MicroRNAs (miRNAs) regulate the synthesis of cytokines in response to Toll-like receptor (TLR) activation. Our recent microarray study comparing normal and inflamed human dental pulps showed that miRNA-181 (miR-181) family is differentially expressed in the presence of inflammation. Prior studies have reported that the dental pulp, which is composed primarily of TLR4/2 + fibroblasts, expresses elevated levels of cytokines including interleukin-8 (IL-8) when inflamed. In this study, we employed an in-vitro model to determine the role of the miRNA-181 family in the TLR agonist-induced response in human fibroblasts. TLR4/2 + primary human dental pulp fibroblasts were stimulated with lipopolysaccharide from Porphyromonas gingivalis (PgLPS), a known oral pathogen, and IL-8 and miR-181 expression measured. An inversely proportional relationship between IL-8 and miR-181a was observed. In-silico analysis identified a miR-181a-binding site on the 3’ untranslated region (UTR) of IL-8, which was confirmed by dual-luciferase assays. MiR-181a directly binds to the 3’UTR of IL-8, an important inflammatory component of the immune response, and modulates its levels. This is the very first report demonstrating miR-181a regulation of IL-8.

INTRODUCTION

MicroRNAs (miRNAs) have emerged as important post-transcriptional regulators of gene expression in diverse biological processes, including inflammation, metabolism and healing. These key regulators of inflammation have been linked to homeostatic response to inflammatory stimuli by Toll-like receptor (TLR)-4 pathway activation and in various TLR-mediated immune responses to bacterial infection where miRNAs either suppressed inflammatory response or reduced inflammatory triggers.

Our recent microarray report showed differential expression of miRNAs in human dental pulp tissues that were clinically diagnosed to have pulpal inflammation (pulpitis). The dental pulp is the tissue located in the middle of the tooth that contains mostly connective tissues, nerves and blood vessels for protection, nourishment and innervation of the tooth. Pulpitis is a relatively common and painful dental disease that represents an immune response to bacterial infection. Pulpitis and its sequela, periapical periodontitis have been directly linked to the pathogenicity of the dental pulp. In our study that compared normal and inflamed human dental pulps, we reported the differential expression of 36 miRNAs. In another in-vivo study examining inflamed tissues surrounding the apices of teeth, 24 miRNAs were differentially expressed. The miR-181 family, which regulates a wide range of gene targets, was differentially expressed in both of these tissues. They have also been recently implicated in TLR-induced in-vivo increase in cytokine levels. However, there have been no reports as yet on the role of these miRNAs in regulating the immune response in human fibroblasts.

In this study, we utilized an in-vitro infection model to determine the role of miR-181 family in regulating immune response to bacteria using primary cultures of human pulpal fibroblasts. These cells express TLRs, a class of proteins that recognize conserved pathogen structures, triggers innate immune responses and primes antigen-specific adaptive immunity. TLR-4, for example, recognizes bacterial lipopolysaccharide (LPS) that stimulates pulp cells to produce cytokines and chemokines.

The miR-181 family has been shown to control inflammation under physiological and pathological conditions, which are essentially the outcome of its role in regulating various key aspects of growth, development and activation. MiR-181b, for example, targets importin-0 that consequently was reported to inhibit downstream canonical nuclear factor κB signaling pathway in endothelial cells, reduce vascular inflammation in vivo, decrease lung inflammation in endotoxemia mice and promote survival in LPS-induced septic mice. In addition, miR-181a regulates osteopontin-mediated vascular smooth muscle function that may be a novel therapeutic approach to modulate osteopontin that is found in abundance in atherosclerotic plaques.

These validated roles of the miR-181 family show its relevance in other highly vascular and immunocompetent tissues in humans like the dental pulp. Interleukin-8 (IL-8), like the miR-181 family, is also linked to TLR activation. IL-8 is a potent chemoattractant that is increased not only in major inflammatory conditions such as hepatitis, chronic obstructive pulmonary disease and periodontitis, but also in pulpitis and in in-vitro models of pulpal infection. Its essential involvement and causative role in acute inflammation by recruiting and activating neutrophils have been firmly established. Both IL-8 and miR-181 family have been associated with other inflammatory conditions and inflammatory responses of various cell types.

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ORIGINAL ARTICLE
MiRNA-181a regulates Toll-like receptor agonist-induced inflammatory response in human fibroblasts

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MicroRNAs (miRNAs) regulate the synthesis of cytokines in response to Toll-like receptor (TLR) activation. Our recent microarray study comparing normal and inflamed human dental pulps showed that miRNA-181 (miR-181) family is differentially expressed in the presence of inflammation. Prior studies have reported that the dental pulp, which is composed primarily of TLR4/2 + fibroblasts, expresses elevated levels of cytokines including interleukin-8 (IL-8) when inflamed. In this study, we employed an in-vitro model to determine the role of the miRNA-181 family in the TLR agonist-induced response in human fibroblasts. TLR4/2 + primary human dental pulp fibroblasts were stimulated with lipopolysaccharide from Porphyromonas gingivalis (PgLPS), a known oral pathogen, and IL-8 and miR-181 expression measured. An inversely proportional relationship between IL-8 and miR-181a was observed. In-silico analysis identified a miR-181a-binding site on the 3’ untranslated region (UTR) of IL-8, which was confirmed by dual-luciferase assays. MiR-181a directly binds to the 3’UTR of IL-8, an important inflammatory component of the immune response, and modulates its levels. This is the very first report demonstrating miR-181a regulation of IL-8.

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The purpose of this study is to determine whether oral pathogens modulate the expression of miR-181 family and to correlate this expression with the production of IL-8.

RESULTS

MiR-181-a, -b and IL-8 expressions are modulated by Pg LPS

To examine whether TLR activation affects the expression of miR-181 family in human dental pulp fibroblasts (HDPF), cells were challenged with varying concentrations of Pg LPS over 8 h. Figure 1a shows that miR-181a expression in fibroblasts is influenced by Pg LPS in a time- and dose-dependent manner. Compared with the 1-h time point, the expression of miR-181a decreased at 4 h. At the 8-h time point, the expression level either remained relatively similar (10 ng ml\(^{-1}\) LPS) or increased slightly (100 ng ml\(^{-1}\) and 1 μg ml\(^{-1}\) LPS). This result is not surprising as a time- and dose-dependent stimulation by Pg LPS has been shown in other cell types.\(^{28,29}\) MiR-181b expression was only noted in 1 μg ml\(^{-1}\) Pg LPS (Figure 1b), whereas miR-181c was not detected in this study. A concentration of 1 μg ml\(^{-1}\) Pg LPS was used in the subsequent experiments, as this dose had been widely used in other studies\(^{28,29}\) and had shown the most consistent result in the miR-181a and -b experiments, which were responsive only to 1 μg ml\(^{-1}\) Pg LPS.

Induction of pro-inflammatory cytokines is a key feature of TLR signaling. We monitored the levels of IL-1, IL-6, IL-8 and tumor necrosis factor-α in the supernatants of LPS-stimulated HDPF. Among the cytokines assayed in this study, only IL-8 was detectable in the cell culture supernatant (Figure 2). IL-1, IL-6 and tumor necrosis factor-α were all below minimum detectable dose. Interestingly, IL-8 was secreted in a time- and dose-dependent manner.

IL-8 and miR-181a and -b show an inversely proportional relationship

From the dose- and time-dependent data, we observed an inversely proportional relationship between miR-181a gene expression and IL-8 protein levels (Figure 3a). The same relationship was observed between IL-8 and miR-181b (Figure 3b). A decrease in the miR-181a expression corresponded with an increase in IL-8 levels in culture supernatants.

IL-8 is modulated by miR-181a

To investigate the possible mechanistic role of miR-181-mediated regulation of IL8, we scanned its 3′UTR for potential miR-181-binding sites. Bioinformatics analysis identified a novel miRNA-binding region spanning 346–368 nts of IL8 3′UTR (Figure 4a). Importantly, the seed sequence is conserved in all the four miRNAs of miR-181 family. To validate functional miRNA-target interactions, dual luciferase assays were performed. HEK293 cells transfected with miR-181a show reduced luciferase activity compared with scramble miRNA and miR-302a (Figure 4b). miR-302a also targets IL-8;\(^{31}\) however, in this study, it did not demonstrate a negative regulation of IL-8. This finding further confirms the potential of miR-181a in regulating IL-8. Increasing the miR-181a mimic concentration to 5 pmol had no further significant impact on the luciferase activity (Figure 4b). Thus, miR-181a appears to directly regulate IL8 levels by interacting with the 3′UTR.

DISCUSSION

MiR-181 is a critical miRNA that was largely thought to regulate lymphocyte development and homeostasis, among other important functions.\(^{32}\) However, recent studies have also revealed its role in immunoregulation.\(^{2,33}\) In this study, we have shown that miR-181 family is expressed in non-leukocytic cells and that IL-8 and miR-181a and -181-b expression is influenced by a TLR agonist in a time- and dose-dependent manner. Moreover, the inversely proportional relationship between miR-181a and IL-8 is likely due in some part, to miR-181a directly binding to the 3′UTR of IL-8. This relationship suggests a possible modulatory role of miR-181a and miR-181b on IL-8 expression.
The findings above reveal the important role of miRNA in the regulatory networks governing the response of various cell types to microbial insults. Fibroblasts are primarily structural in function but their ability to respond to infection as first or second line of defense (after epithelial cells) is critical in limiting the spread of infection and in wound healing.34 The presence of TLRs in fibroblasts confers immunocompetency on these cells,32 giving merit to the importance of studying how their response is regulated. As part of the initial responders to microbial entry, the capability of fibroblasts to recruit professional immune cells to the site of infection can be a double-edged sword that may halt or propagate the infectious process.35

The polymicrobial etiology of pulpitis and the clinical implications associated with it make this disease an ideal model of immunoregulation. As a more Gram negative, anaerobic bacterial species penetrate the pulpdentin interface, pulpal cells, predominantly fibroblasts recognize the conserved microbial patterns through TLRs.12 Consequently, various inflammatory mediators are expressed that initiate and enhance the inflammatory process. We mimicked this response by utilizing an in-vitro model using HDFP stimulated with Pg-LPS. Our results agree with previous studies on the immunocompetence of pulpal fibroblast.7,12,15,17 In this study, the detectable levels of IL-8 in Pg-LPS-stimulated cells may recruit circulating immune cells (for example, neutrophils) to the site of infection can be a double-edged sword that may halt or propagate the infectious process.35

Inflammation of the pulp and periapical tissues is commonly associated with pain, and approximately 90% of dental emergency visits with pain as the chief complaint are attributable to activation of pulpal or periapical nociceptors.38 The prevalence of periapical disease in the United States is estimated to be about 4.1%.39 Despite the prevalence of endodontic disease and the great discomfort associated with it, the fundamental molecular aspects of its pathogenesis are still not fully understood. The current literature on pulpal immune response to microbial infection continues to expand; however, very little is known on the regulatory mechanism behind pulpal disease.

A critical barrier to progress in treatment of pulpitis is the incomplete understanding of the regulatory network governing this disease. This study provides evidence that the dental pulp possesses immunocompetent cells with an active regulatory network capable of responding to microbial insults and modulating inflammatory response. Furthermore, the pathogenesis of pulpitis and its immunoregulation can serve as a model for other microbial diseases that provide less opportunity for ex-vivo or in-vitro studies because of their inaccessibility or critical location like the brain, lungs or eyes.

We confirmed the regulation of IL-8 by miR-181a, a miRNA that has been shown to be differentially expressed in inflamed tissues.5,19,26,27 MiRNAs post-transcriptionally regulate gene expression by targeting specific messenger RNAs for degradation or translational repression.40 Their cytoplasmic levels directly influence the protein bioavailability of their targeted genes. We have shown in this study that the level of IL-8 is inversely proportional with secreted IL-8 level (Figure 3). We confirmed the repression of IL-8 by miR-181a using Luciferase assay (Figure 4b) and in-silico alignment analysis (Figure 4a). This pattern of negative regulation by miRNAs has been shown in other studies.41

With the understanding that IL-8 can be directly regulated with a miRNA, a targeted therapy to control inflammation can be further investigated not only in dental pulp where treatment to pulpitis is currently limited to pulp extirpation but also in other organs or tissue systems as well. This treatment method is already in progress in other fields.42

**MATERIALS AND METHODS**

Dental pulp tissue collection and culture

The study was performed with the approval of and compliance to the guidelines set by the Institutional Regulatory Board of the UNC Office of Human Research Ethics. Dental pulp tissue from a clinically normal tooth of human origin was collected and cultured according to the guidelines set by the Institutional Regulatory Board of the UNC Office of Human Research Ethics. The study was performed with the approval of and compliance to the institutional regulatory board of the UNC Office of Human Research Ethics.
three healthy donors was collected immediately after extraction for orthodontic reason. The pulp tissue was obtained under informed consent.

Fibroblasts cultures were established using a previously published protocol by Adachi et al. In brief, minced pieces of pulp tissues were explanted into 35-mm culture dishes containing Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma, St Louis, MO, USA). 1×10^6 cells were then challenged with 1 μg/ml LPS (Porphyromonas gingivalis W83 LPS (Lipid A); Dr David A. Scott, University of Louisville, KY, USA) for 1, 4 and 8 h. Confluent primary cultures were harvested and stored at −80 °C until use.

Reverse transcription-quantitative PCR (RT-qPCR)

For RT-qPCR, RNA was isolated from cell lysates using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) and was reverse transcribed with High-Capacity cDNA Archive Kit (Applied Biosystems or ABI, Grand Island, NY, USA) according to the manufacturer’s instructions. cDNA was subjected to RT-qPCR using TaqMan inventoried primers and probe sets and the 7500 Fast Real-Time PCR System (both from ABI). Normalization was performed using RNU-6B primer (ABI). Relative quantification values were analyzed using Excel spreadsheet. The experiments were carried out in three independent experimental setups.

ELISA

IL-1β, IL-6, IL-8 and tumor necrosis factor-α were measured by ELISA using commercially available kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. The absorbance was read at 450 nm.

Luciferase assay

IL-8 3′UTR was amplified using forward (5′-GCACCTCGATGTTGGGTTCTCTGTTAGGG-3′) and reverse primers (5′-ATCGCCGTCGACGTTGAGTTTTGGCGTGTG-3′) and the pGL3-IL8 construct (kind gift of Dr Richard Pestell Jefferson Medical College, Philadelphia, PA, USA) as template.44 The amplified fragment (~950 bp) was digested by Xhol and NotI and cloned into psiCHEKTM2 vector (Promega, Madison, WI, USA). The transfection was performed as described previously.44 Briefly, actively growing Human Embryonic Kidney 293 cells (HEK cells) were seeded in a 96-well plate at a density of 2 × 10^3 cells per well in well complete Dulbecco’s modified Eagle’s medium. The next day, cells were co-transfected using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) with 80 ng of IL8 construct and different concentrations (2 and 5 pmol) of miR-181a mimics. To confirm the negative regulation of IL-8 by miR-181a, two controls were used: (1) scramble oligonucleotides (Qiagen), which are claimed to have no potential binding sites on human genes and (2) miR-302-4, which has potential binding with IL-8.31 Thirty-six hours after transfection, luciferase activities were measured by Lumat (Turner BioSystems, Madison, WI, USA). The fold change in luciferase activity was calculated as previously described.45

Statistical analysis

One-way analysis of variance with Tukey’s post-hoc analysis or t-test was employed where statistical difference is noted in the study using Graph Pad (GraphPad Inc; La Jolla, CA, USA). P-values < 0.05 were considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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