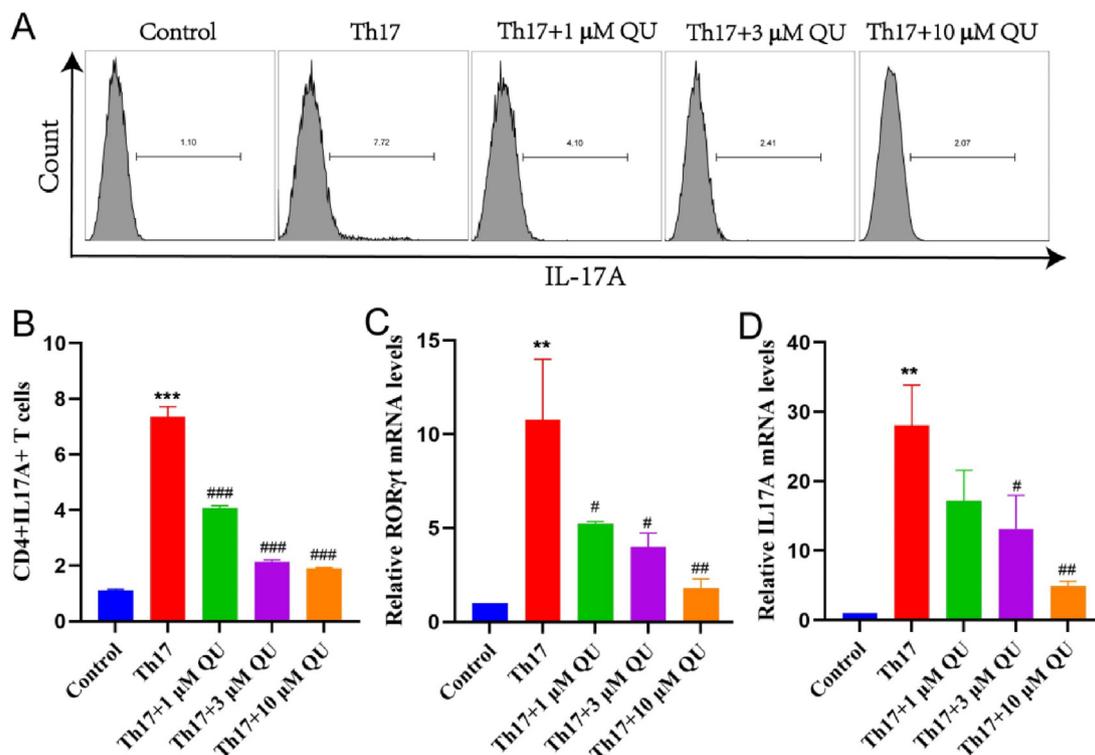
Fig. 3. QU represses Th17 cell differentiation in vitro. CD4$^+$T cells were isolated and purified from mouse spleens and induced to differentiate into Th17 cells for 3 days in the presence or absence of QU. (A—B) The proportion of CD4$^+$IL17A$^+$ T cells in each group was detected by flow cytometry. (C—D) The mRNA levels of RORγt and IL-17A were measured via qPCR. (**$p < 0.01$, ***$p < 0.001$ vs. control. #$p < 0.01$, ##$p < 0.01$, ###$p < 0.001$ vs. Th17).
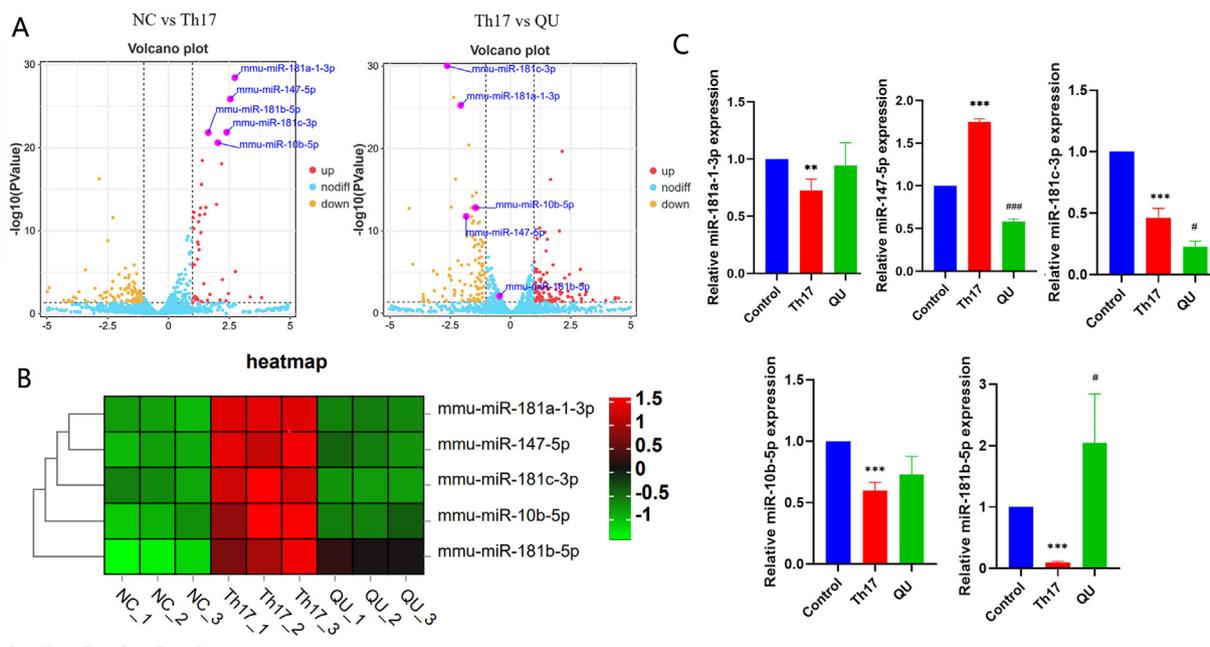

Fig. 4. The key miRNAs were screened. Cells from the Th0 (NC group), Th17, and QU groups were collected for miRNA sequencing. (A) The five most differentially expressed miRNAs are shown in the volcano plot. (B) The top five differentially expressed miRNAs are shown in the heatmap. (C) The top five miRNAs was detected by qPCR. (**$p < 0.01$, ***$p < 0.001$ vs. control. #$p < 0.01$, ###$p < 0.001$ vs. Th17.).

Clip3 were highly inversely related (Fig. 6 A), the in situ hybridization showed that miR-147—5p with Clip3 were co-located in cells of Periodontal tissue (Supplementary Fig. 1A), and a dual-luciferase reporter gene assay (Fig. 6 B) showed that the relative fluorescence intensity of the psiCHECK-2-Clip3 (WT) group was significantly

lower than that of the psiCHECK-2-Clip3 (MUT) group, indicating that miR-147—5p regulated the 3′-UTR of Clip3. To reveal the relationship between miR-147—5p and Clip3, Th17 cells were transfected with mimics-NC or miR-147—5p. qPCR revealed that the mRNA level of Clip3 in the miR-147—5p group was significantly
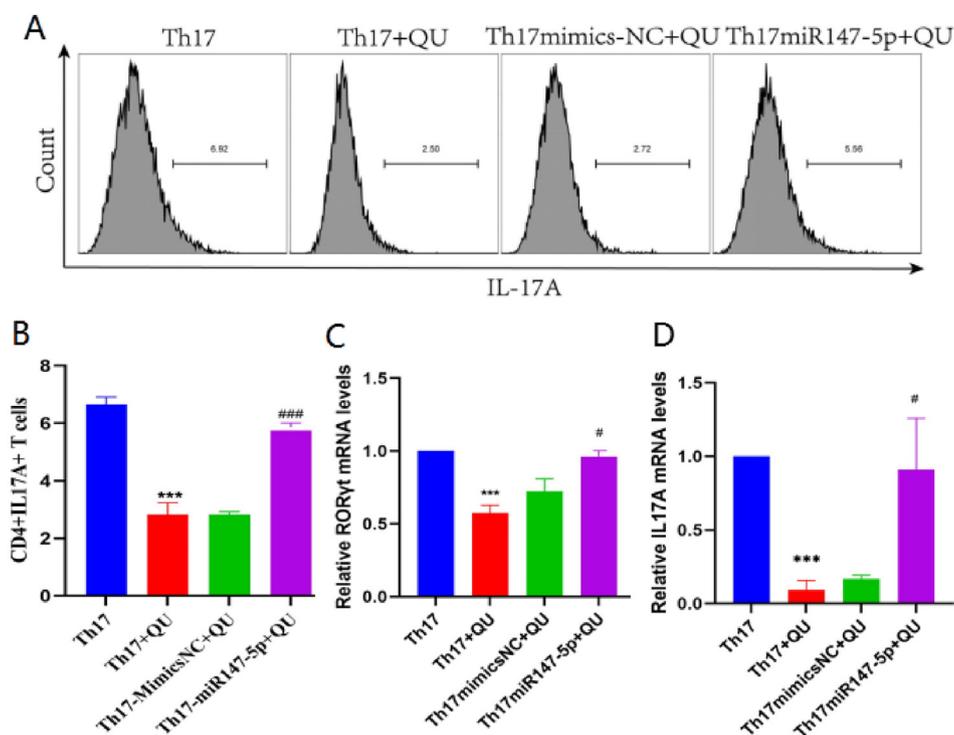
Fig. 5. QU inhibited the differentiation of Th17 cells through miR-147—5p. MiR-147—5p mimics or the negative control (NC) were transfected into cells, after which Th17 differentiation was induced for 3 days in the presence of QU. (A) The proportion of CD4$^+$IL17A$^+$ T cells was detected by flow cytometry. (B) Statistical analysis of the CD4$^+$IL17A$^+$ T cells in each group. (C—D) The mRNA levels of RORγt and IL-17A was detected via qPCR. (***$p < 0.001$ vs. Th17 cells. $^#p < 0.01$, $^{###}p < 0.001$ vs. Th17mimicsNC + QU).

lower than that in the mimics-NC group (Fig. 6C), suggesting that Clip3 is the target gene of miR-147—5p and is negatively regulated.

To further determine whether miR-147—5p could inhibit the differentiation of CD4$^+$T cells into Th17 cells by regulating Clip3, we overexpressed miR-147—5p and Clip3 to observe the changes in Th17 cells. First, pcDNA-Clip3 was transfected into Th17 cells with liposomes, and the results showed that more than 90% of the cells were successfully transfected (Supplementary Fig. 1B) and that the mRNA level of Clip3 in the pcDNA-Clip3 group was significantly increased (Supplementary Fig. 1C). We subsequently divided the cells into five groups: Th17, Th17 + QU, Th17-miR-147—5p + QU, Th17-miR-147—5p + pcDNA3.1 + QU and Th17-miR-147—5p + pcDNA3.1-Clip3 + QU. The results of flow cytometry (Fig. 6 D, E) showed that mimics transfected with miR-147—5p reversed the decrease in the proportion of Th17 cells treated with QU, while the overexpression of Clip3 antagonized the effect of miR-147—5p and further reduced the proportion of Th17 cells. Moreover, the same trend was observed for the mRNA levels; that is, the qPCR results showed that the overexpression of miR-147—5p reversed the low mRNA levels of IL-17 and RORγt induced by QU treatment, and pcDNA3.1 Clip3 treatment further decreased the mRNA levels of IL-17 and RORγt (Fig. 6 F, G). These results suggested that QU inhibits the differentiation of CD4$^+$T cells into Th17 cells through downregulating miR-147—5p to promote Clip3 activation.

4. Discussion

Periodontitis is an inflammatory disease characterized by periodontal soft tissue destruction and alveolar bone resorption that destroys periodontal tissue mainly by affecting the host immune system and bone homeostasis and can eventually lead to periodontal ligament loss, gingival retraction and alveolar bone resorption [20—22]. At present, how to promote periodontal tissue repair and regeneration while controlling infection is a difficult and scientific problem that needs to be solved in the treatment of periodontitis. Notably, the results of this study showed that QU inhibits the differentiation of Th17 cells by regulating the miR-147—5p/Clip3 axis, to reduce inflammation and promote alveolar bone repair, which provides a new perspective for the use of QU in the treatment of periodontitis.

We administered QU to SD rats with via periodontitis intragastric administration once a day for one month. Our results showed that, compared with periodontitis, QU significantly promoted the repair of alveolar bone injury, improved gingival tissue structure and inhibited inflammation, which are consistent with the findings of previous studies on the ability of QU to promote bone injury repair and inhibit inflammation [23—25]. In an aged mouse model, QU reduces inflammation in adipose tissue and improves systemic metabolic function [26]; QU inhibits neutrophil-mediated inflammation and polarization of M1 macrophages [27]; and QU improves renal fibrosis and inflammation through the IL-33/ST2 pathway [28]. In addition, as a powerful antioxidant, QU significantly promotes bone healing [29], maintains the balance of the internal environment of the inflammatory cascade, and protects against the occurrence and development of osteoarthritis [30]. These results suggested that QU promotes the repair and regeneration of alveolar bone injury and has great potential for treating periodontitis.

Increasing amounts of research evidence show that [31—33] excessive activation of the host immune response directly activates osteoclasts and leads to alveolar bone loss, while differently polarized CD4$^+$T cells regulate the host immune response; in particular, the inflammatory factors produced by Th17 cells play important roles in the occurrence and development of periodontal tissue destruction. Therefore, we used immunohistochemical staining and flow cytometry to observe the changes in Th17 cells in each group to explore whether QU interferes with Th17 cells. The results showed that, compared with that in the control group, the
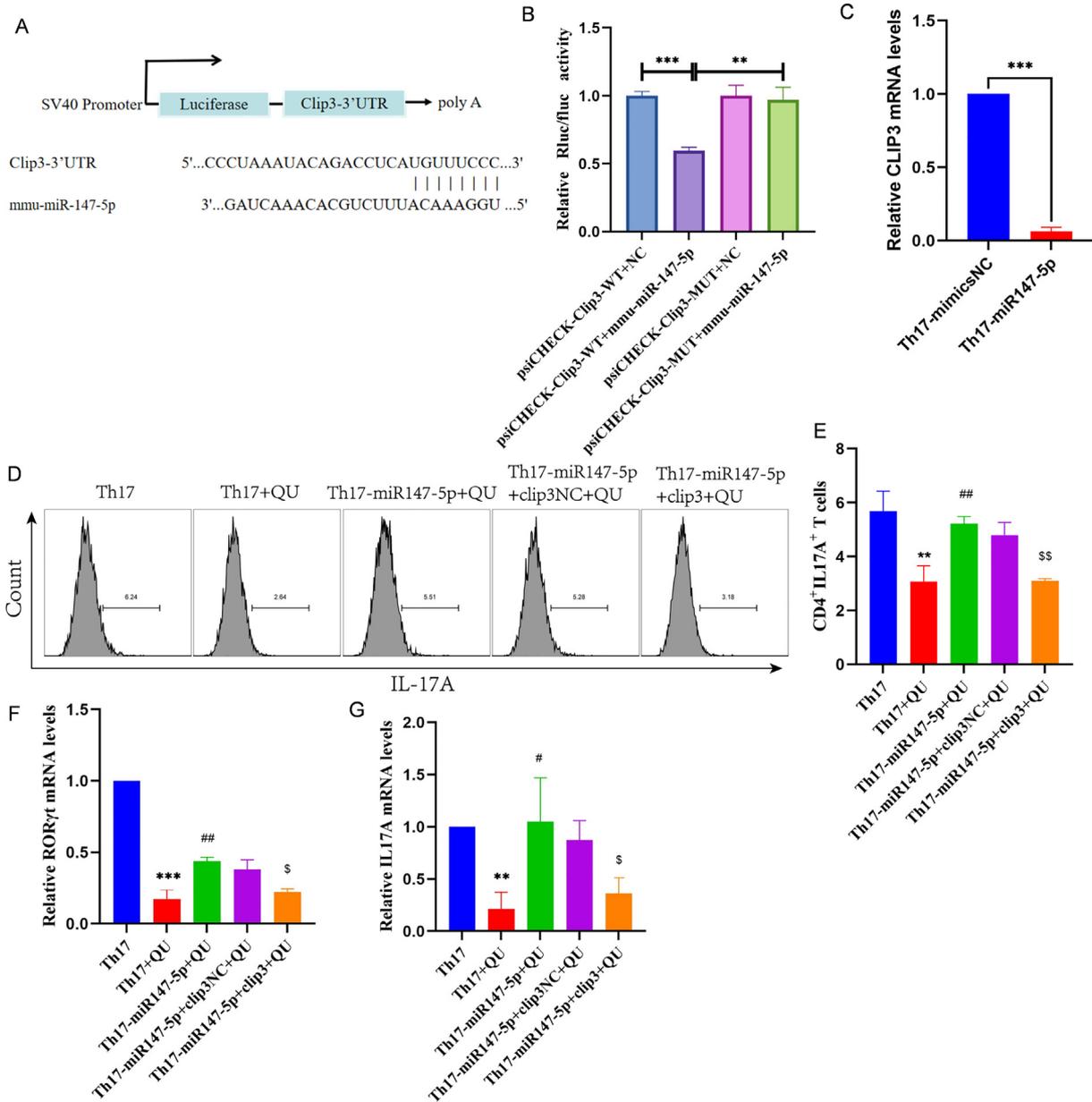
Fig. 6. QU restrains Th17 cell differentiation through miR-147—5p/Clip3 axis. (A) TargetScan was used to predict the binding site and complementary sequence of miR-147—5p in the 3′UTR of Clip3. (B) Dual-luciferase reporter experiments verified that miR-147—5p targeted Clip3. (C) After miR-147—5p mimics were transfected into cells, the expression of Clip3 was detected via qPCR. (D—E) MiR-147—5p mimics and pcDNA3.1 Clip3 were transfected into cells, after which Th17 differentiation was induced for 3 days in the presence of QU, the proportion of CD4$^+$IL17A$^+$T cells was detected via flow cytometry. (F—G) The mRNA levels of RORγt and IL-17A was measured by qPCR. (**$p < 0.001$, ***$p < 0.001$ vs. Th17 cells. $^\#p < 0.05$, $^{\#\#}p < 0.01$ vs. Th17+QU. $^\$p < 0.05$, $^{\$\$}p < 0.01$ vs. Th17-miR147—5p + clip3NC + QU).

number of Th17 cells in the periodontitis group increased but decreased significantly after intragastric administration of QU, which is consistent with previous reports that QU inhibits Th17 cell differentiation and reduces inflammation to improve disease progression [34—36]. In mice with allergic rhinitis, QU was found to improve rhinitis symptoms by reducing the percentage of Th17 cells and inhibiting the expression of inflammation-related factors [37]. In a study of rheumatoid arthritis, it was found that QU inhibited the maturation and production of osteoclasts stimulated by IL-17, macrophage colony stimulating factor or RANKL and reduced the production of Th17 cells and IL-17, but did not affect Treg cells [38]. QU significantly inhibited the polarization of Th17 cells in an arthritis mouse model [39]. Furthermore, our in vitro experiments also showed that 1, 3, and 10 μM QU

significantly inhibited the differentiation of CD4$^+$T cells into Th17 cells and decreased the mRNA levels of IL17A and RORγt. These results suggested that QU inhibits periodontitis by reducing the number of Th17 cells to promote alveolar bone repair and regeneration.

To reveal the role of microRNAs in the inhibitory effect of QU on the differentiation of CD4$^+$T cells into Th17 cells, microRNA sequencing and bioinformatics analysis were performed. Our results showed that mmu-miR-147—5p was downregulated in the control group, upregulated in the Th17 cells, and downregulated after QU intervention. At present, there are no related reports about whether mmu-miR-147—5p regulates the differentiation of Th17 cells in periodontitis. Therefore, mmu-miR-147—5p was a key candidate for further research. After the mimics-mediated

overexpression of mmu-miR-147—5p, the mRNA levels of RORγt and IL17A were upregulated, and the proportion of Th17 cells was significantly increased, while the QU treatment significantly downregulated RORγt and IL17A and decreased the proportion of Th17 cells. These results suggest that QU inhibits the differentiation of Th17 cells by downregulating miR-147—5p.

To further explore the downstream molecular mechanism by which miR-147—5p is regulated by QU, we first used the bioinformatics online prediction software TargetScan to predict the target gene of miR-147—5p. The results showed that miR-147—5p and the Clip3 gene were highly inversely related, suggesting that Clip3 may be a potential target of miR-147—5p. Clip3 involve in radiation-induced bone injury [40], and relative to the dryness and glycolytic activity of cells [41]; however, this phenomenon has not been reported in periodontitis. To exclude the target genes previously studied in periodontitis, we selected Clip3 as a candidate for further research. The results of in situ hybridization showed miR-147—5p with Clip3 were co-located in cells of Periodontal tissue, and the dual-luciferase reporter assay showed that the relative fluorescence intensity in the psiCHECK-2-Clip3 (WT) group was significantly lower than that in the psiCHECK-2-Clip3 (MUT) group, indicating that miR-147—5p could regulate the 3′-UTR of Clip3. To verify the relationship between miR-147—5p and Clip3, mimics-NC and miR-147—5p were transfected into Th17 cells, and the expression of the target gene Clip3 was detected by qPCR. The results showed that the mRNA level of Clip3 in the miR-147—5p group was lower than that in the mimics-NC group. These findings suggested that Clip3 is the target of miR-147—5p and that miR-147—5p can directly and negatively regulate Clip3. To further determine whether QU inhibits the differentiation of Th17 cells through the miR-147—5p/Clip3 axis, we overexpressed miR-147—5p and Clip3 to observe the changes in Th17 cells. Interestingly, our results showed that mimics transfected with miR-147—5p reversed the decrease in the proportion of Th17 cells treated with QU, while overexpression of Clip3 antagonized the effect of miR-147—5p, and further reduced the proportion of Th17 cells. Moreover, the same change trend was observed at the mRNA level; that is, the overexpression of miR-147—5p reversed the decrease in the mRNA levels of IL-17 and RORγt induced by QU treatment, and overexpressed Clip3 further decreased the levels of IL-17 and RORγt. These results suggest that QU inhibits the differentiation of CD4+T cells into Th17 cells by downregulating miR-147—5p to promote Clip3 activation.

5. Conclusion

QU inhibits the differentiation of CD4+T cells into Th17 cells by regulating the mmu-miR-147-5p/Clip3 axis, to reduce the inflammatory response and promote the repair of damaged alveolar bone in rats with periodontitis.

Ethical approval and consent to participate

Experimenta animal production licence number is SCXK (Dian) K2018-0002, and the use of SD rats were approved by the experimental animal ethics committee of Kunming Medical University, the approval number was kmmu20231531.

Consent for publication

Not applicable·

Availability of data and materials

All data are included in this research article.

Authors' contributions

AYY and HHB designed the research. AYY, ZRY, LW, WCX and JLX performed the experiments, and AYY and RXB wrote the manuscript. ZMZ and HHB assisted with the literature searches and revised the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.04.016.

References

[1] Gasparoni LM, Alves FA, Holzhausen M, Pannuti CM, Serpa MS. Periodontitis as a risk factor for head and neck cancer. Med Oral Patol Oral Cir Bucal 2021;26: e430—6.

[2] Hajishengallis G, Chavakis T. Local and systemic mechanisms linking periodontal disease and inflammatory comorbidities. Nat Rev Immunol 2021;21: 426—40.

[3] Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet 2015;386:743—800.

[4] Ruterbusch M, Pruner KB, Shehata L, Pepper M. In vivo CD4(+) T cell differentiation and function: revisiting the Th1/Th2 paradigm. Annu Rev Immunol 2020;38:705—25.

[5] Huang N, Dong H, Luo Y, Shao B. Th17 cells in periodontitis and its regulation by A20. Front Immunol 2021;12:742925.

[6] Dutzan N, Abusleme L. T helper 17 cells as pathogenic drivers of periodontitis. Adv Exp Med Biol 2019;1197:107—17.

[7] Kamali AN, Noorbakhsh SM, Hamedifar H, Jadidi-Niaragh F, Yazdani R, Bautista JM, et al. A role for Th1-like Th17 cells in the pathogenesis of inflammatory and autoimmune disorders. Mol Immunol 2019;105:107—15.

[8] Sifnaios E, Mastorakos G, Psarra K, Panagopoulos ND, Panoulis K, Vitoratos N, et al. Gestational diabetes and T-cell (Th1/Th2/Th17/treg) immune profile. In Vivo 2019;33:31—40.

[9] Mahmoud HA, Solahaye KS, Aghajani H, Frozannia K, Pournasrollah A, Sadegh R, et al. Quercetin decreases Th17 production by down-regulation of MAPK- TLR4 signaling pathway on T cells in dental pulpitis. J Dent 2018;19: 259—64.

[10] Yang Y, Zhang X, Xu M, Wu X, Zhao F, Zhao C. Quercetin attenuates collagen-induced arthritis by restoration of Th17/Treg balance and activation of Heme Oxygenase 1-mediated anti-inflammatory effect. Int Immunopharmacol 2018;54:153—62.

[11] Yang Y, Shi GN, Wu X, Xu M, Chen CJ, Zhou Y, et al. Quercetin impedes Th17 cell differentiation to mitigate arthritis involving PPARgamma-driven transactivation of SOCS3 and redistribution corepressor SMRT from PPARgamma to STAT3. Mol Nutr Food Res 2022:e2100826.

[12] Chen G, Ye Y, Cheng M, Tao Y, Zhang K, Huang Q, et al. Quercetin combined with human umbilical cord mesenchymal stem cells regulated tumour necrosis factor-alpha/interferon-gamma-stimulated peripheral blood mononuclear cells via activation of toll-like receptor 3 signalling. Front Pharmacol 2020;11:499.

[13] Kargar M, Torabizadeh M, Purrahman D, Zayeri ZD, Saki N. Regulatory factors involved in Th17/Treg cell balance of immune thrombocytopenia. Curr Res Transl Med 2023;71:103389.

[14] Akbari KV, Khonakdar TA, Mishan MA, Tafazoli A, Bardania H, Zarpou S, et al. Effects of quercetin on microRNAs: a mechanistic review. J Cell Biochem 2019;120:12141—55.

[15] Naqvi AR, Slots J. Human and herpesvirus microRNAs in periodontal disease. Periodontol 2000 2021;87:325—39.

[16] Huang J, Xu X, Wang X, Yang J, Xue M, Yang Y, et al. MicroRNA-590-3p inhibits T helper 17 cells and ameliorates inflammation in lupus mice. Immunology 2022;165:260—73.

[17] Lin J, Tang J, Lin J, He Y, Yu Z, Jiang R, et al. YY1 regulation by miR-124-3p promotes Th17 cell pathogenicity through interaction with T-bet in rheumatoid arthritis. JCI Insight 2021;6.

[18] Gu D, Nan Q, Miao Y, Yang H, Li M, Ye Y, et al. KT2 alleviates ulcerative colitis by reducing Th17 cell differentiation through the miR-302c-5p/STAT3 axis. Eur J Cell Biol 2022;101:151223.

[19] Yan K, Zhang F, Ren J, Huang Q, Yawalkar N, Han L. MicroRNA-125a-5p regulates the effect of Tregs on Th1 and Th17 through targeting ETS-1/STAT3 in psoriasis. J Transl Med 2023;21:678.

[20] Li Y, Chen Y, Cai G, Ni Q, Geng Y, Wang T, et al. Roles of trained immunity in the pathogenesis of periodontitis. J Periodontal Res 2023;58:864—73.

[21] Alghamdi B, Jeon HH, Ni J, Qiu D, Liu A, Hong JJ, et al. Osteoimmunology in periodontitis and orthodontic tooth movement. Curr Osteoporos Rep 2023;21:128—46.

[22] Larvin H, Kang J, Aggarwal VR, Pavitt S, Wu J. Periodontitis and risk of immune-mediated systemic conditions: a systematic review and meta-analysis. Community Dent Oral Epidemiol 2023;51:705—17.

[23] Wang Y, Che L, Chen X, He Z, Song D, Yuan Y, et al. Repurpose dasatinib and quercetin: targeting senescent cells ameliorates postmenopausal osteoporosis and rejuvenates bone regeneration. Bioact Mater 2023;25:13—28.

[24] Zhou P, Yan B, Wei B, Fu L, Wang Y, Wang W, et al. Quercetin-solid lipid nanoparticle-embedded hyaluronic acid functionalized hydrogel for immunomodulation to promote bone reconstruction. Regen Biomater 2023;10: rbad025.

[25] Chiang MC, Tsai TY, Wang CJ. The potential benefits of quercetin for brain Health: a review of anti-inflammatory and neuroprotective mechanisms. Int J Mol Sci 2023;24.

[26] Islam MT, Tuday E, Allen S, Kim J, Trott DW, Holland WL, et al. Senolytic drugs, dasatinib and quercetin, attenuate adipose tissue inflammation, and ameliorate metabolic function in old age. Aging Cell 2023;22:e13767.

[27] Wang Y, Wan R, Peng W, Zhao X, Bai W, Hu C. Quercetin alleviates ferroptosis accompanied by reducing M1 macrophage polarization during neutrophilic airway inflammation. Eur J Pharmacol 2023;938:175407.

[28] Chen HY, Chiang YF, Hong YH, Shieh TM, Huang TC, Ali M, et al. Quercetin ameliorates renal injury and pyroptosis in lupus nephritis through inhibiting IL-33/ST2 pathway in vitro and in vivo. Antioxidants 2022;11.

[29] Yurteri A, Yildirim A, Celik ZE, Vatansev H, Durmaz MS. The effect of quercetin on bone healing in an experimental rat model. Jt Dis Relat Surg 2023;34:365—73.

[30] Wang H, Yan Y, Pathak JL, Hong W, Zeng Q, Qian D, et al. Quercetin prevents osteoarthritis progression possibly via regulation of local and systemic inflammatory cascades. J Cell Mol Med 2023;27:515—28.

[31] Kitamoto S, Nagao-Kitamoto H, Jiao Y, Gillilland MR, Hayashi A, Imai J, et al. The intermucosal connection between the mouth and gut in commensal pathobiont-driven colitis. Cell 2020;182:447—462.e14.

[32] Leng S, Xu W, Wu L, Liu L, Du J, Yang F, et al. NLRP3 disturbs Treg/Th17 cell balance to aggravate apical periodontitis. J Dent Res 2023;102:656—66.

[33] Pan W, Wang Q, Chen Q. The cytokine network involved in the host immune response to periodontitis. Int J Oral Sci 2019;11:30.

[34] Zhou L, Tan J, Dai Y, Zhu K, Xiao Y, Wu D, et al. Jiawei danxuan koukang alleviates arecoline induced oral mucosal lesions: network pharmacology and the combined ultra-high performance liquid chromatography (UPLC) and mass spectrometry (MS). Drug Des Dev Ther 2023;17:3085—101.

[35] Kim HR, Kim BM, Won JY, Lee KA, Ko HM, Kang YS, et al. Quercetin, a plant polyphenol, has potential for the prevention of bone destruction in rheumatoid arthritis. J Med Food 2019;22:152—61.

[36] Tang M, Zeng Y, Peng W, Xie X, Yang Y, Ji B, et al. Pharmacological aspects of natural quercetin in rheumatoid arthritis. Drug Des Dev Ther 2022;16:2043—53.

[37] Ke X, Chen Z, Wang X, Kang H, Hong S. Quercetin improves the imbalance of Th1/Th2 cells and Treg/Th17 cells to attenuate allergic rhinitis. Autoimmunity 2023;56:2189133.

[38] Kim HR, Kim BM, Won JY, Lee KA, Ko HM, Kang YS, et al. Quercetin, a plant polyphenol, has potential for the prevention of bone destruction in rheumatoid arthritis. J Med Food 2019;22:152—61.

[39] Yang Y, Shi GN, Wu X, Xu M, Chen CJ, Zhou Y, et al. Quercetin impedes Th17 cell differentiation to mitigate arthritis involving PPARgamma-driven transactivation of SOCS3 and redistribution corepressor SMRT from PPARgamma to STAT3. Mol Nutr Food Res 2022;66:e2100826.

[40] Guo Z, Wang Z, Liu Y, Wu H, Zhang Q, Han J, et al. Carbon dots from lycium barbarum attenuate radiation-induced bone injury by inhibiting senescence via METTL3/clip3 in an m(6)a-dependent manner. ACS Appl Mater Interfaces 2023;15:20726—41.

[41] Kang H, Lee S, Kim K, Jeon J, Kang SG, Youn H, et al. Downregulated CLIP3 induces radioresistance by enhancing stemness and glycolytic flux in glioblastoma. J Exp Clin Cancer Res 2021;40:282.