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MicroRNA Microarray-Based Identification of Involvement of miR-155 and miR-19a in Development of Oral Lichen Planus (OLP) by Modulating Th1/Th2 Balance via Targeting eNOS and Toll-Like Receptor 2 (TLR2)

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: A wide range of microRNAs (miRNAs) have been shown to play a significant role in disease regulation. The objective of this study was to explore the role of miR-155 and miR-19a in the regulation of oral lichen planus (OLP).

Material/Methods: Microarray assay, real-time PCR, Western blot assay, computational analysis, luciferase assay, ELISA, and immunohistochemistry analysis were carried out to investigate the role of miR-155 and miR-19a in OLP.

Results: According to microarray assay and real-time PCR results, the expression of miR-155 was most significantly decreased among the 16 candidate miRNAs in the OLP group, whereas the expression of miR-19a was most significantly increased. MiR-155 and miR-19a directly targeted endothelial nitric oxide synthase (eNOS) and TLR2, respectively, since only the cells co-transfected with miR-155/wild-type eNOS 3'UTR or cells co-transfected with miR-19a/wild-type TLR2 3'UTR exhibited decreased luciferase activity. In addition, the expression of TLR2 was highly upregulated in OLP, whereas the expression of eNOS was significantly downregulated. A negative correlation was found between miR-19a and TLR2 mRNA, with a coefficient value of –0.40. Similarly, a negative correlation was found between miR-155 and eNOS mRNA, with a coefficient value of –0.54. A lower level of NO, IL-4, IL-5, and IL-10 was observed in OLP, which was also accompanied by a higher level of TNF-α and IFN-γ. Finally, the upregulation in miR-155 directly decreased the expression of eNOS and further inhibited the production of NO. Downregulation of miR-19a directly increased the expression of TLR2. The inhibition of NO production and the enhancement in TLR2 expression synergistically increased the production of TNF-α and IFN-γ, while decreasing the levels of IL-4, IL-5, and IL-10.

Conclusions: In this study, the peripheral blood mononuclear cells (PBMCs) from subjects with or without OLP were collected and their gene expression profiles were compared. It was found that OLP changed the expression profile of miR-155 and miR-19a, which in turn directly affected the production of eNOS and TLR2, respectively. In addition, by synergistically inducing an imbalance between Th1 and Th2, the simultaneous deregulation of miR-155/eNOS and miR-19a/TLR2 was responsible for an elevated risk of OLP.

MeSH Keywords: Inflammation • MicroRNAs • Nitric Oxide • Nitric Oxide Synthase Type III • Toll-Like Receptor 2

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Background

Oral lichen planus (OLP) is a type of chronic T cell-induced inflammation in oral mucosa that occurs in 1–2% of adults [1] and is more common in women [2]. The manifestations of OLP include blisters, ulcers, erythema, and white plaque [3]. OLP mainly affects the gingival, tongue, and buccal mucosa, although other intraoral sites can also be affected [4]. Local reaction to OLP may be triggered by cytokines secreted from lymphocytes [5]. Although the cause of OLP remains unclear, it has been hypothesized that OLP is a type of T cell-induced autoimmune disorder, in which CD8+ T cells induce the apoptosis of oral epithelial cells [6]. Several types of cytokines, such as Th1 and Th2 cytokines that are generated by subgroups of CD4+ Th cells, can medicate the different immune reactions during OLP. Traditionally, IL-4/IL-5 and IL-2/IFN-γ are classified as Th2 and Th1 cytokines, respectively. However, as a growing number of different cytokines have been discovered and investigated, it is known now that certain cytokines, which are not necessarily generated by CD4+ T cells, can also enhance the production of either Th2 or Th1 cytokines [7]. For instance, some studies have classified IL-6 as a type of Th2-related cytokine, while other studies have classified IL-1, IL-8, and TNF-alpha as Th1-related cytokines [8]. In a previous study investigating the simultaneous production of Th2 and Th1 cytokines, Simark-Mattsson et al. demonstrated that the cells producing TNF-α, TGF-beta 1, IL-2, IL-4, and IL-10 were present in all biopsy samples. They also confirmed the presence of IL-10 and IFN-γ messages in the culture of T cells collected from each subject [9]. Previous studies have analyzed data from 57 articles involving 19 676 patients, and calculated the malignant potential of OLP using a pooled proportion. They revealed that the overall PP was 1.1%, and suggested that a higher risk of malignant transformation OLP was observed in alcoholics, smokers, and HCV-infected patients in comparison with the subjects without these risk factors [10].

It has been shown that the activation of DCs by TLR2 and TLR4 can lead to generation of various cytokines, which eventually trigger different Th1 and Th2 reactions, respectively [11]. Some researchers suggested that during the afferent phase, treatment with a TLR2 agonist can aggravate the airway inflammation, whereas others believed that the treatment with a TLR2/4 or TLR2 agonist reduces airway inflammation [12–14]. However, a recent report showed that treatment with a TLR2 agonist during the afferent phase reduced allergic inflammation in the airway [15]. Nevertheless, all these studies have attributed the actions of TLR2 or TLR2/4 agonists to their regulation of the Th1/Th2 balance [16].

As a family of small endogenous (about 22 nt in length) noncoding RNAs, microRNAs (miRs or miRNAs) can bind to their target mRNA transcripts at the 3' untranslated region (3'UTR), thus inhibiting target gene expression at the translational level or leading to target mRNA degradation [17]. Currently, about 1000 human miRNAs have been identified and are believed to regulate the expression of as much as 30% of human genes. Therefore, miRNAs are implicated in the modulation of many biological functions, such as cell growth, differentiation, and apoptosis. In an miRNA microarray analysis performed with OLP patients and healthy subjects, the expression profiles of about 70 miRNAs demonstrated substantial changes (more than a 2-fold difference) [18]. In a follow-up study, the expression of 9 miRNAs (miR-21, 26b, 121, 137, 146a, 155, 203, 375, and 4484) showed significant changes in OLP patients [19].

Endothelial nitric oxide synthase (eNOS) and its catalytic product, NO, have been shown to suppress inflammatory reactions by maintaining a balance between Th1 and Th2 [20]. On the other hand, TLR2 has been found to promote inflammatory reactions by inducing Th1/Th2 imbalance [21,22]. In addition, it is believed that Th1/Th2 imbalance participates in the pathogenesis of OLP [23]. In the present study, the peripheral blood mononuclear cells (PBMCs) from subjects with or without OLP were collected and used to perform an miRNA microarray analysis. Subsequently, the expression of candidate miRNAs was measured and compared between the 2 groups. The role of the most significantly upregulated miRNA, miR-155, and the most significantly downregulated miRNA, miR-19a, as well as their target genes, was further investigated in cultured monocytes and PBMCs.

Material and Methods

Subjects

The study was conducted according to the Declaration of Helsinki. We enrolled 22 patients clinically diagnosed with OLP and 19 people free of any health problems. Data regarding age, sex, clinical classification (erosive, reticular, and/or plaque-like, atrophic), and lesion site (buccal mucosa, buccal mucosa and gingiva) were collected prior to the study. We excluded patients with any systemic diseases, gingival inflammation or suspected restoration-related reactions, and who have received any systemic or topical medications within the 3 months prior to the initiation of the research. All patients provided signed informed consent for participating in the study. In addition, this study was approved by the Ethics and Research Committees of China Medical University.

Isolation and culturing of PBMCs

Peripheral blood samples were stored at –80°C before a Ficoll-Paque (Sigma-Aldrich, St Louis, MO) PLUS centrifuge was utilized to isolate PBMCs from 10 mL of peripheral blood samples.

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Following the centrifugation, PBMCs were collected from the interphase layer and washed 4 times in DMEM (Dulbecco's modified Eagle's medium) (GIBCO, Carlsbad, CA). Subsequently, the PBMCs were cultured in a 37°C humidified atmosphere of 5% CO_2 in DMEM (Dulbecco's modified Eagle's medium) (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Inc., Grand Island, NY), 100 mg/mL streptomycin sulfate, and 100 U/mL penicillin sodium. The growth medium was changed once every 2 days until the cells were cloned, which were then transferred into a new dish and continuously cultured until the confluence reached 80%. Subsequently, the cells were passaged again and the cells from the second passage were used in the following experiments.

MicroRNA microarray

The microarray expression profiling assay was performed using 5 μg of total RNA extracted from PBMCs. During the assay, a MiRCURYTM Array Labeling kit (Exiqon, Vedbaek, Denmark) was utilized to fluorescently label the target miRNAs (miR-223-3p, miR-186, miR-423, miR-181a, miR-155, miR-375, miR-133a, miR-497, miR-92, miR-1469, miR-564, miR-1304, miR-296, miR-346, miR-19a, and miR-122), which were then hybridized on an miRNA microarray chip (Exiqon, Vedbaek, Denmark). The hybridization data were collected using a GenePix 4000B laser scanner (Axon Instruments, Foster City, CA), and the GenePix 4.0 software (Axon Instruments, Foster City, CA) was used to digitize and analyze the images. Three independent tests were performed.

RNA isolation and real-time PCR

TRizol™ reagent (Invitrogen, Burlington, ON, Canada) was used to extract the total RNA from THP-1 cells and tissue samples following a standard protocol. Subsequently, chloroform was added into the lysate and the mixture was centrifuged at 4°C and 13 000× g for 15 min. In the next step, the pellet was treated by isopropanol and washed by ethanol before being resuspended in RNAse-free water. The RNA concentration was determined by UV spectrophotometry. A reverse transcription kit (Applied Biosystems Inc, USA) was utilized to synthesize the cDNAs of eNOS and TLR2 from 2 μg of total RNA, and a housekeeping gene, 18SRNA, served as the internal control. The expression of miR-155/miR-19a and eNOS/TLR2A was measured using SYBR green dye on a light cycler (Roche Diagnostics, PQ). The relative mRNA expression of miR-155/miR-19a and eNOS/TLR2 was determined by calculating the values of cycle threshold (Ct), and U6 was utilized as the internal control in such calculations. Relative expressions of miR-223-3p, miR-186, miR-423, miR-181a, miR-155, miR-375, miR-133a, miR-497, miR-92, miR-1469, miR-564, miR-1304, miR-296, miR-346, miR-19a, and miR-122 were also compared between the 2 groups. Three independent reactions were run for each target miRNA.

Cell culture and transfection

THP-1 cells were obtained from the Chinese Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in a 37°C humidified atmosphere of 5% CO_2, in DMEM (Dulbecco's modified Eagle's medium) (GIBCO, Carlsbad, CA) supplemented with 10% FBS (Life Technologies, Inc., Grand Island, NY), 100 mg/mL streptomycin sulfate, and 100 U/mL penicillin sodium. The cells were then activated with LPS. When the cells reached 80% confluence, Lipofectamine 2000 (Invitrogen, CA was used to transfect miR-155 mimics/inhibitors and miR-19a mimics/inhibitors into LPS-treated THP-1 cells. Three independent experiments were carried out.

Cell proliferation assay

A total of 4×10^3 THP-1 cells were seeded into each well of a 24-well plate in 100 μL of medium supplemented with 10% FCS. Subsequently, 10 μg/well of a proliferation reagent, CCK-8 (Yeasen, Shanghai, China), was added and the plate was incubated at 37°C for 4 h in 5% CO_2. A Spectra Max M5 (Molecular Devices, MD, US) instrument was utilized to measure the absorbance of the samples. Three independent measurements were carried out.

Luciferase assay

According to the instructions provided by the supplier, a High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) was utilized to perform RT-PCR (reverse transcription) and to synthesize eNOS cDNA containing the miR-155 binding sites. A similar approach was used to synthesize TLR2 cDNA containing the miR-19a binding site. In addition, the 3'-UTR of the target genes (eNOS, TLR2) and a fragment of eNOS/TLR2-3'-UTR mutant (control) were inserted into pmiR-REPORT vectors (RiboBio, Guangzhou, China) to develop corresponding luciferase reporter constructs. Lipofectamine 2000 (Invitrogen, CA) was used to co-transfect the PTENCE cells with the WT-eNOS/TLR2 3'-UTR vector or mut-eNOS/TLR2 3'-UTR vector, in conjunction with the control (Ribobio, Guangzhou, China) or miR-155/miR-19a mimics. At 48 h after transfection, the luciferase activity of transfected cells was measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The activity of Renilla luciferase was used as the endogenous control. Each assay was run in triplicates.

Western blot analysis

In order to analyze the protein expression of eNOS and TLR2, cell lysates were prepared in an ice-cold lysis buffer (pH 7.4) containing 1% NP-40, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl, 150 mM NaCl, and protease inhibitors (Roche, Indianapolis, IN). The total protein was separated by 10–15% w/v sodium

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dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently electro-transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). In the next step, the membrane was blocked for 60 min in phosphate-buffered saline (PBS) containing 5% nonfat dry milk, followed by incubation at 4°C for 12 h in PBS containing polyclonal primary antibodies against β-actin (1: 10 000 dilution, Santa Cruz, CA), eNOS or TLR2 (1: 5000 dilution, Santa Cruz, CA). Subsequently, the membrane was washed in PBS twice and incubated at room temperature for 2 h with secondary antibodies conjugated to HRP (horseradish peroxidase) (1: 12 000 dilution, Santa Cruz, CA). A chemiluminescent kit (Pierce, Waltham, MA) was used to visualize the antigen-antibody complexes, with the protein level of eNOS and TLR2 quantified by Quantity One software (Bio-Rad Life Science, Shanghai, China). All experiments were repeated at least 3 times.

ELISA

A radioimmunoprecipitation assay buffer was used to isolate the total protein from THP-1 cells and tissue samples, and a BCA protein assay kit (Pierce, Rockford, IL) was used to determine the concentration of proteins. The levels of NO, IL-4, IL-5, IL-10, TNF-α, and IFN-γ were measured at 450 nm using an ELISA kit (R&D Systems, Minneapolis, MN). Three independent measurements were performed.

Immunohistochemistry assay

The tissue samples were cut into 4-μm^3 sections and blocked by a blocking reagent (Protein Block Serum-Free, Dako Cytomation, Glostrup, Denmark) in an autoclave and underwent non-specific reaction at 100°C for 20 min. Subsequently, the sections were incubated at 4°C for 12 h with rabbit anti-eNOS or anti-TLR2 monoclonal antibodies (1: 800 dilution, Abcam, Cambridge, MA), followed by another 30 min of incubation at room temperature with HRP (horseradish peroxidase)-conjugated secondary antibodies (1: 1000 dilution, Histofine, Simple stain MAX-PO; Nichirei, Tokyo, Japan). After counter-staining the cytoplasm using hematoxylin, the Dako REAL™ EnVision™ Detection System (Dako, Glostrup, Denmark) was used to visualize the immune-complexes. All tests were repeated 3 times.

Statistical analysis

All data are presented as mean ± SD. SAS software version 6.1 (SAS institute, Cary, NC) was utilized to carry out all statistical analyses. The differences between treated and untreated groups were analyzed using one-tailed Williams' tests, or one-tailed Shirley-Williams' tests with Holm's correction. Two-way analysis of variance (ANOVA) was used to evaluate the effect of miR-155/miR-19a on the expression of eNOS/TLR2. A *P* value of less than 0.05 was considered statistically significant.

Table 1. Demographic and clinicopathological characteristics of the recruited subjects.

Characteristic	OLP (n=22)	Control (n=19)	*P* value
Age (years)			
Mean ±SD	45.5 ± 15.8	43.7 ± 10.7	0.814
Range	18–79	20–69	
Gender			
Female/Male	14/8	12/7	0.672
Clinical classification			
Erosive	12	–	
Reticular	10	–	
Location			
Cheek	13	–	
Tongue	8	–	
Gingiva	1	–	

Results

Characteristics of the participants

A total of 41 subjects were enrolled in this study, including 22 OLP patients and 19 healthy subjects. The demographic and clinicopathological features of the participants, such as age, sex, clinical classification (erosive, atrophic), and lesion location (cheek, tongue, and gingiva), were recorded and listed in Table 1. Unpaired *t* tests were used to compare the subjects from the 2 groups, and no difference was observed between them with respect to age and sex.

Different microarray expression profiles of miRNAs in the 2 groups

To identify whether miRNAs were potentially involved in the development of OLP, a microarray study was conducted to compare the miRNA expression profiles between the 2 groups. As the result, 16 miRNAs (miR-223-3p, miR-186, miR-423, miR-181a, miR-155, miR-375, miR-133a, miR-497, miR-92, miR-1469, miR-564, miR-1304, miR-296, miR-346, miR-19a, and miR-122) were identified as potential candidates for subsequent functional analysis. In addition, real-time PCR was performed to confirm the microarray results. As shown in Figure 1, the expression of miR-155 was most significantly downregulated in the OLP group, whereas the expression of miR-19a was most significantly upregulated.

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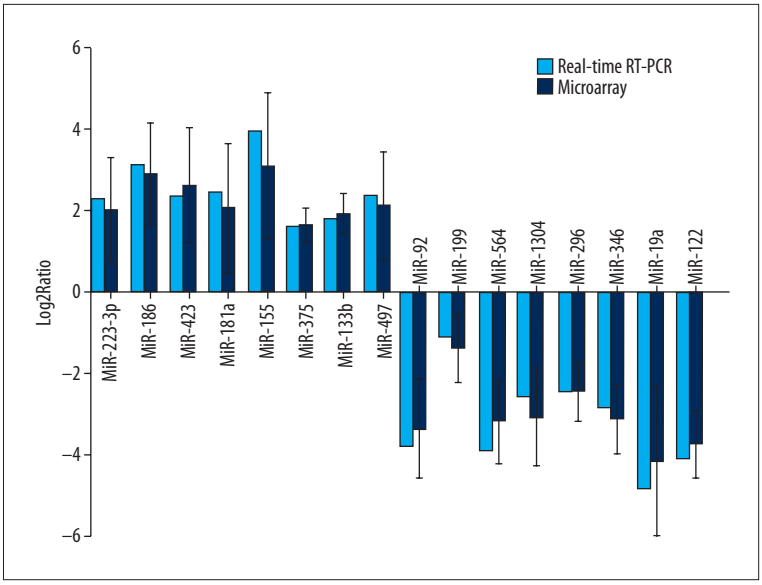

Figure 1. Microarray assay and real-time PCR were used to investigate miRNAs involved in OLP, and we found that miR-155 was most downregulated and miR-19a was most upregulated in the OLP group.

miR-155 and miR-19a directly targeted the genes of eNOS and TLR2, respectively

Two computational tools, DIANA-microT and TargetScan, were utilized to identify the target genes of miR-155 and miR-19a. As shown in Figure 2, miR-155 (Figure 2C) and miR-19a (Figure 2E) can bind to the 3'UTR of eNOS and TLR2 mRNA, respectively, suggesting that eNOS and TLR2 act as the molecular targets of miR-155 and miR-19a. To confirm whether the predicted binding sites of miR-155 and miR-19a were functional, a luciferase assay was performed. In the THP-1 cells co-transfected with miR-155 and the constructs containing wild-type eNOS 3'UTR (Figure 2D), as well as in the cells co-transfected with miR-19a and the constructs containing wild-type TLR2 3'UTR (Figure 2F), a lower luciferase activity was observed as compared to that in the control, suggesting that eNOS and TLR2 were direct target genes of miR-155 and miR-19a, respectively.

The relationship between miR-155 and eNOS, and the relationship between miR-19a and TLR2

As shown in Figure 3, the mRNA level of TLR2 (Figure 3A) and eNOS (Figure 3B) in the OLP group was much higher than that in the controls. Therefore, real-time PCR was carried out to study the correlation between miR-155 and eNOS, as well as the correlation between miR-19a and TLR2. We found that the mRNA level of TLR2 (Figure 3C) was negatively correlated with the level of miR-19a, with a coefficient value of –0.40. Similarly, the mRNA level of eNOS (Figure 3D) was negatively correlated with the level of miR-155, with a coefficient value of 0.54.

Different levels of TLR2 and eNOS proteins in the 2 groups

Immunohistochemistry assays were performed to measure the protein expression of eNOS and TLR2. As shown in Figure 4, the protein level of eNOS (Figure 4A, 4B) in the OLP group was much lower than that in the control group, whereas the protein level of TLR2 (Figure 4C, 4D) in the OLP group was much higher than that in the control group.

Different levels of inflammatory cytokines in the 2 groups

It is well known that inflammation is functionally involved in the pathogenesis of OLP. Therefore, in this study, ELISA assays were performed to measure the level of inflammatory cytokines, such as NO, IL-4, IL-5, IL-10, TNF-α and IFN-γ, in the collected samples. As shown in Figure 5, the levels of NO (Figure 5A), IL-4 (Figure 5B), IL-5 (Figure 5C), and IL-10 (Figure 5D) were all significantly decreased in the OLP group, whereas the levels of TNF-α (Figure 5E) and IFN-γ (Figure 5F) were significantly increased in the OLP group.

Effect of modified expression of miR-155 and miR-19a on production of inflammatory cytokines in cultured monocytes

THP-1 cells were stimulated by LPS before being transfected with a miR-155 mimic/inhibitor, a miR-19a mimic/inhibitor, or a scramble control. Initially, the cells were transfected with the miR-155 mimics alone, the miR-19a inhibitors alone, or both constructs, and the treatment by the miR-19a inhibitors alone showed no significant effect on the level of NO. In contrast, treatment by the miR-155 mimics alone or co-transfection with both miR-19a inhibitors and miR-155 mimics significantly reduced the production of NO (Figure 6A). In addition,

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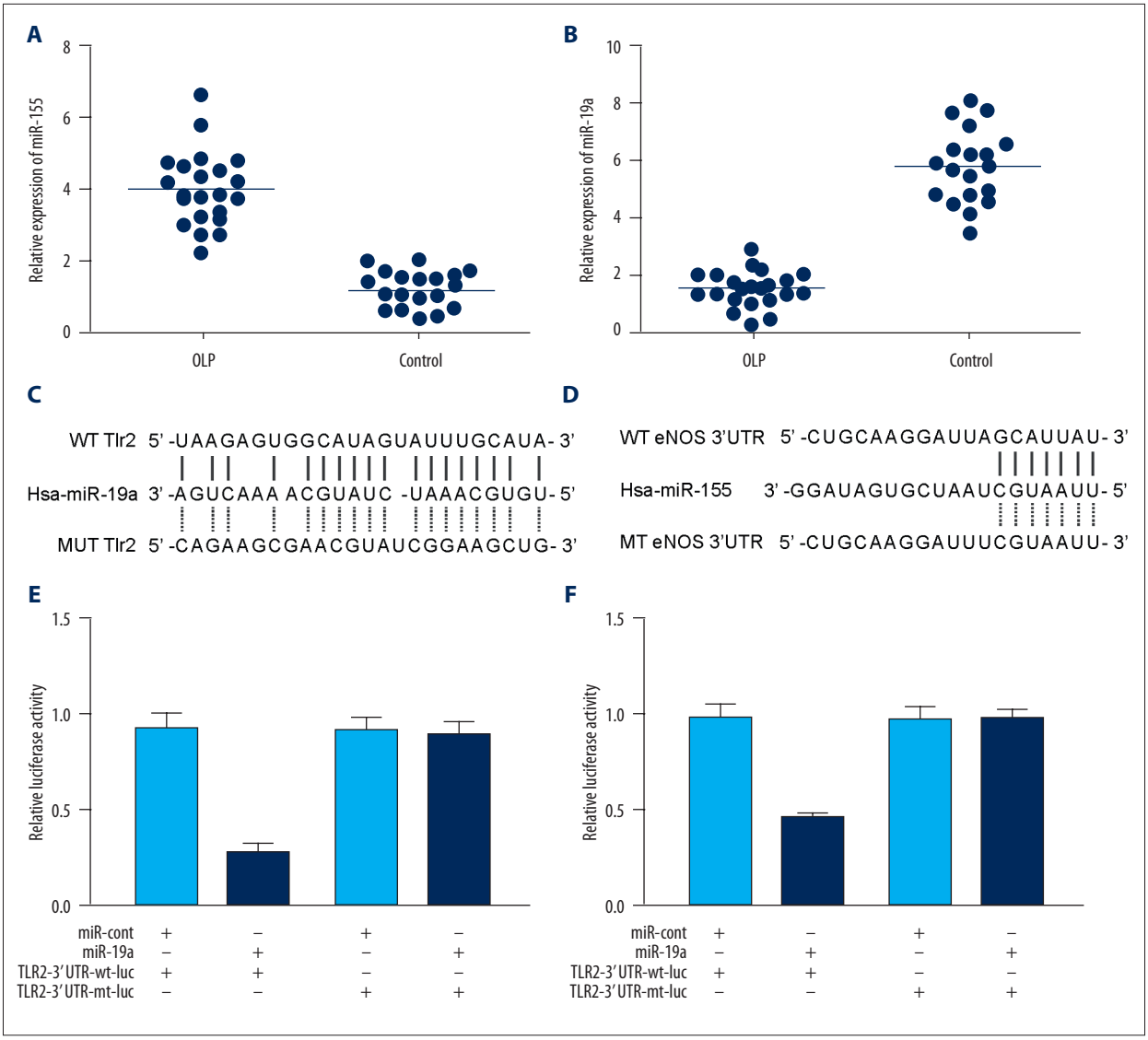

Figure 2. MiR-155 and miR-19a directly targeted eNOS and TLR2, respectively. (**A**) MiR-155 had low expression in the OLP group. (**B**) MiR-19a was highly expressed in the OLP group. (**C**) Schematic comparison of the "seed sequence" in 3' UTR of eNOS and miR-155. (**D**) MiR-155 clearly inhibited luciferase activity of wild-type eNOS 3'UTR but not that of mutant eNOS 3'UTR. (**E**) Schematic comparison of the "seed sequence" in 3' UTR of TLR2 and miR-19a. (**F**) MiR-19a clearly inhibited luciferase activity of wild-type TLR2 3'UTR but not that of mutant TLR2 3'UTR.

the transfection of miR-155 mimics or miR-19a inhibitors alone reduced the production of IL-4 (Figure 6B), IL-5 (Figure 6C), and IL-10 (Figure 6D), while the presence of both constructs at the same time synergistically reduced the production of these cytokines to an even lower level. Transfection of miR-155 mimics alone or miR-19a inhibitors alone enhanced the production of TNF-α (Figure 6E) and IFN-γ (Figure 6F), while the presence of both constructs at the same time synergistically increased the production of these cytokines to an even higher level. Subsequently, we found that the transfection of miR-19a inhibitors alone, with or without the presence of miR-155 mimics, substantially enhanced the expression of TLR2, whereas the transfection of miR-155 mimics alone showed little effect on the expression of TLR2 (Figure 6G). On the other hand, the transfection of miR-155 mimics alone, with or without the presence of miR-19a inhibitors, reduced the expression of eNOS, whereas the transfection of miR-19a inhibitors alone showed little effect on the expression of eNOS (Figure 6H).

In the next step, the cells were transfected with miR-155 inhibitors alone, miR-19a mimics alone, or both constructs, and the treatment by miR-19a mimics alone showed no significant effect on the level of NO. However, treatment by miR-155 inhibitors alone or the co-transfection with both miR-19a mimics

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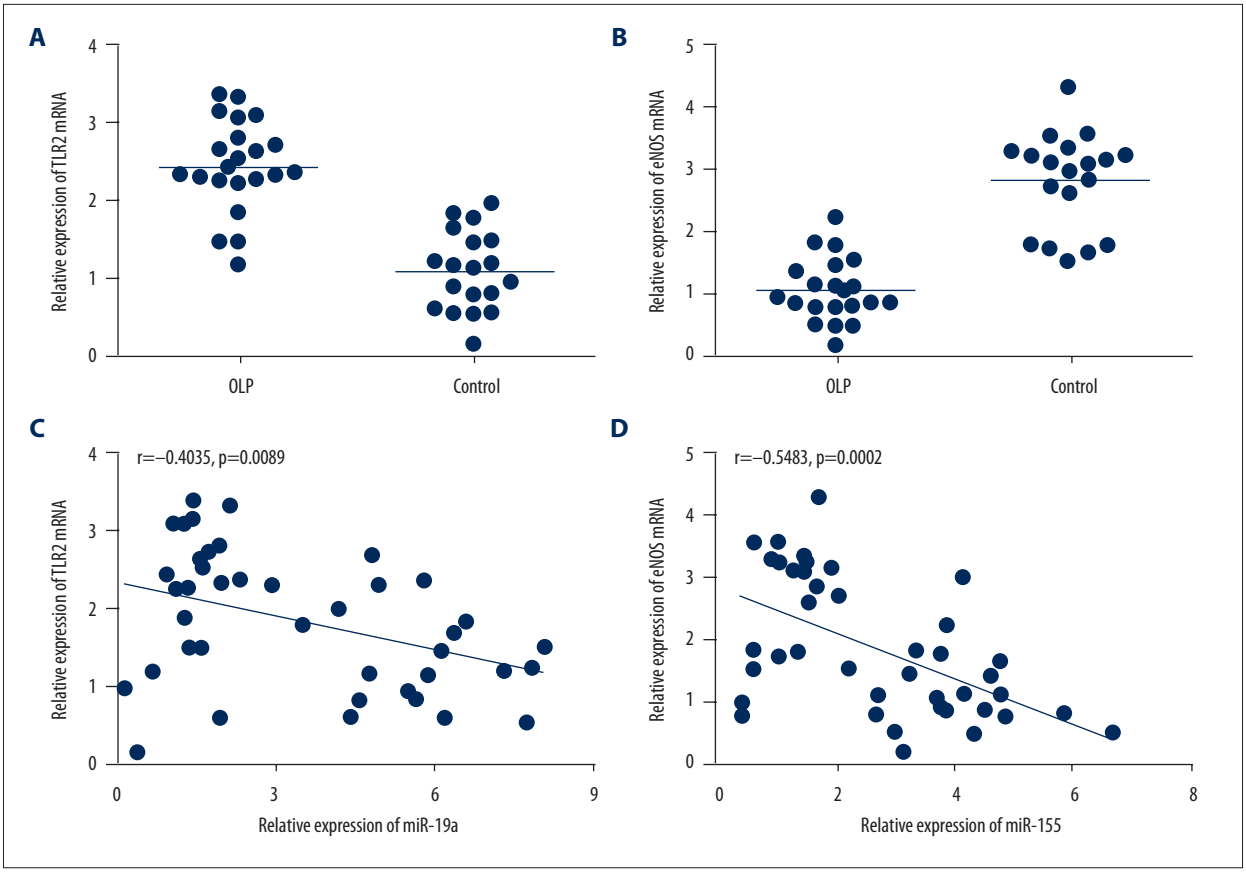

Figure 3. (**A**) TLR2 mRNA level in the OLP group was much higher. (**B**) eNOS mRNA level in the OLP group was much lower. (**C**) The negative correlation between miR-19a and TLR2 was confirmed. (**D**) The regulatory relationship between miR-155 and eNOS was negative.

and miR-155 inhibitors significantly promoted the production of NO (Figure 7A). In addition, the transfection of miR-155 inhibitors or miR-19a mimics alone increased the production of IL-4 (Figure 7B), IL-5 (Figure 7C), and IL-10 (Figure 7D), while the presence of both constructs at the same time synergistically increased the production of these cytokines to an even higher level. Transfection of miR-155 inhibitors or miR-19a mimics alone reduced the production of TNF-α (Figure 7E) and IFN-γ (Figure 7F), while the presence of both constructs at the same time synergistically reduced the production of these cytokines to an even lower level. Subsequently, we found that the transfection of miR-19a mimics alone, with or without the presence miR-155 inhibitors, substantially inhibited the expression of TLR2, whereas the transfection of miR-155 inhibitors alone showed little effect on the expression of TLR2 (Figure 7G). On the other hand, the transfection of miR-155 inhibitors alone, with or without the presence of miR-19a mimics, enhanced the expression of eNOS, whereas the transfection of miR-19a mimics alone showed little effect on the expression of eNOS (Figure 7H).

Discussion

As a type of T cell-induced inflammation in oral mucosa [24], OLP is characterized by a range of immune reactions that are mediated by different cytokines, such as Th1 and Th2 cytokines. For example, IFN-γ is involved in the constitutive production of major histocompatibility class II molecules, as well as the activation and maturation of CD8+ T cells, thus leading to the apoptosis of keratinocytes and the chronic behavior of OLP [25]. On the other hand, IL-4 is required for the differentiation of Th2 cells and hence plays a critical role in the modulation of both humoral immune responses and antibody productions [26]. In addition, due to its immunological effect, the balance of Th1/Th2 cytokines plays an important role in the pathogenesis and progression of OLP [24].

Immune cells are conventionally divided into 2 distinct subgroups (Th2 and Th1) based on their expression of cytokines [23]. The cells in the Th2 subgroup express IL-4, IL-5, IL-10, and IL-13, and are crucial for antibody production and the induction of humoral immune responses [27]. Cells in the Th1 subgroup express tumor necrosis factor-alpha (TNF-α),

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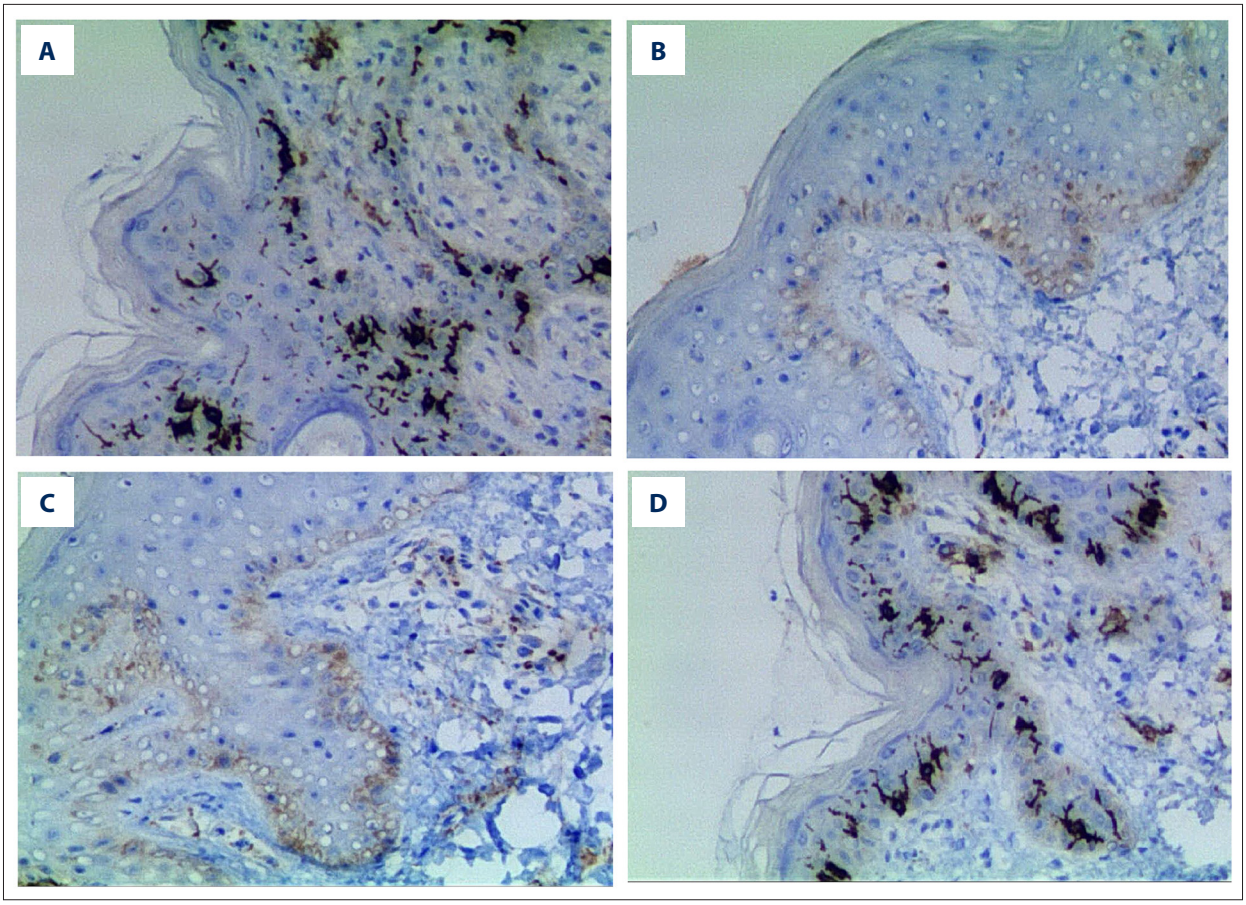

Figure 4. TLR2 and eNOS protein levels differed in various groups. (**A, B**) eNOS protein level in OLP group was much lower. (**C, D**) TLR2 protein level in OLP group was much higher.

interleukin-2 (IL-2), and interferon-gamma (IFN-γ), all of which are powerful mediators in the stimulation of cytotoxic T lymphocytes (CTL) and macrophages, and hence can induce immune reactions in local cells. In addition, IL-4 and IFN-y are regarded as typical cytokines generated by Th1 and Th2 cells, respectively [28]. Recently, a study has indicated that ILW, IFN-y, IL-9, IL-17, and IL-22 are all involved in systemic and oral infections [29].

In the present study, 22 OLP patients and 19 healthy controls were studied to identify the miRNAs differentially expressed in OLP. Using both a microarray assay and real-time PCR, we found that the expression of miR-155 was most significantly downregulated in the OLP group, whereas the expression of miR-19a was most significantly upregulated. A previous report on miR-155 investigated its effect in endothelial cells (ECs) and identified the gene of eNOS as a target of miR-155. By binding to the 3'UTR of eNOS mRNA, miR-155 can reduce the stability of eNOS mRNA and inhibit its expression. In addition, the deletion of miR-155 can impair the cytokine-medicated reduction in eNOS expression, thus decreasing the generation of NO [30,31]. As a critical activator for many progrowth signaling cascades in cancer [32], NO can induce both cGMP-independent and cGMP-dependent Th1-Th2 immune shift, and therefore plays a critical role in triggering tumor reactions upon radiation-induced injury [33]. For instance, post-IR and NOS-suppressed tumors showed an increased level of IL-12p40 Th1, IFN-γ, and IL-2. More importantly, as a type of receptor-mediated cytokine, IL-2 can interact with IL-12Rβ2 to increase the level of IL-12 and IFN-γ during the differentiation of Th1 cells [34]. On the other hand, tumors that underwent irradiation alone showed an elevated level of IL-10, IL-3, IL-4, and IL-5 as the result of Th2 induction. Intriguingly, the level of IL-2 was also increased in irradiated tumors, and hence might be associated with an increased level of IL-4 and IL-5 during Th2 cell differentiation, which in turn is dependent on IL-4Rα [34]. It has been shown that miR-155 was heavily involved in the immune mechanisms induced by CD4+ T cells. For example, miR-155 promotes Th1 differentiation of active CD4+ T cells, whereas the deletion of bic gene disrupts the Th1/Th2 balance in CD4+ T cells [35]. The stimulation of cells by PGN on gram-positive bacteria is mainly mediated by TLR2, which also leads to the stimulation of transcription factor NF-κB and modulates the transcription of different cytokines implicated in immune responses. These cytokines provide a polarizing

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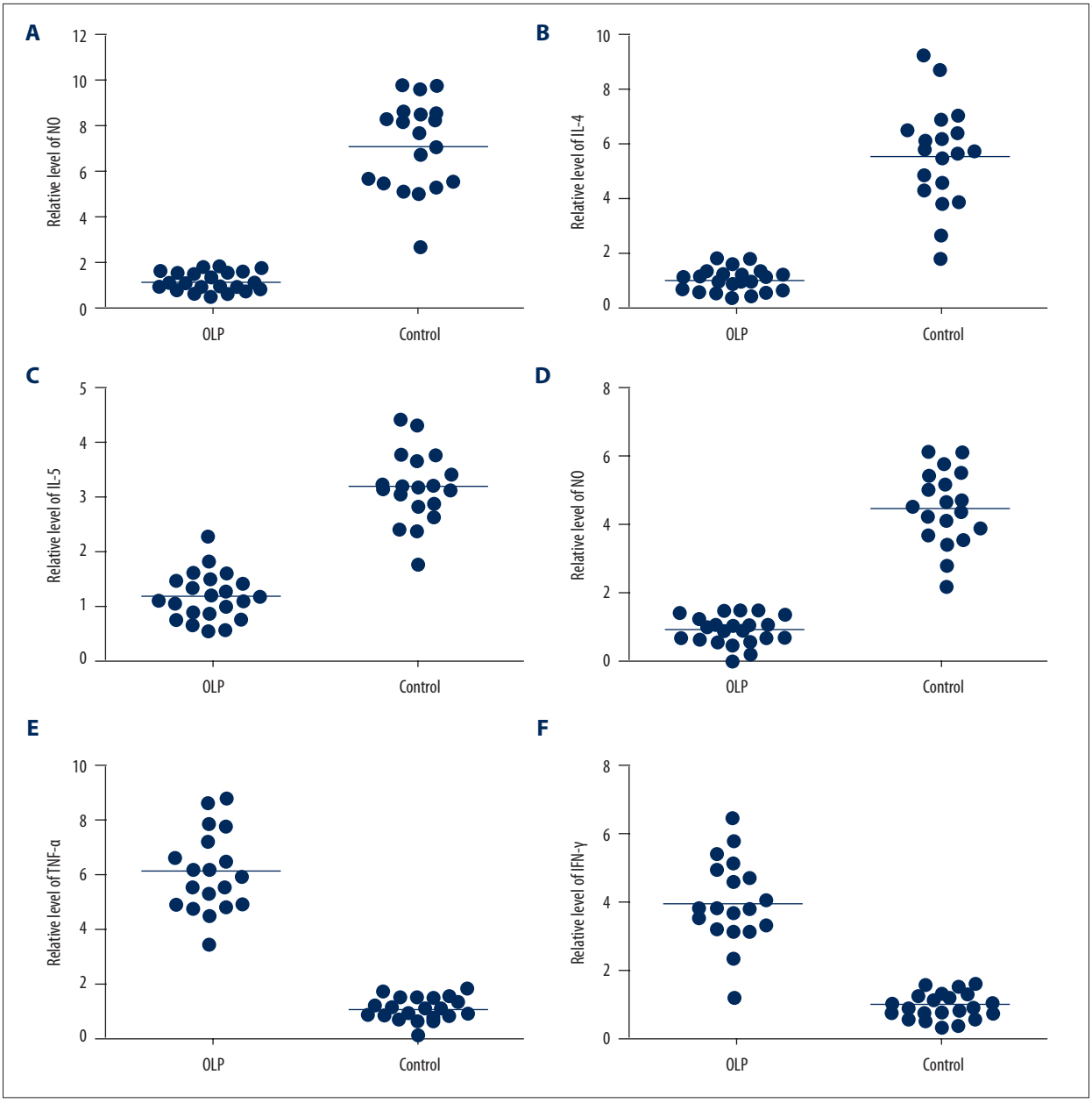

Figure 5. Levels of inflammatory cytokines differed in various groups. (**A**) NO level was much lower in PBMC isolated from OLP patients. (**B**) IL-4 level was downregulated in PBMC isolated from OLP patients. (**C**) IL-5 level was decreased in PBMC isolated from OLP patients. (**D**) IL-10 level was much lower in PBMC isolated from OLP patients. (**E**) TNF-α level was much higher in PBMC isolated from OLP patients. (**F**) IFN-γ level was increased in PBMC isolated from OLP patients.

message containing 1 of the 3 distinct signals necessary for the stimulation of T cells. Among these cytokines, the expression of IL-12 is tailored to a Th1-type response, whereas the expression of IL-4 and IL-10 primarily stimulates a Th2-type response [36]. In the present study, THP-1 cells were transfected with miR-155 mimics/inhibitors, miR-19a mimics/inhibitors, or a scramble control, and we confirmed that miR-155 regulated the expression of eNOS and the production of NO. In addition, the expression of TLR2 was regulated by miR-19a. The production of TLR2, but not TLR4, is highly upregulated in the cells of spinous layers and the infiltrating monocytes in OLP tissues. In addition, the expression of TLR2 is much higher in the PBMCs of OLP subjects as compared to those from healthy subjects [37]. In the present study, the tissue samples from OLP patients showed reduced miR-19a expression and elevated TLR2 expression. In a recent study, OLP patients demonstrated the reduced expression of eNOS, indicating that the elevated NO production, which was triggered by

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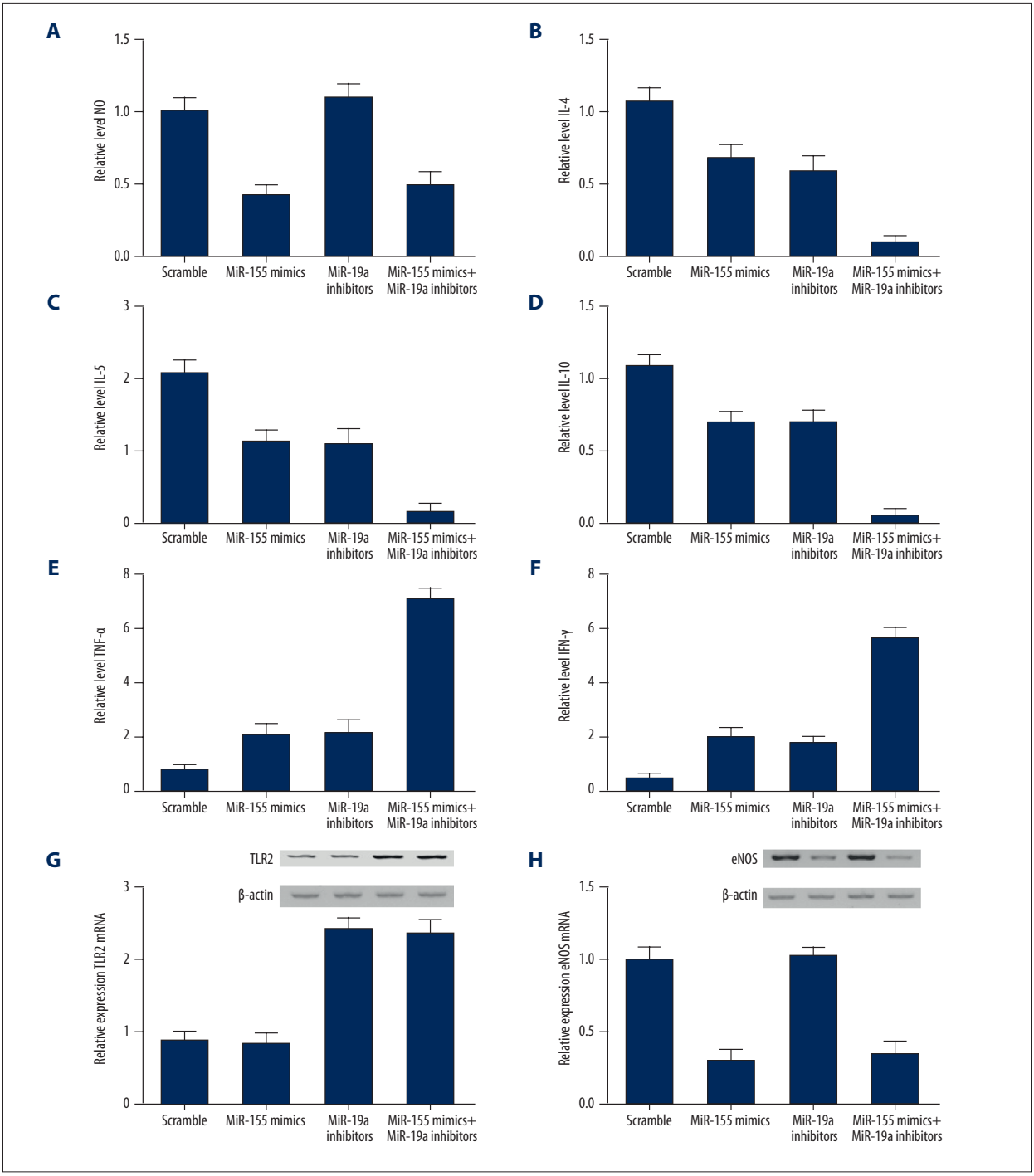

Figure 6. The effect of alternation of expression of miR-155 and miR-19a on the production of inflammatory cytokines. (**A**) miR-155 mimic and miR-155 mimic along with miR-19a inhibitor suppressed NO level, and miR-19a inhibitor had no effect on NO level. (**B**) miR-155 mimic, miR-19 inhibitor inhibited IL-4 expression, and transfection with both synergically reduced IL-4 level. (**C**) miR-155 mimic and miR-19 inhibitor inhibited IL-5 expression, and transfection with both synergically reduced IL-5 level. (**D**) miR-155 mimic and miR-19 inhibitor inhibited IL-10 expression, and transfection with both synergically reduced IL-10 level. (**E**) miR-155 mimic and miR-19 inhibitor enhanced TNF-α expression, and transfection with both synergically improved TNF-α level. (**F**) miR-155 mimic and miR-19 inhibitor enhanced IFN-γ expression, and transfection with both synergically improved IFN-γ level. (**G**) miR-19a inhibitor and miR-19a inhibitor plus miR-155 mimic increased TLR2 level, and miR-155 mimic had no effect on TLR2 level. (**H**) miR-155mimic and miR-19a inhibitor plus miR-155 mimic decreased eNOS level, and miR-19a inhibitor had no effect on eNOS level.

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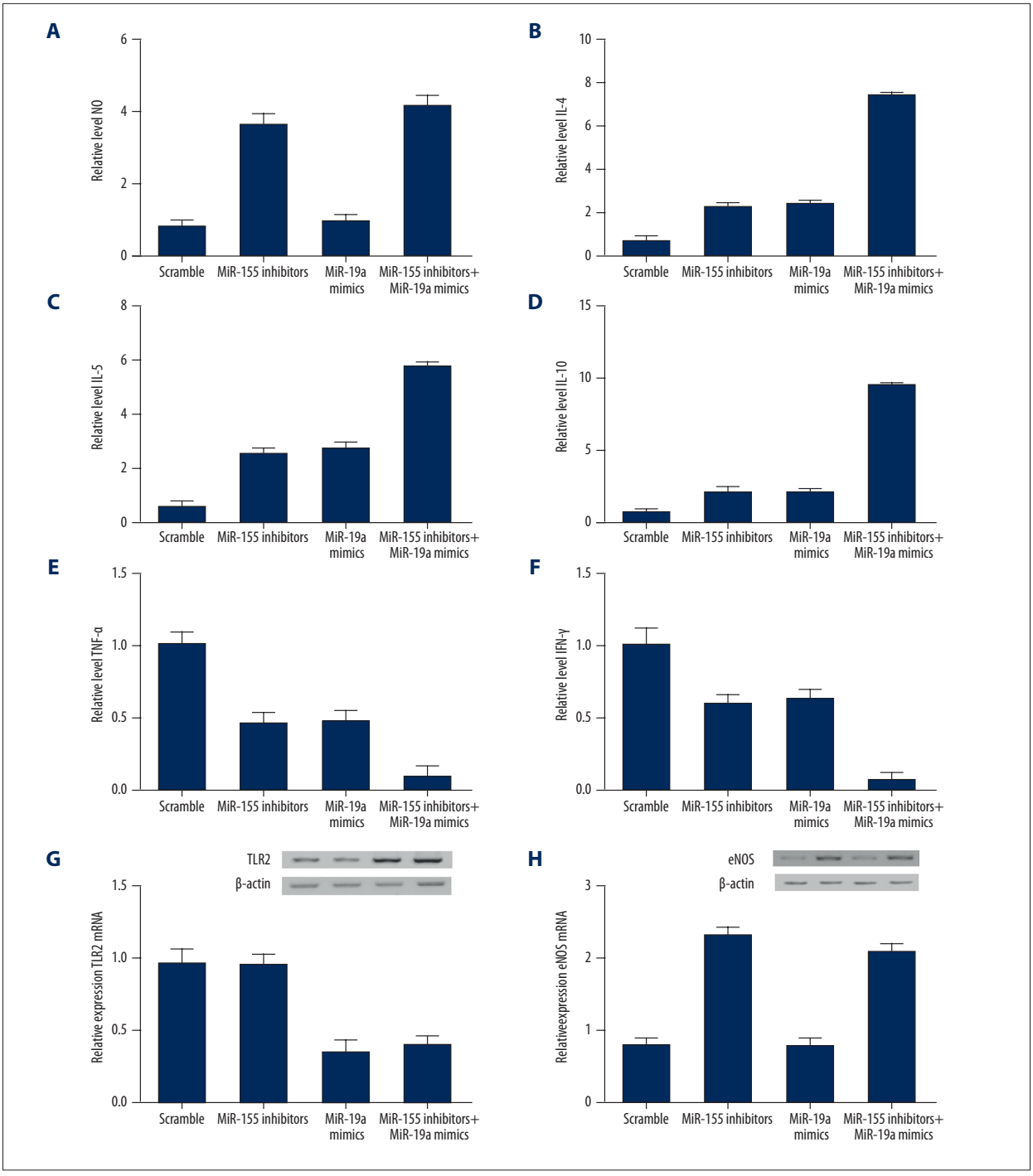

Figure 7. The effect of alternation of expression of miR-155 and miR-19a on the production of inflammatory cytokines. (**A**) miR-155 inhibitor and miR-155 inhibitor along with miR-19a mimic suppressed NO level, and miR-19a mimic had no effect on NO level. (**B**) miR-155 inhibitor and miR-19 mimic upregulated IL-4 expression, and transfection with both synergically increased IL-4 level. (**C**) miR-155 inhibitor and miR-19 mimic upregulated IL-5 expression, and transfection with both synergically increased IL-5 level. (**D**) miR-155 inhibitor and miR-19 mimic upregulated IL-10 expression, and transfection with both synergically increased IL-10 level. (**E**) miR-155 inhibitor and miR-19 mimic downregulated TNF-α expression, and transfection with both synergically decreased TNF-α level. (**F**) miR-155 inhibitor and miR-19 mimic downregulated IFN-γ expression, and transfection with both synergically decreased IFN-γ level. (**G**) miR-19a mimic and miR-19a mimic plus miR-155 inhibitor decreased TLR2 level, and miR-155 inhibitor had no effect on TLR2 level. (**H**) miR-155 inhibitor and miR-19a mimic plus miR-155 inhibitor increased eNOS level, and miR-19a mimic had no effect on eNOS level.

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an increased level of iNOS and eNOS, can exert a certain immune and circulatory effect on the pathogenesis of OLP [38]. In the present study, the consistent downregulation in eNOS and NO, as well as the upregulation in miR-155, was observed in the OLP group. In addition, we found that the mRNA level of TLR2 (Figure 3C) and eNOS (Figure 3D) was negatively correlated with the level of miR-19a and miR-155, respectively. A recent study has demonstrated the involvement of a series of miRNAs in OLP [39]. It has been shown that 20 miRNAs were downregulated in OLP patients, with an additional 46 miRNAs differentially expressed between OLP patients and healthy controls [40,41]. In other studies, knockdown of miR-27b and miR-125b has been implicated in the onset of OLP [18,41,42], whereas the upregulation of miR-146a, miR-155, miR-203, miR-21, and miR-4484 contributed to OLP by modulating immune responses [18,41,43,42,44]. In addition, aberrant expression of miR-375 was observed in tissue samples of OLP [45].

In the present study, the transfection of THP-1 cells by miR-155 mimics alone or miR-19a inhibitors alone reduced the production of IL-4 (Figure 6B), IL-5 (Figure 6C), and IL-10 (Figure 6D), whereas the presence of both miR-155 mimics and miR-19a inhibitors at the same time synergistically reduced the production of these cytokines to an even lower level. Transfection of miR-155 mimics alone or miR-19a inhibitors alone enhanced the production of TNF-α (Figure 6E) and IFN-γ (Figure 6F), whereas the presence of both miR-155 mimics and miR-19a inhibitors at the same time synergistically increased the production of these cytokines to an even higher level. Furthermore, the transfection of miR-155 inhibitors alone or miR-19a mimics alone increased the production of IL-4 (Figure 7B), IL-5 (Figure 7C), and IL-10 (Figure 7D), whereas the presence of miR-155 inhibitors and miR-19a mimics at the same time synergistically increased the production of these cytokines to an even higher level. In addition, the transfection of miR-155 inhibitors alone or miR-19a mimics alone reduced the production of TNF-α (Figure 7E) and IFN-γ (Figure 7F), whereas the presence of miR-155 inhibitors and miR-19a mimics at the same time synergistically reduced the production of these cytokines to an even lower level. This observation is consistent with our hypothesis that the simultaneous deregulation of miR-19a/TLR2 and miR-155/eNOS contributes to the development of OLP in a synergistic fashion.

There are certain limitations in this study. Firstly, the sample size was relatively small due to limited time and funding. Secondly, the conclusion of this study should be further tested in human tissue samples and animal models. Therefore, further studies employing a larger sample size and populations with different genetic backgrounds are warranted. In addition, functional studies using animal models or human tissue samples should also be conducted to confirm the results of the present study.

Conclusions

In this study, PBMCs from subjects with or without OLP were collected to compare their gene expression profiles. We found that OLP changed the expression profiles of miR-155 and miR-19a, which in turn directly affected the production of eNOS and TLR2, respectively. In addition, by synergistically inducing the imbalance between Th1 and Th2, the simultaneous deregulation of miR-155/eNOS and miR-19a/TLR2 was shown to be responsible for elevated risk of OLP.

Conflict of interest

None.

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