

ORIGINAL RESEARCH

MIR-181A-5P ATTENUATES OVALBUMIN-INDUCED ALLERGIC INFLAMMATION IN NASAL EPITHELIAL CELLS BY TARGETING IL-33/P38 MAPK PATHWAY

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Songliang Long, MB¹, Hua Zhang, MM^{2,3}¹ Department of Otorhinolaryngology, Hunan Children's Hospital, Changsha, China² Clinical Nursing Teaching and Research Section, The Second Xiangya Hospital, Central South University, Changsha, Hunan, 410001, China³ Department of Gastroenterology, The Second Xiangya Hospital, Central South University, Changsha, Hunan, 410001, China

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ABSTRACT

Purpose: Chronic inflammation of the nasal mucosal tissues plays an important role in the pathogenesis of allergic rhinitis (AR). Aberrantly-expressed micro ribonucleic acid (miRNA) has been found to have strong associations with the inflammatory reactions in allergic diseases; however, its functional significance and molecular mechanism in AR remains unclear. The purpose of this study is to determine the functional role and mechanism of miR-181a-5p in AR.

Methods: Allergic inflammatory reaction was induced by ovalbumin in human nasal epithelial cell line RPMI-2650. The anti-inflammatory effects of miR-181a-5p were evaluated by examining pro-inflammatory cytokines (interleukin (IL)-1 β , IL-6, tumor necrosis factor- α (TNF- α)) in the culture of RPMI-2650 cells stimulated by ovalbumin, using quantitative real-time reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay. Luciferase assay and gain-of-function assay were used to investigate the association of miR-181a-5p and IL-33/p38 MAPK axis.

Results: MiR-181a-5p was significantly downregulated in mucosal tissues of AR patients and in RPMI-2650 cells treated with ovalbumin. The overexpression of miR-181a-5p showed prominent suppression of inflammatory cytokine production in RPMI-2650 cells with the stimulation of ovalbumin. MiR-181a-5p directly targeted, and negatively regulated IL-33 to suppress the activation of p38 MAPK signalling.

Conclusion: The results suggest that miR-181a-5p restricted allergic inflammation through inhibition of IL-33/p38 MAPK pathway, indicating miR-181a-5p may play an anti-inflammatory role in AR.

Correspondence to: Hua Zhang Email: Zhanghua@126-web.net

INTRODUCTION

Allergic rhinitis (AR) is a chronic or seasonal inflammatory reaction of nasal mucosa induced by allergens [1,2]. Micro ribonucleic acids (miRNAs) are a class of endogenous non-coding small molecules that play an important role in the posttranscriptional regulation [3]. Since it was first discovered in nematodes in 1993, miRNA has attracted extensive attention. Many studies have shown that miRNA is involved in physiological processes such as body development, differentiation and immune regulation, and its expression and dysfunction may lead to tumorigenesis [4,5]. miRNAs has been shown to play a crucial role in controlling the inflammatory process and are considered promising biomarkers [6].

More detailed knowledge of the role of specific miRNAs in the pathogenesis of AR may provide new treatments for this disease. Huang et al. reported that miR-181a-5p could significantly inhibit the release of LPS-induced inflammatory factors in macrophages [7]. Zhao et al. found that miR-181a-5p alleviated pulmonary hypertension and lung tissue inflammation induced by monoclonine in rats [8]. Li et al. showed that bone marrow mesenchymal stem cells via secreting miR-181a-5p alleviated acute pancreatitis by regulating the PTEN/Akt/TGF- β 1 signalling pathway [9]. Gao et al. also showed that Cirrhivep2 was involved in microglial activation and inflammatory response through miR-181a-5p/SOCS2 signaling pathway [10]. Several studies demonstrated that the expression

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of miR-181a was significantly lower in the nasal mucosa of AR patients, which was correlated with the severity of the disease, and the development, differentiation and activation of T-helper cells [11-13].

This study attempts to analyze the expression of miR-181a-5p in AR and to clarify its effect on ovalbumin (OVA)-induced allergic inflammation of nasal mucosa epithelial cells. The bioinformatics algorithm starBase (<http://starbase.sysu.edu.cn>) predicted that miR-181a-5p had binding sites with the inflammatory cytokine interleukin (IL)-33 3'UTR and pro-inflammatory effects of IL-33 in AR have been documented [14-16]. In addition, IL-33 has been shown to be involved in regulating the p38 MAPK inflammatory signalling pathway [17-19]. Therefore, we speculated that miR-181a-5p could inhibit the activation of p38 MAPK inflammatory signalling pathway by targeting the expression of IL-33, thus alleviating the allergic inflammatory response of nasal mucosa epithelial cells.

MATERIALS AND METHODS

Patients

Fifteen patients diagnosed with AR (10 males, five females; mean: 38.2 ± 10.2 years, range: 25-61 years) were enrolled from The Second Xiangya Hospital of Central South University, China from September 2017 to July 2018. All patients were required to meet the following inclusion criteria: 1) diagnosed as AR based on symptoms, duration of symptoms onset, skin prick test, serum IgE antibody test and aeroallergen nasal provocation test in accordance with Chinese Society of Allergy Guidelines for Diagnosis and Treatment of AR; 2) age ≥ 18 years; and 3) volunteered to participate in the present study and agreed to the collection of nasal mucosa tissues. Patients were excluded if they had a solid tumor, bronchial asthma, chronic rhinosinusitis, nasal polyposis, chronic obstructive pulmonary disease, a nasal infection or other active infections. The healthy control group comprised 15 cases undergoing nasal septum correction (12 males, three females; mean: 40.3 ± 12.0 years, range: 27-62 years) with no history of AR, rhinosinusitis, asthma, aspirin intolerance or other systemic diseases. Notably, no research subject (AR or control) had been treated with specific immunotherapy, or received antibiotics, steroids, antihistamines or immune drugs in the four weeks prior to recruitment. The nasal biopsy samples were taken during their allergic season. After intraoperative local anesthesia, inferior turbinate mucosal tissues were collected under nasal endoscope and were prepared for RNA isolation.

All the participants provided informed consent. The study was carried out with the approval of the Ethics Committee of The Second Xiangya Hospital of Central

TABLE 1.
DEMOGRAPHICS OF ALLERGIC RHINITIS PATIENTS AND HEALTHY CONTROLS.

Characteristics	AR (n=15)	Controls (n=15)	P
Age (years), mean $\pm$ SD	38.2 ± 10.2	40.3 ± 12.0	>0.05
Gender (male/female)	10/5	12/3	>0.05
Serum IgE (IU/mL), mean $\pm$ SD	301.0 ± 150.8	22.4 ± 10.3	<0.01
INSS, mean $\pm$ SD			
Rhinorrhea score	1.9 ± 0.7	-	-
Itching score	1.8 ± 0.5	-	-
Sneezing score	2.2 ± 0.7	-	-
Congestion score	2.0 ± 0.7	-	-
TNSS, mean $\pm$ SD	7.9 ± 1.6	-	-

Abbreviations: AR, allergic rhinitis; IgE, immunoglobulin E; INSS, Individual nasal symptom score; SD, standard deviation; TNSS, total nasal symptom score.

South University. Demographics of AR patients and controls are summarized in Table 1.

Cell culture and OVA treatment

Human nasal epithelial RPMI-2650 cells (CCL-30™; ATCC®, Manassas, VA, USA) were incubated in Eagle's Minimum Essential Medium (30-2003; ATCC) containing 10% fetal bovine serum in a humidified incubator containing 5% CO₂ at 37°C. At 70%-80% confluent, the medium was replaced with serum-depleted media, and the cells were used for subsequent experiments. To mimic allergic response, RPMI-2650 cells were treated with OVA (A5503; Sigma-Aldrich, St Louis, MO, USA) at a dose of 2 mg/mL.

Cell transfection

Employing Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA), transfection was performed in RPMI-2650 cells with pcDNA3.1-IL-33, miR-181a-5p mimic, and the negative controls synthesized from GenePharma Co., Ltd. (Shanghai, China) following OVA challenge.

Luciferase reporter assay

The 3'-UTR fragments of IL-33 containing the predicted wild-type or mutated miR-181a-5p binding sites were amplified, and sub-cloned into the pmirGLO Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). When RPMI-2650 cells reached about 70% confluence, these luciferase reporter plasmids were co-transfected with either miR-181a-5p mimic or miR-NC. After 48h transfection, cells were subjected to luciferase assays.

qRT-PCR analysis

Total RNA was derived from nasal tissues and RPMI-2650 cells using the TRIzol™ Reagent (Invitrogen) and reverse-transcribed into cDNA. The thermocycling conditions were as follows: 95°C for 15 min; 95°C for 10 s; 60°C for 20 s and 72°C for 20 s, for a total for 40 cycles. The expression levels of miR-181a-5p, IL-33, IL-1β, IL-6, and TNF-α were determined by quantitative real time polymerase chain reaction (qRT-PCR) analysis, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 served as the internal references. The specific primers were listed as follows: miR-181a-5p (Forward 5'-GGGCAGCCTTAAGAGGA-3'; Reverse 5'-CAGTGCCTGTCGTGGA-3'), IL-33 (Forward 5'-GTGACGGTGTGATGGTAAGATGT-3'; Reverse 5'-CACTCCAGGATCAGTCTTGCAT-3'), IL-1β (Forward 5'-ATGCCTCGTGTGCTGAC-3'; Reverse 5'-TCCCGACCATGCTGTTCC-3'), IL-6 (Forward 5'-TCCAGCCAGTTGCCTTCTTG-3'; Reverse 5'-AGCCACTCTTCTGTGACTC-3'), TNF-α (Forward 5'-CCAGAACTCCAGGCGGTGTC-3'; Reverse 5'-GGCTACGGCTTGCTCACTCG-3'), GAPDH (Forward 5'-TGTGGGCATCAATGGATTGG-3'; Reverse 5'-ACACCATGTATTCCGGGTCAAT-3'), U6 (Forward 5'-CTCGCTTCGGCAGCACA-3'; Reverse 5'-AACGCTTCACGAATTTCGCT-3').

CCK-8 assay

Cell viability was assessed by a Cell Counting Kit-8 (CCK-8, Beyotime Biotechnology, Shanghai, China). Briefly, cells were seeded in 96-well plate with 5,000 cells/well, and the CCK-8 solution was added to the culture medium. The cultures were incubated for 1 h at 37°C in humidified 95% air and 5% CO₂. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

ELISA assay

The concentration of IL-1β, IL-6 and TNF-α in cell culture supernatant was measured using the commercial enzyme-linked immunosorbent (ELISA) kits purchased from Beyotime Biotechnology (PI305 for IL-1β; PI330 for IL-6; PT518 for TNF-α).

Western blot

The total protein was extracted from cultured cells using RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and quantified with a BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Proteins (30 µg) were separated by electrophoresis on 12% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were then incubated with primary antibodies (1:1,000 dilution) bought from Cell Signaling Technology (Boston, MA, USA) against IL-33 (#88513), p38 MAPK (#8690), p-p38 MAPK (#4511) and GAPDH (#5174) overnight at 4°C and secondary antibodies

(1:2000 dilution; #7074) for 1 h at room temperature. These bands were then visualized by enhanced chemiluminescence.

Statistical analysis

All data were expressed as the means ± standard deviation (SD). Statistical analysis was performed by GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Comparisons between groups were performed using t-test or one-way ANOVA, followed by Tukey's *post hoc* test. *P* < 0.05 was considered to be statistically significant.

RESULTS

Decreased expression of miR-181a-5p in nasal mucosa of AR patients and OVA-induced RPMI-2650 cells

As demonstrated by qRT-PCR analysis, miR-181a-5p in the nasal mucosa of AR patients was significantly lower than that in the healthy controls, suggesting that miR-181a-5p might participate in the development of AR (Figure 1A). Subsequently, we evaluated the expression of miR-181a-5p in OVA-induced AR cell model. Results showed that exposure to OVA led to the downregulation of miR-181a-5p in RPMI-2650 cells, compared to the untreated cells (Figure 1B).

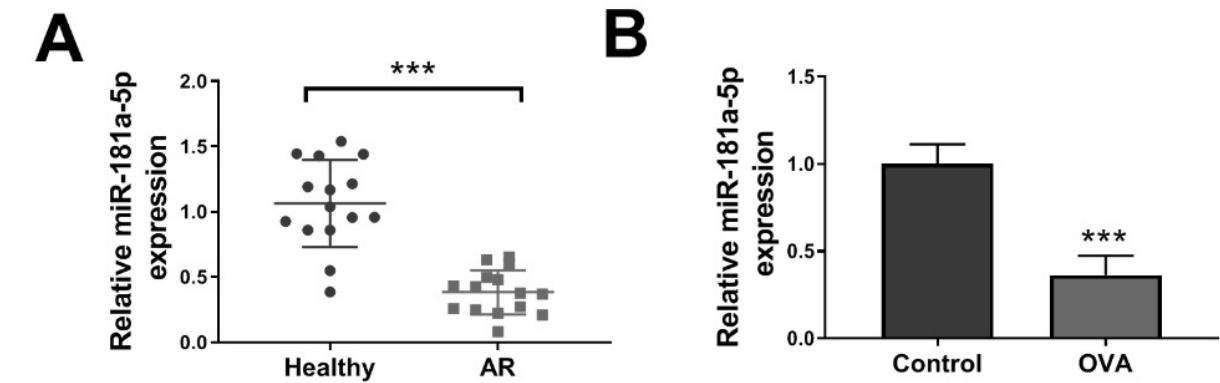
Effects of miR-181a-5p overexpression on OVA-induced secretion of inflammatory cytokines in RPMI-2650 cells

qPCR analysis was also performed to assess the transfection efficiency and showed that the expression of miR-181a-5p was markedly elevated in RPMI-2650 cells transfected with miR-181a-5p mimic compared with the miR-NC group (Figure 2A). CCK-8 assay showed that cell viability was significantly reduced following treatment with OVA in RPMI-2650 cells. To reveal whether miR-181a-5p exerted anti-inflammatory response to AR, the levels of proinflammatory cytokines were detected via qRT-PCR and ELISA. As a result, OVA treatment significantly increased the mRNA expression of IL-1β, IL-6 and TNF-α, whereas the upregulation of miR-181a-5p inhibited these mRNA expression levels in OVA-stimulated RPMI-2650 cells (Figures 2B-2D). Likewise, the protein levels of IL-1β, IL-6 and TNF-α were increased in OVA-treated RPMI-2650 cells, while enforced expression of miR-181a-5p alleviated the production of IL-1β, IL-6 and TNF-α (Figures 2E-2G).

IL-33 is negatively regulated by miR-181a-5p

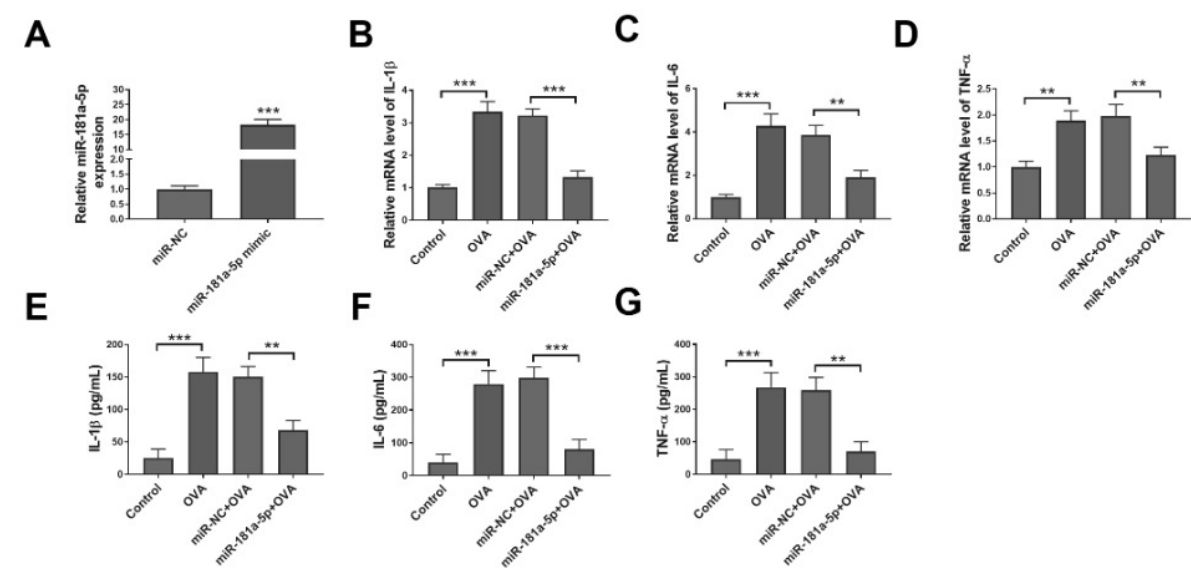
To uncover the molecular mechanism by which miR-181a-5p functioned in the progression of inflammatory responses, bioinformatics algorithm (starBase) was used to search for the potential targets of miR-16. As shown in Figure 3A, the complementary sequences of miR-181a-5p

FIGURE 1.
MIR-181A-5P IS DOWNREGULATED IN NASAL MUCOSA OF AR PATIENTS AND OVA-INDUCED RPMI-2650 CELLS.



(A) Relative miR-181a-5p expression in the nasal mucosa of AR patients (n=15) and healthy controls (n=15) were determined by qRT-PCR analysis. (B) The differential expression of miR-181a-5p was determined in human nasal epithelial RPMI-2650 cells with or without OVA stimulation using qRT-PCR. Values are presented as means $\pm$ SD and are representative of three individual experiments. ***P<0.001.

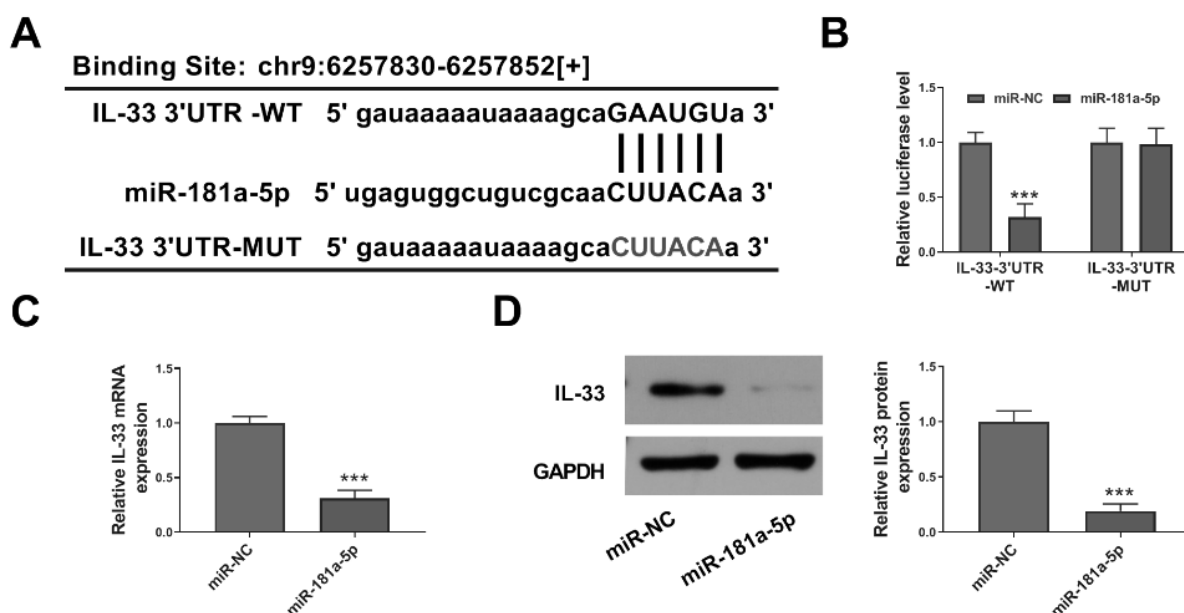
FIGURE 2.
MIR-181A-5P OVEREXPRESSION ALLEVIATES OVA-INDUCED SECRETION OF INFLAMMATORY CYTOKINES IN RPMI-2650 CELLS.



A) qRT-PCR was performed to determine the expression of miR-181a-5p in RPMI-2650 cells after miR-181a-5p mimic transfection. The mRNA expression of IL-1 β (B), IL-6 (C) and TNF- α (D) were determined by qRT-PCR in RPMI-2650 cells after miR-181a-5p mimic transfection prior to treatment with OVA (2 mg/ml). The protein levels of IL-1 β (E), IL-6 (F) and TNF- α (G) were determined by ELISA in RPMI-2650 cells after miR-181a-5p mimic transfection prior to treatment with OVA (2 mg/ml). Values are presented as means $\pm$ SD and are representative of three individual experiments. **P<0.01; ***P<0.001.

were observed in the 3'-UTR of IL-33 mRNA. To determine whether miR-181a-5p could directly target IL-33, the luciferase reporter assay was used. Our data showed that the miR-181a-5p mimic significantly decreased the luciferase activities of IL-33 with wild type 3'-UTR but not mutant IL-33 (Figure 3B). Further, we examined whether miR-181a-5p could modulate the expression of IL-33 in RPMI-2650 cells. According to the results of qRT-PCR and immunoblotting, overexpression of miR-181a-5p observably reduced the mRNA and protein levels of IL-33 (Figure 3C-3D).

FIGURE 3.
IL-33 IS NEGATIVELY REGULATED BY MIR-181A-5P.



(A) The predicted miR-181a-5p binding site on IL-33. (B) Luciferase activity in RPMI-2650 cells co-transfected with miR-181a-5p mimic and luciferase reporters containing IL-33 wild type or MUT 3'-UTR. (C, D) miR-181a-5p mimic or miR-NC were transfected into RPMI-2650 cells, then the mRNA and protein levels of IL-33 were detected by qRT-PCR and western blotting. Values are presented as means $\pm$ SD and are representative of three individual experiments. *** $P < 0.001$.

miR-181a-5p relieves inflammatory phenotypes by targeting IL-33

RPMI-2650 cells were co-transfected with the miR-181a-5p mimic and pcDNA3.1-IL-33 and then exposed to OVA. Transfection efficiency was detected by western blotting, and we observed that IL-33 was upregulated after transfection with IL-33 overexpression plasmid (Figure 4A). As shown in Figures 4B-4D, the restoration expression of IL-33 reversed the inhibitory effects of miR-181a-5p overexpression on IL-1 β , IL-6 and TNF- α mRNA levels in OVA-treated RPMI-2650 cells. Also, IL-33 overexpression abolished the anti-inflammatory effects induced by miR-181a-5p mimic as evidenced by increasing the protein levels of IL-1 β , IL-6 and TNF- α (Figures 4E-4G).

Effects of miR-181a-5p on IL-33/p38 MAPK signalling pathway in OVA-induced RPMI-2650 cells

Since p38 MAPK signalling pathway is known to play a critical role in AR [20, 21], we sought to determine whether the p38 MAPK pathway was implicated in the protection of miR-181a-5p in AR. As shown in Figure 5, OVA treatment significantly increased the protein expression level of p-p38, while overexpression of miR-181a-5p reduced the phosphorylated expression of p38 MAPK. IL-33 upregulation reversed this effect of the miR-181a-5p mimic. Thus, our data demonstrated that miR-181a-

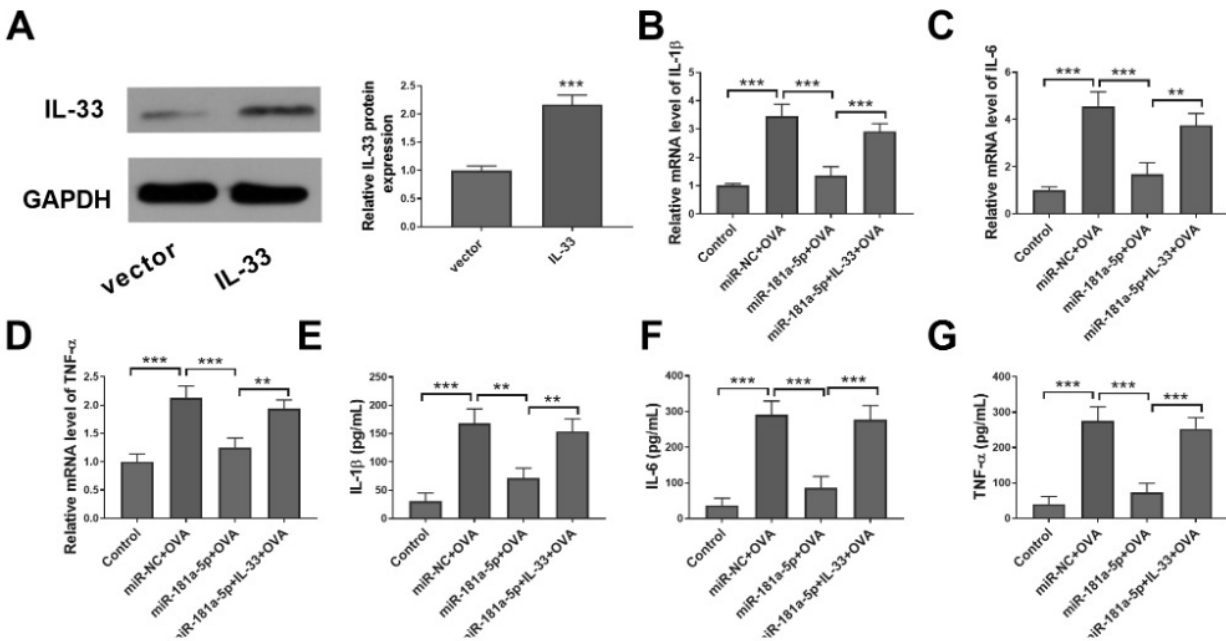
5p limited the activation of the p38 MAPK pathway by downregulating IL-33 expression.

DISCUSSION

In this study, miR-181a-5p was found to be downregulated in the nasal mucosal tissues from AR patients and in the OVA-induced cell model of AR. Furthermore, we reported for the first time that miR-181a-5p targeted IL-33 and suppressed IL-33 expression, thereby inhibiting the activation of p38 MAPK signalling, leading to ameliorate OVA-induced allergic inflammation.

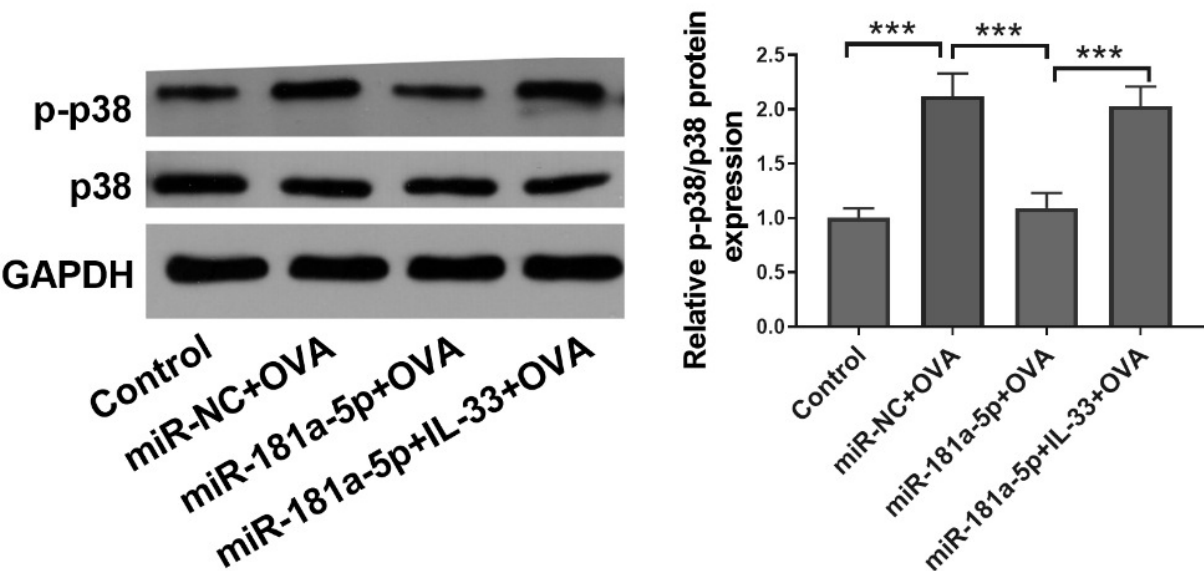
Convincing evidence has indicated that miRNAs represent promising therapeutic targets for allergic diseases, such as miR-146a [22], miR-466a-3p [23] and miR-345-5p [24]. Recent studies have confirmed that miR-181a-5p acts as a tumor suppressor by targeting anti-oncogenes, and has the great capacities to inhibit the proliferation, immigration and invasion of diversified tumor types, including non-small cell lung cancer [25,26], colorectal cancer [27,28], and breast cancer [29]. In addition, miR-181a-5p has been found to be protective during vascular remodeling in rats with MCT-induced pulmonary arterial hypertension and its overexpression inhibits the inflammatory response in TNF- α -stimulated rat pulmonary arterial endothelial cells [8]. Decreased miR-181a-5p expression was observed in nasal mucosa

FIGURE 4.
MIR-181A-5P RELIEVES INFLAMMATORY PHENOTYPES BY TARGETING IL-33.



(A) The protein levels of IL-33 in RPMI-2650 cells transfected with IL-33 overexpression vector. The mRNA expression of IL-1β (B), IL-6 (C) and TNF-α (D) were determined by qRT-PCR in RPMI-2650 cells after miR-181a-5p or IL-33 overexpression prior to treatment with OVA (2 mg/ml). The protein levels of IL-1β (E), IL-6 (F) and TNF-α (G) were determined by ELISA in RPMI-2650 cells after miR-181a-5p or IL-33 overexpression prior to treatment with OVA (2 mg/ml). Values are presented as means ± SD and are representative of three individual experiments. **P<0.01; ***P<0.001.

FIGURE 5.
MIR-181A-5P INHIBITS THE ACTIVATION OF THE IL-33/P38 MAPK AXIS.



(A) The protein levels of p-p38 and p38 in RPMI-2650 cells after miR-181a-5p and (B) IL-33 overexpression prior to treatment with OVA (2 mg/ml). Values are presented as means ± SD and are representative of three individual experiments. ***P<0.001.

of AR patients, which was reported by both previous [11] and current studies. The role of miR-181a-5p in allergic inflammation was also investigated. IL-1 β is a pro-inflammatory cytokine produced by activated monocytes and macrophages and plays pivotal roles in the maturation, activation, proliferation of immune cells [30]. IL-6 is one of the essential pleiotropic cytokines that produces diverse biological activities, which is responsible for inflammatory responses [31]. TNF- α is a cytotoxic protein with proinflammatory properties, which is mainly secreted by adipocytes, activated microglia, macrophages, B cells and T cells, and implicated in the development of inflammatory diseases, including AR [32]. Here, we also found that miR-181a-5p expression levels were reduced by OVA treatment in RPMI-2650 cells. Moreover, miR-181a-5p upregulation reduced IL-1 β , IL-6 and TNF- α cytokine production and enhanced cell viability in OVA-stimulated RPMI-2650 cells. The results implied the anti-inflammatory effect of miR-181a-5p in AR.

IL-33 is a member of the IL-1 family cytokines that not only participates in gene transcription regulation as a nuclear factor, but also plays an essential role in the pathogenesis of allergic diseases, such as AR, asthma and atopic dermatitis [14,33]. Upon IL-33 binding to its receptor ST2, several classical inflammatory pathways are activated, especially the ERK1/2, p38 MAPK, JNK, NF- κ B signalling pathways, which potently drive the production of Th2-associated cytokines and chemokines, resulting in allergic inflammation [34,35]. Inhibition of the IL-33/p38 MAPK signalling pathway may thus be a beneficial approach for controlling the pathologic process of AR. In our study, we found miR-181a-5p could directly target the 3'-UTR of IL-33 and negatively regulate the expression of IL-33. Furthermore, IL-33 could rescue the inhibition of pro-inflammatory cytokines mediated by miR-181a-5p. Based on the above results, we concluded that miR-181a-5p targeted IL-33 and inhibited OVA-induced airway inflammatory response in AR by inhibiting the activation of p38 MAPK signalling pathway.

Limitations

Our study has two important limitations: small sample size and the lack of *in vivo* experiments.

CONCLUSION

We demonstrated that miR-181a-5p exerted its anti-inflammatory activity in OVA-stimulated human nasal epithelial cells through targeting IL-33 and inhibiting p38 MAPK signalling. We proposed that the miR-181a-5p/IL-33/p38 MAPK axis might represent a therapeutical strategy of preventing airway inflammation.

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