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miR-146a regulates inflammatory cytokine production in *Porphyromonas gingivalis* lipopolysaccharide-stimulated B cells by targeting IRAK1 but not TRAF6

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Abstract

It has been suggested that microRNAs (miRs) are involved in the immune regulation of periodontitis. However, it is unclear whether and how miRs regulate the function of B cells in the context of periodontitis. This study is to explore the role of miR-146a on the inflammatory cytokine production of B cells challenged by *Porphyromonas gingivalis* (*P. gingivalis*) lipopolysaccharide (LPS). Primary B cells were harvested from mouse spleen. Quantitative real-time polymerase chain reaction (qPCR), enzyme-linked immunosorbent assay (ELISA) were used to detect the expression of inflammatory cytokines in B cells in the presence or absence of *P. gingivalis* LPS and/or miR-146a. Bioinformatics, luciferase reporter assay and overexpression assay were used to explore the binding target of miR-146a. Our results showed that miR-146a level in B cells was elevated by *P. gingivalis* LPS stimulation, and the mRNA expressions of interleukin (IL)-1β, 6 and 10, and IL-1 receptor associated kinase-1 (IRAK1), but not TNF receptor associated factor 6 (TRAF6), were also upregulated. The expression levels of IL-1β, 6, 10 and IRAK1 were reduced in the presence of miR-146a mimic, but were elevated by the addition of miR-146a inhibitor. MiR-146a could bind with IRAK1 3′ untranslated region (UTR) but not TRAF6 3′-UTR. Overexpression of IRAK1 reversed the inhibitory effects of miR-146a on IL-1β, 6 and 10. In summary, miR-146a inhibits inflammatory cytokine production in B cells through directly targeting IRAK1, suggesting a regulatory role of miR-146a in B cell-mediated periodontal inflammation.

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Keywords

B cell; miR-146a; LPS; IRAK1

1. Introduction

Periodontitis is featured with gingival inflammation, alveolar bone resorption, tooth loosening and migration, eventually leading to tooth loss. A complex immune/inflammatory cascade plays an important role in the process of periodontitis, in which immune cells and cytokines are involved [1]. It is now clear that inflammatory responses to chronic periodontal infections involve activated immune B cells, which helps protect against microbial insults but also contribute to bone pathogenesis in periodontitis [2–4]. A pathogenic role of B cells in periodontitis has been confirmed in B cell-deficient mice whose alveolar bone loss was protected after bacterial infection [2]. Our previous study showed that *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*)-binding B cells triggered the resorption of alveolar bone [3].

MicroRNAs (miRs) are small molecular RNAs (about 22 nucleotides), which could downregulate gene expression via binding with 3′-untranslated region (UTR) of target genes. Studies have shown that miRs participate in the physiological and pathological process, playing a vital role in cancer, inflammation, immune responses, and metabolic disorders [5–7]. It has been demonstrated that miRs function as an important regulator in periodontitis [8, 9]. Our previous studies demonstrated that miR-146a regulated the cytokine secretion in human gingival fibroblasts and periodontal ligament cells [10, 11]. Recent studies from others delineated that miR-128 controlled the inflammatory response in macrophages after stimulated with *porphyromonas gingivalis* (*P. gingivalis)* [12], and miR-24 acted as a negative regulator in the polarization and plasticity of macrophages activated by *P. gingivalis* LPS or *A. actinomycetemcomitans* LPS [13].

It has been suggested that miR-146a could regulate the function of B cells in disease. Contreras et al reported that miR-146a could modulate B-cell oncogenesis through early growth response-1 [14]. Loss of miR-146a led to the accumulation of T follicular helper cells and the germinal center B cells, which enhanced the maturation of germinal centers response [15]. In myasthenia gravis, knockdown of miR-146a reduced numbers of memory B cells, B-1 cells and plasma cells, and decreased the activation of B cells [16]. However, the effect of miR-146a on B cells in periodontitis is unclear.

P. gingivalis, one of the key periodontal pathogens, could induce periodontal inflammation and bone resorption through activation and infiltration of T and B cells [17, 18]. *P. gingivalis* LPS has been confirmed to contribute to periodontal tissue destruction through the induction of inflammatory mediators by periodontal cells [10, 11]. The purpose of this study is to determine the effect of miR-146a on the cytokine production by *P. gingivalis* LPS-challenged B cells, and to elucidate the mechanism of miR-146a-mediated regulation of B cell function.

2. Material and methods

2.1. Mouse B cell extraction and culture

C57BL/6 mice (8–10 weeks), purchased from the Jackson Laboratory (USA), were sacrificed and dissected according to the guidelines of the Institutional Animal Care and Use at the Forsyth Institute. The spleens were harvested and gently grinded in complete medium (Iscove's Modified Dulbecco's Medium with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.25 μg/ml Amphotericin B) (Gibco by life technologies, Carlsbad, CA, USA). The cell suspension were collected and centrifuged at 1500 rpm for 5 min. The pellet was lyzed using 1 ml ACK lysis buffer (Gibco by life technologies, Carlsbad, CA, USA) to remove red blood cells. Single cell suspension was centrifuged and resuspended in phosphate buffer saline (PBS). Afterward, B cells were isolated by using Pan B cell isolation kit, filtered with LD column (Miltenyi Biotec, Somerville, MA, USA) and centrifuged at 1500 rpm for 5 min. Isolated B cells were resuspended in final culture medium (complete medium, 1×2-mercaptoethanol (Gibco by life technologies, Carlsbad, CA, USA)) and were seeded in 96-well plate at 1×10^6/well.

B cells were treated with miR-146a mimic, miR-146a inhibitor or scramble controls in the presence or absence of 1 μg/ml *P. gingivalis* LPS (Strain ATCC33277, InvivoGen, San Diego, CA, USA). After 24 or 48h, supernatant and B cells were harvested for the subsequent measurements.

2.2. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were extracted with PureLink® RNA Mini Kit (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction. Reverse transcription and qPCR were performed using SuperScript II reverse transcriptase (Invitrogen by Life Technologies, Carlsbad, CA, USA) and LightCycler 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany). The primers used for these genes were as following. IL-1β: 5′-ATGCCTTCCCCAGGGCATGT-3′ (forward), 5′-CTGAGCGACCTGTCTTGGCCG-3′ (reverse); IL-6: 5′-TCCAGTTGCCTTCTTGGGAC-3′ (forward), 5′-GTACTCCAGAAGACCAGAGG-3′ (reverse); IL-10: 5′-GACCAGCTGGACAACATACTGCTAA-3′ (forward), 5′-GATAAGGCTTGGCAACCCAAGTAA-3′ (reverse), IRAK1: 5′-GCCCTTTGGCTCTATTTGGG-3′ (forward), 5′-TCTGAGGCTCATCCAGCAAAG-3′ (reverse); TRAF6: 5′-ATATGACAGCCACCTCCCCT-3′ (forward),5′-TTGGCGTCCATGACCTCTTC-3′ (reverse); glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-CCCCAGCAAGGACACTGAGCAA-3′ (forward), 5′-GTGGGTGCAGCGAACTTTATTGATG-3′ (reverse); Mmu-miR-146a: 5′-GGGTGAGAACTGAATTCCA-3′(forward), 5′-CAGTGCGTGTCGTGGAGT-3′ (universal reverse); U6 small nuclear RNA: 5′-GCTTCGGCAGCACATATACTAAAAT-3′ (forward), 5′-CGCTTCACGAATTTGCGTGTCAT-3′ (reverse). GAPDH and U6 were taken as internal reference to mRNA and miR, respectively. The expression of genes was presented using the $2^{-\Delta\Delta CT}$ method.

2.3. Enzyme-linked immunosorbent assay (ELISA)

To analyze the secretion of IL-1β, IL-6 and IL-10, mouse ELISA MAX™ Standard sets were used (Biolegend, San Diego, CA, USA) and the assays were performed according to the manufacturer's instruction. For intracellular IRAK1 and TRAF6, mouse IRAK1 and TRAF6 ELISA kit (LSBio, Seattle, WA, USA) were used. In brief, collected supernatant (for the detection of IL-1β, IL-6 and IL-10) or cell lysate (for the detection of IRAK1 and TRAF6) was added into the antibody pre-coated 96-well plates, incubated, and washed with PBS including 0.1% Tween. After reaction with primary antibody and secondary antibody, substrate solution was added and incubated for 15–30 min at 37°C. The stop solution was applied to each well and the optical density (OD value) of each well was immediately determined at 450 nm using a microplate reader (Bio-tek, Winooski, VT, USA).

2.4. Transfection

After seeding of 1×10^6 cells per well in 96-well plate, cells were cultured for 2 h at 37°C and 5% CO_2. miR-146a mimic, inhibitor and negative controls (final concentration 10 nM) were mixed with HiPerFect transfection reagent (Qiagen, Germantown, MD, USA). For co-transfection of IRAK1 plasmids (100ng) and miR-146a mimic or scramble RNA (10nM), IRAK1 plasmids or vector only (100ng), Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, CA, USA) were used. The mixture was incubated for 10 min at room temperature to allow the formation of transfection complexes. The cells were transfected with these complexes for 24 h. The cells were then treated with 1 μg/ml *P. gingivalis* LPS and the expressions of cytokines were detected 24 h after *P. gingivalis* LPS stimulation.

2.5. Luciferase reporter assay

We used miR database (miRNA.org) to explore the potential target genes of miR-146a in the NF-κB pathway, and found the two candidate targets as IRAK1 and TRAF6. BCL1 clone 5B1b (ATCC®TIB-197™, ATCC, Manassas, VA, USA) were cultured at 37°C with 5% CO_2. The B cells were transfected with miR-146a mimic (final concentration 40 nM), scramble RNA (final concentration 40 nM), IRAK1 or TRAF6 luciferase plasmid (200 ng), control plasmid (200 ng) (GeneCopoeia, Rockville, MD, USA) with Lipofectamine® 2000 reagent (Invitrogen) according to the manufacturer's protocol. After incubation for 24 h at 37°C, B cells were harvested and resuspended with PBS, then centrifuged at 3000 rpm for 5 min. The pellets were used for luciferase assay with Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol. The data were normalized to the activity of Renilla luciferase in the same cells.

2.6 Mouse model of experimental periodontitis with oral *P. gingivalis* infection

C57BL/6J mice (8–10 weeks old) were randomly divided into 4 groups (n=6): 1) control uninfected, 2) *P. gingivalis* infection only, 3) *P. gingivalis* infection with gingival PBS injection, and 4) *P. gingivalis* infection with gingival miR-146a injection. For groups 2–4, mice were orally infected with live *P. gingivalis* bacteria (ATCC 33277) pre-mixed with an equal volume of sterile 2% (wt/vol) low-viscosity carboxymethylcellulose (CMC). The infection was administered by oral gavage using 1×10^8 live *P. gingivalis* bacteria per animal per day for four consecutive days (days 0 to 3). For groups 3–4, each mouse received palatal

gingival injections of 1 μl/injection of PBS or 100nM miR-146a on the gingival papillae using a 28.5-gauge double-beveled MicroFine needle (Becton, Dickinson). The injections for animals were administered three times on days 5, 9, and 14. Experiments were terminated at day 28.

2.7 Bone morphometric analysis

The maxillae were removed and defleshed by a dermestid beetles colony. After bleaching with 3% hydrogen peroxide, the bone was stained with 1% toluidine blue. Bone resorption measurements were assessed under a microscope (Nikon SMZ745T, Nikon Instruments Inc, Japan). The polygonal area was measured using Image J (NIH) on buccal and palatal surfaces for each segment. The bone resorption area was enclosed coronally by the cemento-enamel junction (CEJ) of the molars, laterally by the exposed distal root of the first molar and the exposed mesial root of the third molar, and apically by the alveolar bone crest.

2.8. Statistical analysis

All experiments were performed at least twice. The data were presented as means ± standard deviation. Multi-grouped data were analyzed by one-way ANOVA, with post hoc test. Two-grouped data were analyzed by student t-test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. P. gingivalis LPS stimulated inflammatory cytokine production in B cells

After *P. gingivalis* LPS treatment, mRNA levels of inflammatory cytokines including IL-1β, IL-6 and IL-10 were increased in B cells compared with non-LPS treated cells ($p < 0.05$) (Fig. 1A–C). To further explore the signaling pathway of inflammatory responses, we checked the expression of IRAK1 and TRAF6. As shown in Fig. 1D, IRAK1 mRNA level was increased after *P. gingivalis* LPS treatment ($p < 0.05$). However, TRAF6 mRNA level did not change significantly after *P. gingivalis* LPS treatment (Fig. 1E, $p > 0.05$). These data suggested that *P. gingivalis* LPS could induce inflammatory cytokine response in B cells.

3.2. MiR-146a expression was induced in B cells by P. gingivalis LPS

To detect miR-146a expression in B cells, qRT-PCR was used after treatment with 1μg/ml *P. gingivalis* LPS. As shown in Fig. 2, miR-146a expression was increased 24 h and 48 h ($p < 0.05$) after *P. gingivalis* LPS stimulation compared with non-*P. gingivalis* LPS treatment. Up-regulation of miR-146a in *P. gingivalis* LPS-stimulated B cells indicates that miR-146a may be involved in the inflammatory response in B cells.

3.3. Addition of miR-146a inhibited inflammatory cytokine production in P. gingivalis LPS-challenged B cells

After transfection with miR-146a mimic or its inhibitor in B cells, the productions of IL-1β, IL-6 and IL-10 were measured following *P. gingivalis* LPS challenge (1μg/ml). As shown in Fig. 3A–C, miR-146a mimic inhibited IL-1β, IL-6 and IL-10 mRNA expression levels in B cells ($p < 0.05$). Meanwhile, miR-146a inhibitor could increase the mRNA expressions of IL-1β, IL-6 and IL-10 in B cells ($p < 0.05$, Fig. 3D–F). Furthermore, we detected the

secreted protein levels of IL-1β, IL-6 and IL-10 in the presence of miR-146a mimic or its inhibitor. The secreted protein productions of IL-1β, IL-6 and IL-10 were inhibited by miR-146a mimic ($p < 0.05$, Fig. 4A–C) and enhanced by miR-146a inhibitor in B cells ($p < 0.05$, Fig. 4D–F) after challenged by *P. gingivalis* LPS. These data demonstrated that miR-146a could regulate the inflammatory cytokine productions in B cells.

3.4. MiR-146a directly targeted IRAK1 through binding with its 3′-UTR

To explore the mechanism of miR-146a inhibitory effect on cytokine secretion, we analyzed the potential target genes of miR-146a from miR database. According to our previous study [10, 11], *P. gingivalis* LPS activated the Toll-like receptor-nuclear factor (NF)-κB signaling pathway. Based on the information, we found two key factors, IRAK1 and TRAF6, which were candidate targets of miR-146a involved in this pathway. Fig. 5A showed that 3′-UTR of IRAK1 and TRAF6 possessed the putative binding sequence for miR-146a. As shown in Fig. 5B and C, the addition of miR-146a could decrease the luciferase activity of IRAK1 luciferase plasmid ($p < 0.05$), but not that of TRAF6 luciferase plasmid ($p > 0.05$), which indicates that miR-146a could directly target IRAK1 3′-UTR.

3.5. Addition of miR-146a inhibited IRAK1 expression in P. gingivalis LPS-challenged B cells

As shown in Fig. 6, addition of miR-146a mimic significantly reduced the mRNA and protein levels of IRAK1 expression ($p < 0.05$) in B cells challenged with *P. gingivalis LPS*, as detected by qRT-PCR (Fig. 6A) and ELISA (Fig. 6B) respectively. The addition of miR-146a mimic also reduced the mRNA (Fig. 6C) and protein levels (Fig. 6D) of TRAF6 expression ($p < 0.05$) in B cells pretreated with *P. gingivalis LPS*. Meanwhile, miR-146a inhibitor significantly increased the mRNA and protein levels of IRAK1 expression in *P. gingivalis LPS*-challenged B cells ($p < 0.05$) (Fig. 6E–F). The expression of TRAF6 was also elevated at both mRNA and protein levels in *P. gingivalis LPS*-challenged B cells in the presence of miR-146a inhibitor (Fig. 6G–H).

3.6. Overexpression of IRAK1 reversed the inhibitory effect of miR-146a on cytokine production

To further determine the effects of miR-146a on IRAK1 pathway, we transfected IRAK1 expression plasmid in B cells. After overexpression of IRAK1, the mRNA levels and the protein secretions of IL-1β, IL-6 and IL-10 were significantly increased in B cells even though in the presence of miR-146a ($p < 0.05$) (Fig. 7A–F). An increased IRAK1 expression at mRNA and protein level was observed by qPCR and ELISA respectively (Fig. 7G–H), confirming the stable transfection of IRAK1 plasmid, Also, the expression levels of TRAF6 were significantly increased after IRAK1 overexpression ($p < 0.05$) (Fig. 7I–J).

3.7 Gingival injection of miR-146a significantly reduced inflammatory bone loss in P. gingivalis infection-associated experimental periodontitis

In order to investigate the effect of miR-146a *in vivo*, the mouse model of *P. gingivalis* infection-induced experimental periodontitis was used and miR-146a was injected into gingival tissues at days 5, 9 and 14 in the total 28 days period. The areas of bone loss around

maxillary second molars were measured in each group (Fig. 8A). The resorption area was significantly increased after *P. gingivalis* infection as compared to the control (Fig. 8B). The resorption area on the miR-146a treatment group was significantly decreased when compared with the PBS treatment group, indicating that miR-146a significantly decreased periodontal bone loss *in vivo* (Fig. 8B).

4. Discussion

B cell inflammatory infiltration is the hallmark of periodontitis [19], which participates in periodontal bone loss in periodontitis [4]. In this study, we determined whether miR-146a plays a role in B cell inflammatory response. We also explored the regulatory mechanism of miR-146a in B cells stimulated with *P. gingivalis* LPS. MiR-146a has been identified as a protective regulator in inflammation through regulating inflammatory cytokine secretion [10, 11]. In this study, *P. gingivalis* LPS could enhance miR-146a expression in B cells, which indicates that B cells might deploy miR-146a as a negative feedback mechanism for protective regulation of cytokine productions when they are activated.

IL-1β participates in the inflammatory responses in periodontitis. Increased IL-1β level in saliva and gingival crevicular fluid is associated with periodontitis progression [20, 21]. However, IL-1β has dual roles in which IL-1β at low doses that are close to physiologically healthy levels promotes the osteogenesis, but at higher doses inhibits osteogenesis of periodontal ligament stem cells which are the main cells to repair alveolar bone [22]. Also, IL-1β could enhance macrophage M1 phenotypes, which is responsible for bone resorption [23]. In our study, *P. gingivalis* LPS could enhance IL-1β expression from B cells (Fig. 1A). The upregulated IL-1β would impair the osteogenesis of periodontal ligament stem cells and accelerate bone resorption, which is not beneficial for bone repair and regeneration. Increased miR-146a could decrease the production of IL-1β (Fig. 4A), which suggested that miR-146a may play a protective role in B cell-mediated osteogenesis impairment and bone resorption through regulation of IL-1β.

As one of main pro-inflammatory cytokines, IL-6 plays an important role in periodontitis, which enhances periodontal inflammation at higher dose. IL-6 level in gingival crevicular fluid showed significant positive correlation with the clinical parameters in periodontitis [21, 24]. Meanwhile, Mahanonda et al mentioned that IL-6 was involved in local B cell responses and was detected in inflamed gingival tissues [19]. *P. gingivalis* LPS could enhance IL-6 secretion in human gingival fibroblasts and periodontal ligament cells [10, 11]. Also, we confirmed that *P. gingivalis* LPS could significantly up-regulate IL-6 in B cells (Fig. 1B), and miR-146a could reduce the production of IL-6 in B cells (Fig. 4B), which is consistent with other researches [10, 11]. These results suggested that miR-146a may protect periodontal tissue from inflammation partially through reducing IL-6 secretion by B cells.

Studies have demonstrated that IL-10 is a potent anti-inflammatory cytokine [25, 26]. IL-10 can maintain bone mass through inhibiting osteoclastic bone resorption and enhancing osteoblastic bone formation. From Zhang et al's research, IL-10 levels were lower in the gingival crevicular fluid in patients with chronic periodontitis and aggressive periodontitis [24]. However, other reports demonstrated that anti-IL-10 antibody could cause Th1-like

immune response and protect mice injected with *P. gingivalis* LPS from abscess induced by *P. gingivalis* [27]. Also, IL-10 was involved in local B cell response and detected in inflamed gingival tissues [19]. IL-10 could be secreted by T cells, B cells and other periodontal cells [10, 26, 28]. Studies demonstrated that miR-146a inhibition did not change IL-10 production in human gingival fibroblasts [10]. In this study, IL-10 production from B cells was inhibited by miR-146a mimic (Fig. 4C) and enhanced by miR-146a inhibitor (Fig. 4F), which indicates that miR-146a has multiple regulatory functions not only on the production of pro-inflammatory but also anti-inflammatory cytokines by B cells. Nonetheless, the overall anti-inflammatory effect of miR-146a was further suggested by the *in vivo* results showing the reduction of *P. gingivalis*-induced inflammatory bone loss after gingival injection of miR-146a (Fig. 8).

According to the previous studies, miR-146a was regulated by NF-κB in human gingival fibroblasts [29], meanwhile, inhibition of miR-146a could decrease toll-like receptor 4 and NF-κB on acetylcholine receptor-specific B cells in peripheral blood mononuclear cells from myasthenia gravis patients [16], which indicates that miR-146a is involved in NF-κB pathway. From bioinformatics analysis miR-146a might target 3′-UTR of IRAK1 and TRAF6. In human periodontal ligament cells and gingival cells, miR-146a targets IRAK1, not TRAF6 [10, 11]. In this present study, overexpression of miR-146a could downregulate IRAK1 and TRAF6 at both mRNA and protein levels (Fig. 6), which is inconsistent with Zhang et al's results [16], showing that knockdown of miR-146a had no effect on IRAK1 and TRAF6 in B cells [16], although there was no LPS-induced activation of B cells in that study. This may suggest that the involvement of miR-146a in B cells is dependent on how cells are activated. We have further demonstrated that miR-146a could directly bind with 3′-UTR of IRAK1, not TRAF6 (Fig. 5). It is suggested that IRAK1 was the key signaling molecule of miR-146a regulatory pathway in B cells during inflammatory responses because overexpressed IRAK1 could reverse the inhibitory effect of miR-146a (Fig. 7). Also, we found that overexpressed IRAK1 could increase TRAF6 level, which explained, to some extent, that IRAK1 inhibition by miR-146a might be accompanied with TRAF6 decrease. However, the mechanism of TRAF6 pathway regulation need to be further investigated.

In conclusion, miR-146a could inhibit inflammatory cytokine secretion in *P. gingivalis* LPS-challenged B cell through directly targeting IRAK1, which may provide a novel immunotherapeutic method to treat periodontitis.

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Abbreviations

miRs

microRNAs

P. gingivalis
Porphyromonas gingivalis

LPS
lipopolysaccharide

PBS
phosphate buffer saline

IL
interleukin

IRAK1
IL-1 receptor associated kinase-1

TRAF6
TNF receptor associated factor 6

UTR
untranslated region

A. actinomycetemcomitans
Aggregatibacter actinomycetemcomitans

GAPDH
glyceraldehyde-3-phosphate dehydrogenase

OD
optical density

ELISA
enzyme-linked immunosorbent assay

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Highlights

1. miR-146a inhibits inflammatory cytokine production in B cells.
2. miR-146a directly targets IRAK1 through binding its 3′ untranslated region.
3. The inhibitory effects of miR-146a on cytokines was neutralized by IRAK1 overexpression.

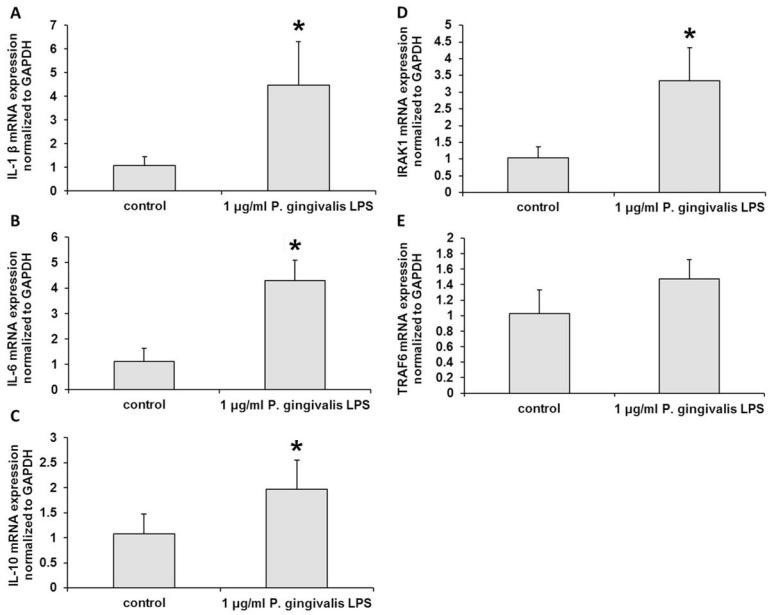

Fig. 1. The inflammatory responses in B cells were activated by*P. gingivalis* LPS

After *P. gingivalis* LPS treatment, mRNA levels of inflammatory cytokines including IL-1β (A), IL-6 (B) and IL-10 (C) were significantly increased in B cells. IRAK1 mRNA level was also increased (D). However, TRAF6 mRNA level was not changed (E). The experiments were performed in triplicate. n=6–8. Data are presented as mean±SD. Student's t-test. * $p < 0.05$ indicates significant difference compared with control group. Control: non-*P. gingivalis* LPS treatment.

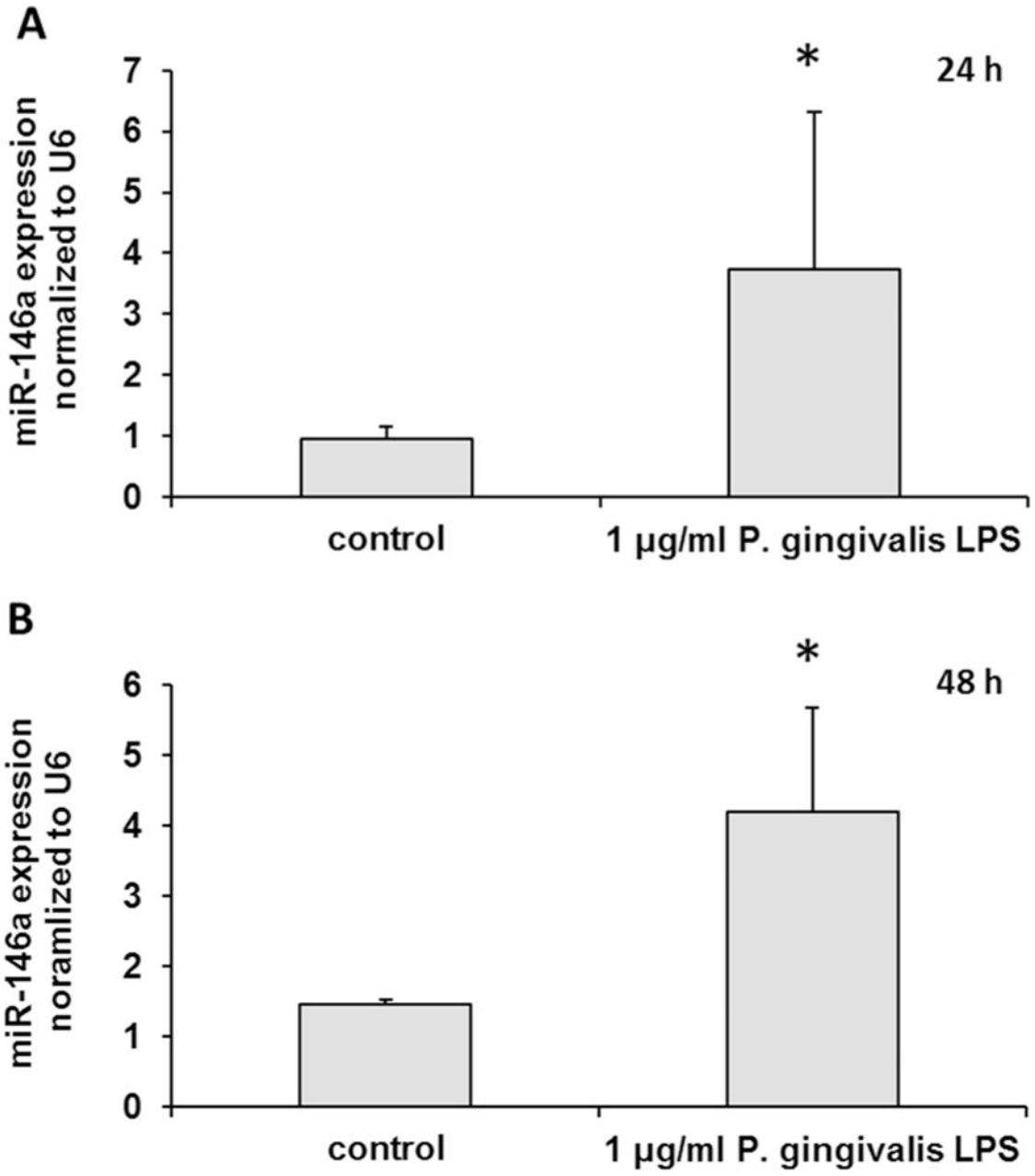

Fig. 2. The expression of miR-146a in B cells was induced by *P. gingivalis* LPS

The expression of miR-146a in B cells was detected by qRT-PCR after *P. gingivalis* LPS-stimulation. The miR-146a expression was increased at both 24 h and 48 h. The experiments were performed in triplicate. n=6–8. Data are presented as mean±SD. Student's t-test. * $p < 0.05$ indicates significant difference compared with control group. Control: non-*P. gingivalis* LPS treatment.

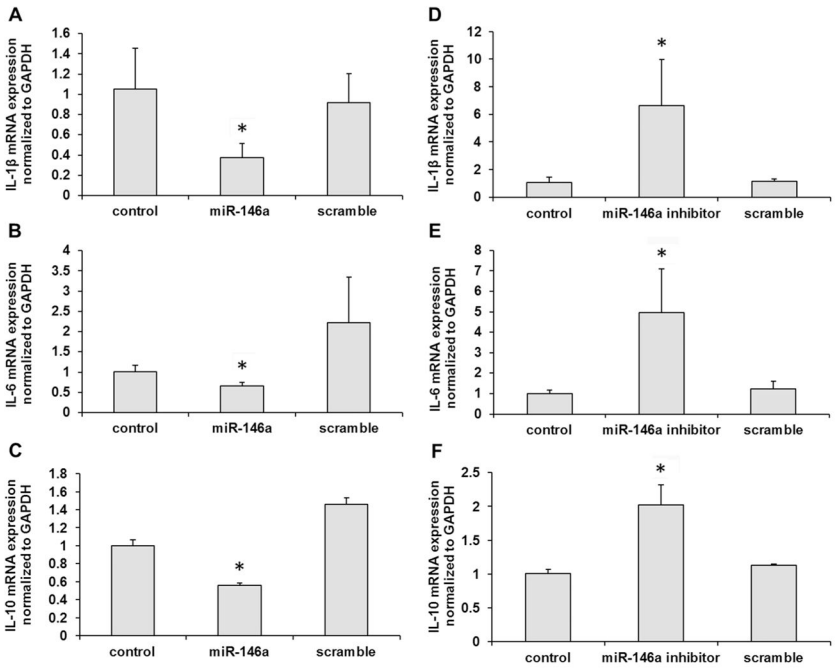

Fig. 3. The mRNA expressions of inflammatory cytokines were inhibited by miR-146a in B cells after challenged with *P. gingivalis* LPS

After overexpression of miR-146a in B cells, mRNA levels of IL-1β (A), IL-6 (B) and IL-10 (C) were significantly decreased. However, after blockade of miR-146a by its inhibitor, the mRNA levels of IL-1β (D), IL-6 (E) and IL-10 (F) were significantly increased. The experiments were performed in triplicate. n=6–8. Data are presented as mean±SD. One-way ANOVA. * $p < 0.05$. Control: no RNA transfection.

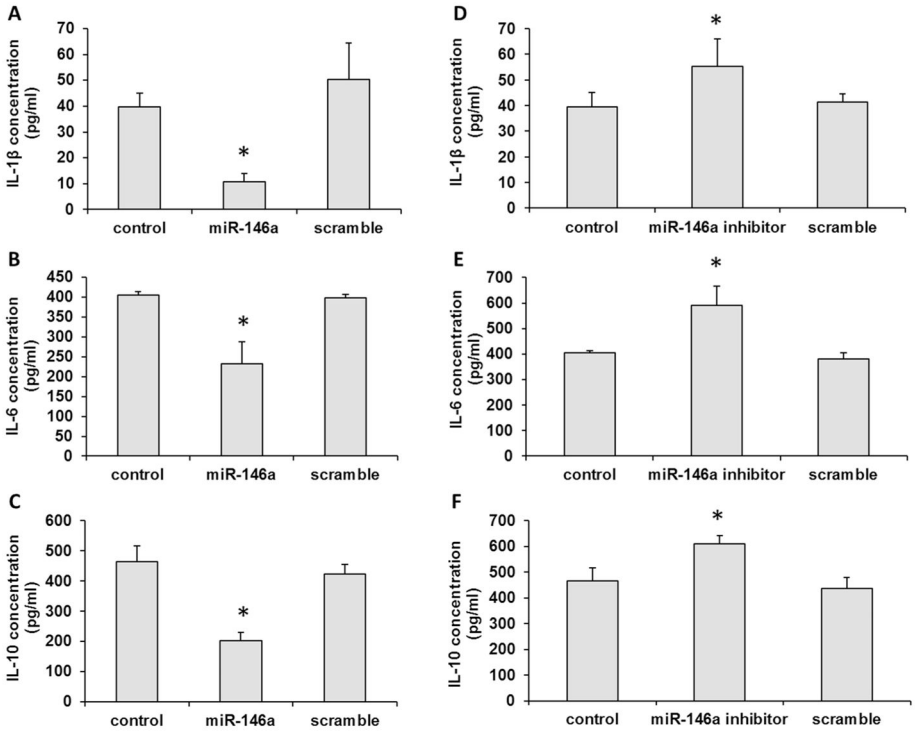

Fig. 4. miR-146a inhibited inflammatory cytokine secretion from in B cells after challenged with *P. gingivalis* LPS

The secretions of IL-1β (A), IL-6 (B) and IL-10 (C) decreased in presence of miR-146a mimic. The productions of IL-1β (D), IL-6 (E) and IL-10 (F) were enhanced by miR-146a inhibitor. The experiments were performed in triplicate. n=6–8. Data are presented as mean ±SD. One-way ANOVA. * $p < 0.05$. Control: no RNA transfection.

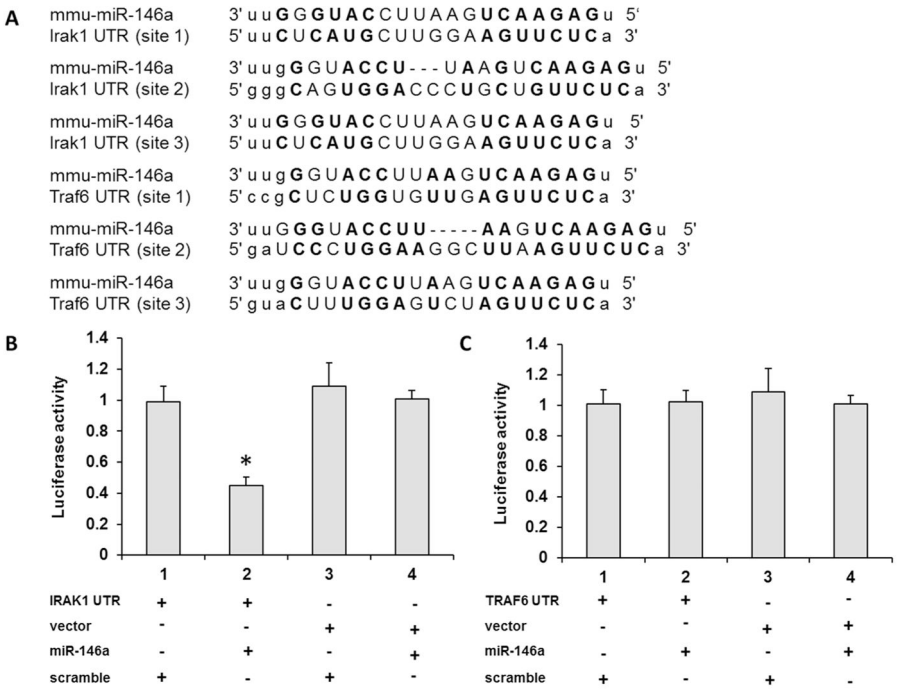

Fig. 5. miR-146a directly targeted IRAK1 through binding with its 3′-UTR

From miRNA target database, miR-146a has the binding sequence in 3′-UTR of IRAK1 and TRAF6 (A). In luciferase assay, miR-146a could decrease the luciferase activity of IRAK1 luciferase plasmid (B), however, not TRAF6 luciferase plasmid (C). The experiments were performed at least three times in triplicate. Data are presented as mean±SD. One-way ANOVA. * $p < 0.05$.

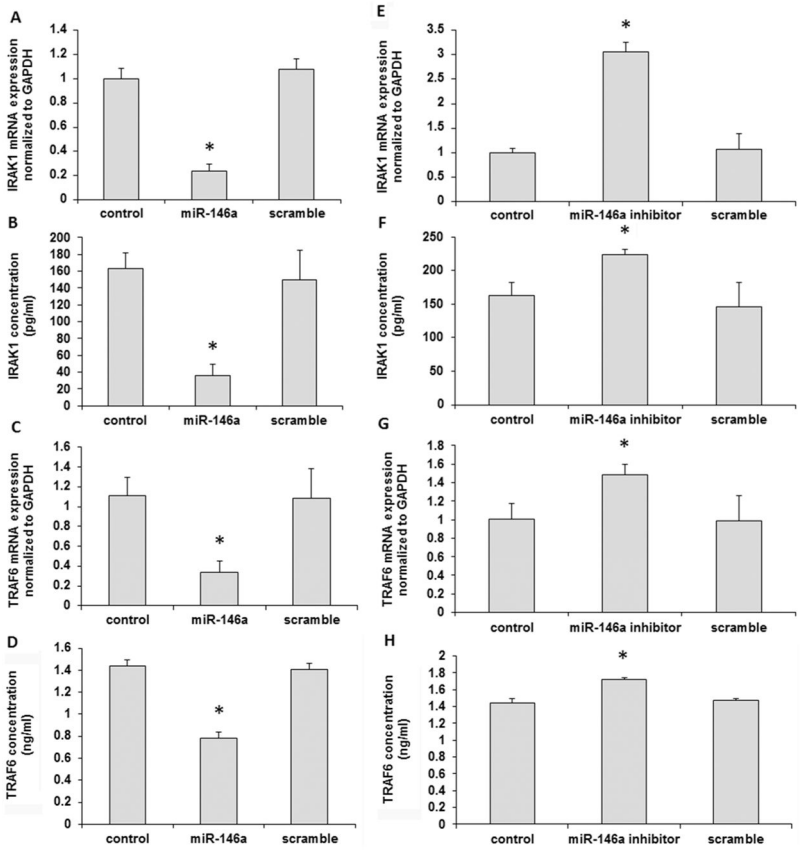

Fig. 6. IRAK1 expression was inhibited by miR-146a in B cells after challenged with *P. gingivalis* LPS

miR-146a mimic could reduce the mRNA and protein levels of IRAK1 as detected by PCR (A) and ELISA (B). Also, addition of miR-146a inhibited TRAF6 expression in B cells at both mRNA (C) and protein level (D). Inhibition of miR-146a by its inhibitor significantly elevated both mRNA and protein levels of IRAK1 expression in B cells (E and F), as well as mRNA (G) and protein (H) levels of TRAF6 expression in B cells. The experiments were performed in triplicate. n=6–8. Data are presented as mean±SD. One-way ANOVA. * $p < 0.05$. Control: no RNA transfection.

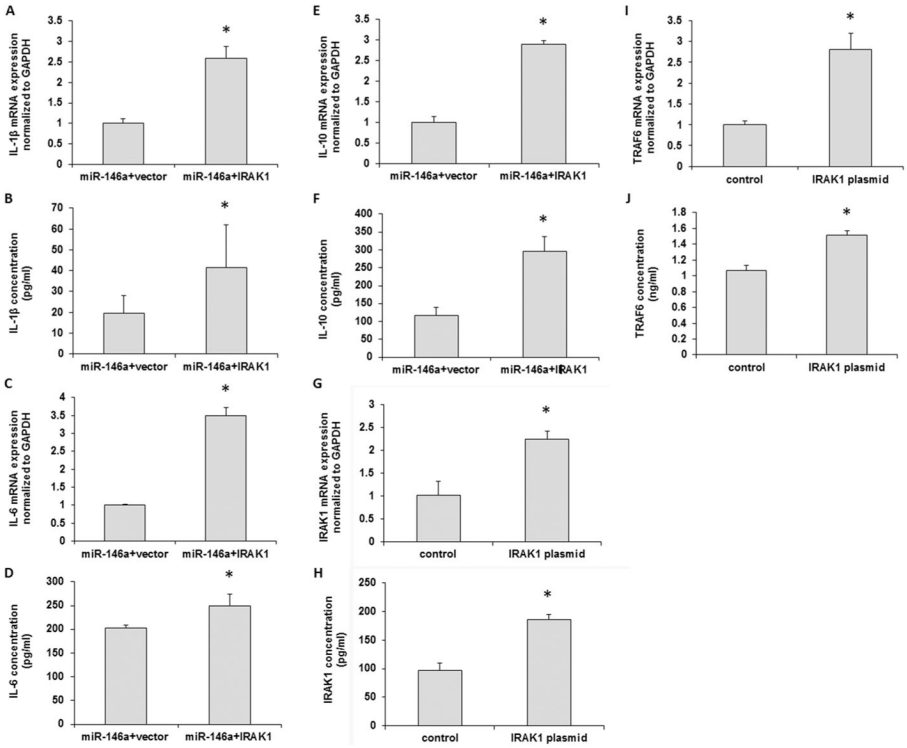

Fig. 7. Overexpression of IRAK1 reversed the inhibitory effect of miR-146a on cytokine production

After overexpression of IRAK1, the mRNA and protein levels of IL-1β (A, B), IL-6 (C, D) and IL-10 (E, F) were significantly increased in B cells pretreated with miR-146a mimic. As expected, after transfection with IRAK1 plasmid, IRAK1 mRNA and protein levels were significantly increased in B cells (G, H). In addition, mRNA and protein levels of TRAF6 expression were also elevated in B cells (I, J). The experiments were performed in triplicate. n=6–8. Data are presented as mean±SD. Student's t-test. $p < 0.05$ indicates significant difference compared with the cells transfected with miR-146a and/or blank vector. Control: cells transfected with blank vector.

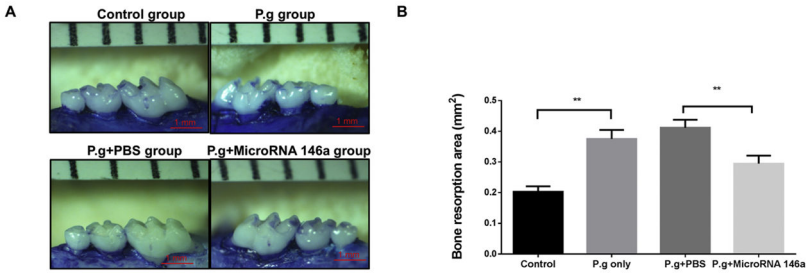

Fig. 8. Gingival injection of miR-146a reduced bone loss in *P. gingivalis* infection-induced experimental periodontitis

Mice were orally infected with live *P. gingivalis* bacteria for 4 consecutive days on day 0 to day 3. Gingival injection of miR-146a or PBS (as control) was performed on days 5, 9 and 14. Maxilla were collected on day 28. (A) The alveolar bone resorption areas around maxillary second molars were measured and (B) analyzed as bone resorption area/mm^2 at the magnification of 30X (means ± SD, n=6, $^{**}p<0.01$).