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MicroRNA-155 plays critical effects on Th2 factors expression and allergic inflammatory response in type-2 innate lymphoid cells in allergic rhinitis

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Abstract. – OBJECTIVE: Allergic rhinitis (AR) is a chronic inflammatory disease. This study aimed to investigate the role of microRNA-155 (miR-155) in type-2 innate lymphoid cells (ILC2s) on AR.

PATIENTS AND METHODS: Nasal mucosa tissues and peripheral blood samples were collected. mRNA expression of miR-155, interleukin-25 (IL-25), and interleukin-33 (IL-33) in nasal mucosa tissues was determined using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The AR model was established by injecting with murine IL-33. The frequency of ILC2s was quantified using flow cytometry. MiR-155 agomir or antagomir was intranasally administrated to mice. MiR-155 and helper T cell 2 (Th2) cytokines were measured with quantitative Real-time PCR (qRT-PCR), enzyme-linked immunosorbent assay (ELISA) or Western blotting, respectively. Hematoxylin and eosin (HE) staining was used for the histopathological examination.

RESULTS: Compared with controls, mRNA levels miR-155 ($p<0.001$), IL-25 ($p<0.05$), and IL-33 ($p<0.001$) were increased in nasal mucosa tissues of AR patients and AR mice, and ILC2s ratios were enhanced in human peripheral blood ($p<0.0001$), which were much higher after intranasal administration with miR-155 agomir ($p<0.0001$). MiR-155 expression of AR mice was significantly reduced after intranasal administration with miR-155 antagomir ($p<0.05$). Frequencies of ILC2s in human peripheral blood significantly correlated with miR-155 ($r=0.4803$, $p=0.0130$). MiR-155 up-regulation markedly increased frequencies of nasal rubbing/sneezing and levels of IL-4, IL-5, IL-9, and IL-13. Pathological changes were worsened after miR-155 agomir and ameliorated after miR-155 antagomir administration. MiR-155 agomir mice ($p<0.001$) showed higher ILC2s, whereas lower in miR-155 antagomir mice compared to AR mice ($p<0.05$).

CONCLUSIONS: MiR-155 played critical effects on Th2 factor expression and allergic inflammatory response in ILC2 cells in AR.

Key Words

MicroRNA-155, Allergic rhinitis, Type-2 innate lymphoid cells, Interleukin.

Introduction

Allergic rhinitis (AR) is one of the most common noninfectious chronic upper respiratory diseases in the world, mainly based on helper T cell 2 (Th2) type immune response[1]. Although it seems to be a simple clinical symptom (repeated sneezing, runny nose, and stuffy nose), it may cause sleep disturbances and decreased attention seriously impacting on the quality of life and work performance[2,3]. AR is the basis of many complications and a major risk factor for poor asthma control[4], implying that AR plays a pivotal role in the overall pathogenesis of allergic inflammation. At present, the clinical treatment of AR is still mainly based on a symptomatic treatment, such as intranasal steroids and antihistamines. The application of specific immunotherapy is still limited[1]. Therefore, it is of foremost importance to study the pathogenesis of AR and understand potential pharmacological agents for the treatment of AR. Previous studies have shown that Th2 is considered to be an invaluable source of cytokines during the immune process. Until the discovery of Group 2 innate lymphoid cells (ILC2s), we learned that these previously unrecognized cytokine secreting cells lack of the surface expression of the common lineage markers associated with T cells, B cells, and other leukocytes, but are capable of producing large amounts of type 2 cytokines [interleukin-4, 5, 13 (IL-4, IL-5, IL-13)] also shown to promote Th2- type inflammation[5-7]. Enrichment of ILC2s was observed in AR patients with elevated serum IgE and airway eosinophilia compared to healthy subjects after allergen challenge[8]. Particularly, nasal cat allergen challenge induced a notably increased percent of peripheral blood chemoattractant receptor-homologous expressed on Th2 lymphocytes receptor (CRTH2)-positive ILC2 in AR patients[9]. Although the study of ILC2s in AR is still in its

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infancy, the results of these researches have fully demonstrated that ILC2s are involved in allergic inflammation by expressing type 2 cytokines. However, its intracellular regulation mechanism remains unclear and requires further exploration. MicroRNAs (miRNAs) belong to a family of small non-coding single-stranded RNAs that involved in the regulation of posttranscriptional gene expression by binding to target mRNAs to promoting mRNA degradation or blocking protein translation[10]. Investigations implicate that microRNAs can affect allergic inflammation by regulating the function of the immune cell. Among immune system-related microRNAs, microRNA-155 (miR-155) is the most closely related to immune regulation and intensively studied in allergic diseases[11]. A previous research[12] indicated that miR-155 plays a vital role in the development of the immune system, the maturation, and differentiation of immune cells, and the maintenance of the immune function. By using miR-155-deficient mice demonstrated that miR-155 plays a central role not only during acute inflammation but also in the chronic inflammatory response[13]. MiR-155 is involved in the local regulation of TH2 responses in allergen-induced eosinophilic airway inflammation, further highlighting the role of miR-155 as a potential target for patients with allergic inflammatory diseases[14]. However, the effect of miR-155 in immune diseases is still unclear. In addition, the intracellular mechanisms by which miR-155 affects the expression of ILC2s and regulates allergic inflammation remain to be fully elucidated. In this work, we examined the effects of miR-155 and ILC2s on allergic inflammation in AR. Our results also suggest that miR-155 affects the expression of ILC2s and regulates ILC2s-mediated inflammation. Accordingly, the present study examined the expression of ILC2s and miR-155 in clinical samples and assessed the relation between the ratio of ILC2s and the expression of miR-155. In addition, the effect of up-regulation and down-regulation of miR-155 expression on ILC2s was examined *in vivo*.

Patients and Methods

Patient Selection and Clinical Data

A total of 54 patients who were diagnosed as "nasal septum deviation" were recruited from the Department of Otorhinolaryngology Head and Neck Surgery at the Second Affiliated Hospital of Nanchang University from September 2017 to June 2018. All patients were diagnosed based on their medical history, nasal endoscopy, an allergen skin prick test, and a specific IgE assay. According to the "Guidelines for the diagnosis and treatment of allergic rhinitis (2015, Tianjin, China)", the 54 patients were divided into AR group (26 patients) and healthy control group (28 patients). Patient characteristics are summarized in Table I. Exclusion criteria for AR and healthy control groups were as follows: 1- systemic glucocorticoids and anti-histamines within one month before surgery; 2- smoking within the past 12 months or a history of >10 packs/y; 3- chronic sinusitis, posterior nasal polyps, fungal rhino-sinusitis, immunodeficiency or cystic fibrosis diagnosis; 4- immunotherapy for any allergens; 5- allergic reaction to any drug within the last 2 weeks 6- pregnancy. Nasal mucosal samples were obtained from inferior turbinate sections during the operation. Written informed consent was obtained from all of the subjects and this investigation was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University (Nanchang, China).

Flow Cytometry Analysis for ILC2s

Peripheral blood mononuclear cells (PBMCs) were isolated from 54 participants using centrifugation through the Ficoll-Paque Plus. PBMCs were surface-labeled for 30 min at 4°C immediately with the following antibodies for ILC2s: FITC-labeled antibodies to lineage cocktail (consisting of CD3, CD14, CD16, CD19, CD20, and CD56); PEcy7-labeled antibodies to CRTH2 and APC-labeled antibodies to CD127. Human ILC2s were

Table I. Demographic and clinical characteristics for patients.

Characteristic	HC (n=26)	AR (n=28)	*p*-values
Sex (male/female)	11/15	14/17	0.5567
Age (years)	28.7 ± 14.4	27 11.3	0.2128
SPT (positive/subjects tested)	0/26	31/31	
Specific IgE (kU/l, mean SD)	<0.35	49.8 ± 17.7	<0.0001
Total IgE (kU/l, mean SD)	85 ± 7.2	98 ± 6.7	<0.0001

SPT: skin prick test.

identified as lineage $^{-}$ CRTH2^{+}CD127^{+} lymphocytes. The nasal mucosal cell suspensions of mice were labeled for 30 min at 4°C immediately with the following antibodies for ILC2s: PerCP-Cy™ 5.5-labeled antibodies to lineage cocktail (consisting of CD3e, CD11b, CD45R, Ly-76, Ly-6G, and Ly-6C); FITC-labeled antibodies to CD45, PE-labeled antibodies to KLRG1 and APC-labeled antibodies to CD90.2. Mice ILC2s were identified as lineage $^{-}$CD45^{+} KLRG1^{+}CD90.2^{+} lymphocytes. The antibodies all from BD Bioscience (Franklin Lakes, NJ, USA). Cell counts were performed using the FACSJazz™ (BD Biosciences, Franklin Lakes, NJ, USA) and all data were analyzed using the FACSDiva software (BD Biosciences, Franklin Lakes, NJ, USA). The proportion of ILC2s was expressed as a percentage of total PBMCs in human and total Lin^{-} cells in mice.

Quantitative Real Time-Polymerase Chain Reaction Real Time (qRT-PCR) and miRNA Assay

The nasal mucosal samples of patients were obtained from inferior turbinate sections. The nasal mucosa tissues of the mice were taken after euthanization. Quickly frozen nasal mucosal samples were kept at –80°C before the extraction of RNA. Total RNA, including miRNA, was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The total amount of RNA was measured with a NanoDrop ND-2000c (Thermo Fisher Scientific, Waltham, MA, USA). Quantification of IL-4, IL-5, IL-9, IL-13, and miR-155 was carried out as described by the manufacturer (Life Technologies, Carlsbad, CA, USA). The target gene expression was normalized between different samples based on the values of RNU48 small nuclear RNA expression. Results were analyzed using SDS 1.4 Software according to the $2^{-\Delta\Delta CT}$ method (Life Technologies, Carlsbad, CA, USA), as described earlier[15]. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as endogenous references for miRNAs and mRNAs, respectively. All primers were synthesized by Invitrogen (Shanghai, China), and their sequences are presented in Table II. Samples were tested in duplicates. The relative expression was calculated using the comparative cycle threshold method[16].

Table II. The primers for qRT-PCR assay.

Genes		Primers sequences (5'-3')
For human		
IL-25	Forward	ATGAGGGAGCGACCCAGATTAG
	Reverse	AGAGGTGTCCTGCCCTTTGCT
IL-33	Forward	CAAAGTGGAAGAACACAGCAAGC
	Reverse	CTGTTGACAGGCAGCGAGTAC
miR-155	Forward	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAACCCCTA
	Reverse	ACACTCCAGCTGGGTTAATGVCTAATCGTGAT
GAPDH	Forward	GGAAGCTTGTCATCAATGGAAATC
	Reverse	TGATGACCCTTTTGGCTCCC
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT
For mouse		
IL-4	Forward	GGTCTCAACCCCCAGCTAGT
	Reverse	GCCGATGATCTCTCTCAAGTGAT
IL-5	Forward	GATCCTCCTGCCTCCTCTTC
	Reverse	CTGAGACCCTGATGCAACG
IL-9	Forward	GGTGACATACATCCTTGCCTCTG
	Reverse	GACGGTGGATCATCCTTCAGA
IL-13	Forward	GCAGCATGGTATGGAGTGTG
	Reverse	GGAATCCAGGGCTACACAGA
GAPDH	Forward	CCTCGTCCCGTAGACAAAATG
	Reverse	TGAGGTCAATGAAGGGGTCGT
miR-155	Forward	GGGCTTAATGCTAATTGTGAT
	Reverse	CAGTGCGTGTCGTGGAGT
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGTTCACGAATTTGCGT

Mice

Specific Pathogen-Free (SPF) female BALB/c mice, 6-8 weeks of age, were purchased from Hunan SJA Laboratory Animal Co. Ltd (Hunan, China). These mice were maintained in horizontal laminar flow cabinets and provided sterile food and water in an SPF facility of Nanchang University of Translational Medicine (Jiangxi, China). The mice were randomly divided into 4 experimental groups (n=6 for each group): control, AR (AR mice treated with saline), miR-155 agomir (AR mice treated with miR-155 agomir), and miR-155 antagomir (AR mice treated with miR-155 antagomir). All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

AR Model Production

The AR model was induced according to Figure 1. Briefly, 24 mice were randomly assigned to two groups: Control group (n=6) and model group (n=18). The model group mice were intranasal challenges with 1 ug recombinant murine IL-33 (Peprotech, Rocky Hill, CT, USA) in 20 µl saline on day 1, 3, and 5. Control mice received 20 µl saline. The mice were briefly anesthetized with isoflurane (Yuyan Instruments, Shanghai, China) before intranasal challenges.

Intranasal Administration of MiR-155 Agomir or MiR-155 Antagomir

MiR-155 agomir and miR-155 antagomir were constructed at the GenePharma Company (Shanghai, China). As displayed in Figure 1, 5 pmol/µl miR-155 agomir diluted in 20 µl saline was intranasally administrated to mice on days 6-12. The antagomir miR-155 group was intranasally administrated with 5 pmol/µl miR-155 antagomir diluted in 20 µl saline, while AR group was treated with an equal amount of saline. The samples were collected 24 h after the final challenge.

Symptom Score

30 min after the final challenge on day 5, AR mice have received the behavioral test as described by Bae et al[17]. The frequencies of rubbing and sneezing were recorded, and scores were calculated as follows: 1-wipes the nose slight for several times or sneezes less than 3 times; 2- repeatedly wipes the nose or sneezes more than 3 times and less than 10 times; 3- keeps rubbing from nose to face or sneezes more than 10 times. The mouse model of AR was regarded as a successful case when the behavioral test scores were >5, while the animals unable to achieve more than 5 scores were excluded from further study. The behavioral test was also performed within 30 min of miR-155 agomir or miR-155 antagomir challenge on day 12.

Enzyme-Linked Immunosorbent Assay (ELISA)

Blood samples were collected from the orbital venous plexus of anesthetized mice at the time of death. The serums were harvested from blood samples by room temperature for 30 min and then centrifuged at 2000 r/min for 10 min at 4°C. Stored at–80°C until use. The cytokines of IL-4, IL-5, IL-9, and IL-13 were measured with ELISA kits from R&D Systems (Minneapolis, MN, USA). All ELISAs were performed according to the manufacturer's instructions. The results were presented as the means of duplicates and expressed as pg/ml.

Western Blot Analysis

Nasal mucosal samples were lysed, and total protein lysates (60 µg/lane) were separated via 12% of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. The membranes were subsequently blocked with 5% non-fat milk in Tris-Buffered Saline and Tween-20 (TBST, maintaining 20 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween-20, pH 7.5) for 2 h at room

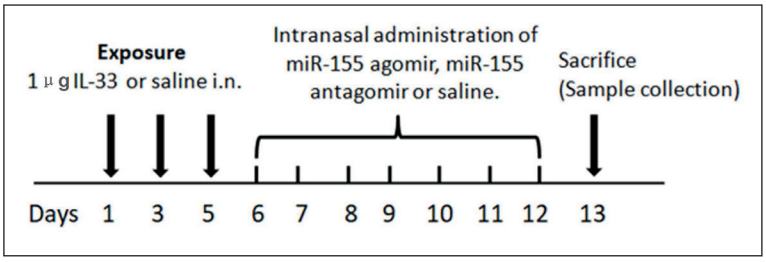

Figure 1. Animal experimental design schedule. Mice were intranasal challenges with recombinant murine IL-33 or saline control on day 1, 3, and 5. After that, miR-155 agomir or miR-155 antagomir were intranasally administrated to mice on days 6-12.

temperature and incubated overnight with rabbit anti-IL-4 (1: 500), rabbit anti-IL-5 (1:500), rabbit anti-IL-9 (1:500), rabbit anti-IL-13 (1:1000), and mouse anti-α-tubulin antibodies (1:1000, Abcam, Cambridge, MA, USA) at 4°C according to the manufacturer's instructions. After washes with Tris-Buffered Saline and Tween 20 (TBST, Beyotime Biotech. Shanghai, China) for 3 times, the membranes were incubated with the secondary antibody and finally processed using an enhanced chemiluminescence (ECL) reaction kit (Cell Signaling Technology, Danvers, MA, USA), followed by exposure to medical film. The relative band densities of the target proteins relative to the α-tubulin band density were quantified using the Bio-Rad Quantity One 1-D analysis software package (Bio-Rad, Hercules, CA, USA).

Histological Analyses

Mice were euthanized under isoflurane anesthesia 24 h after the final intranasal challenge. The heads of the mice were removed and immediately fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 24 h at 4°C. Tissue samples were dehydrated in a series of increasing concentrations of ethanol and finally embedded in paraffin. Nasal mucosa tissues were cut into 4 μm thick sections and stained with hematoxylin and eosin (HE) for histopathological examination.

Statistical Analysis

SPSS software (version 22.0, SPSS Inc., Armonk, NY, USA) was used for all statistical analyses. All data were presented as the mean ± standard error of the mean (SEM). All ILC2s data, miR-155 expression, and Th2-cytokine (IL-4, IL-5, IL-9, IL-13, IL-25, and IL-33) levels were analyzed by using the unpaired *t*-test. Tukey's post-hoc test validated the analysis of variance (ANOVA). It was employed for comparing the data among groups. Linear correlation analysis (Spearman method) was performed between the ratio of ILC2s and expression of miR-155. Differences with *p*-values less than 0.05 were considered to be significant.

Results

Expressing Levels of MiR-155, IL-25, and IL-33 Were Up-Regulated in Nasal Mucosa of AR Patients

We collected the inferior turbinate mucosa of 26 AR patients and 28 healthy controls during operation, and the expressing levels of miR-155, IL-25 and IL-33 in the nasal mucosa were detected by qRT-PCR. Results showed an up-regulation of miR-155 (Figure 2A, 0.5709 ± 0.06833 *vs.* 1.778 ± 0.2467, $p<0.001$), IL-25 (Figure 2B, 0.2276 ± 0.069 *vs.* 0.4338 ± 0.04813, $p<0.05$), and IL-33 (Figure 2C, 2.013 ± 0.781 *vs.* 30.48 ± 5.782, $p<0.001$) in AR, compared with healthy controls.

ILC2s Arose in Peripheral Blood During AR InÐammation

At the same time, we collected the peripheral blood of the above 26 AR patients and 28 healthy controls and then measured the proportion of ILC2 cells by flow cytometry. The gating strategy was shown in Figure 3A. Co-expression of CD127 and CRTH2 clusters in the lineage negative population of PBMCs was the target ILC2s. ILC2s were enriched in AR patients compared with the healthy controls (Figure 3B, $p<0.0001$). The ILC2s ($Lin^-CRTH2^+CD127^+$) percentages in PBMCs in healthy controls (0.03308% ±

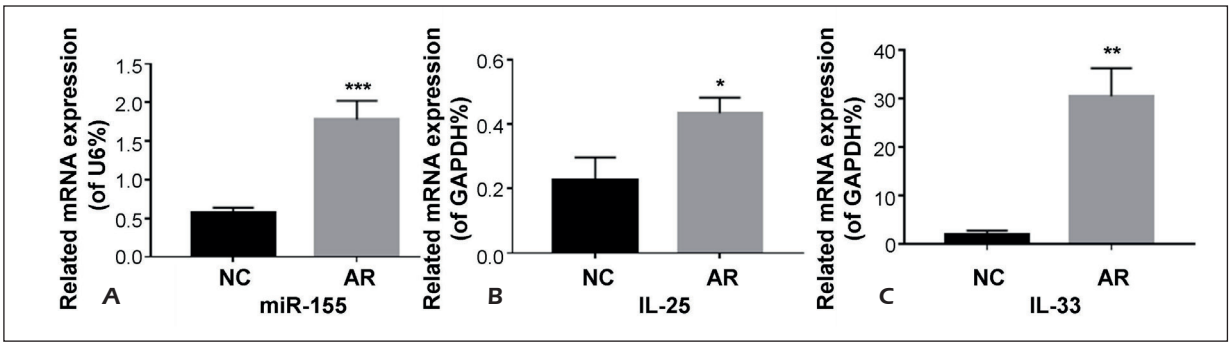

Figure 2. Evaluation for the mRNAs expression of miR-155, IL-25, and IL-33 in nasal mucosa of AR patients. **A**, Statistical analysis for miR-155 expression. **B**, Statistical analysis for IL-25 expression. **C**, Statistical analysis for IL-33 expression. *$p<0.05$; **$p<0.01$; ***$p<0.001$; n=6 in each group.

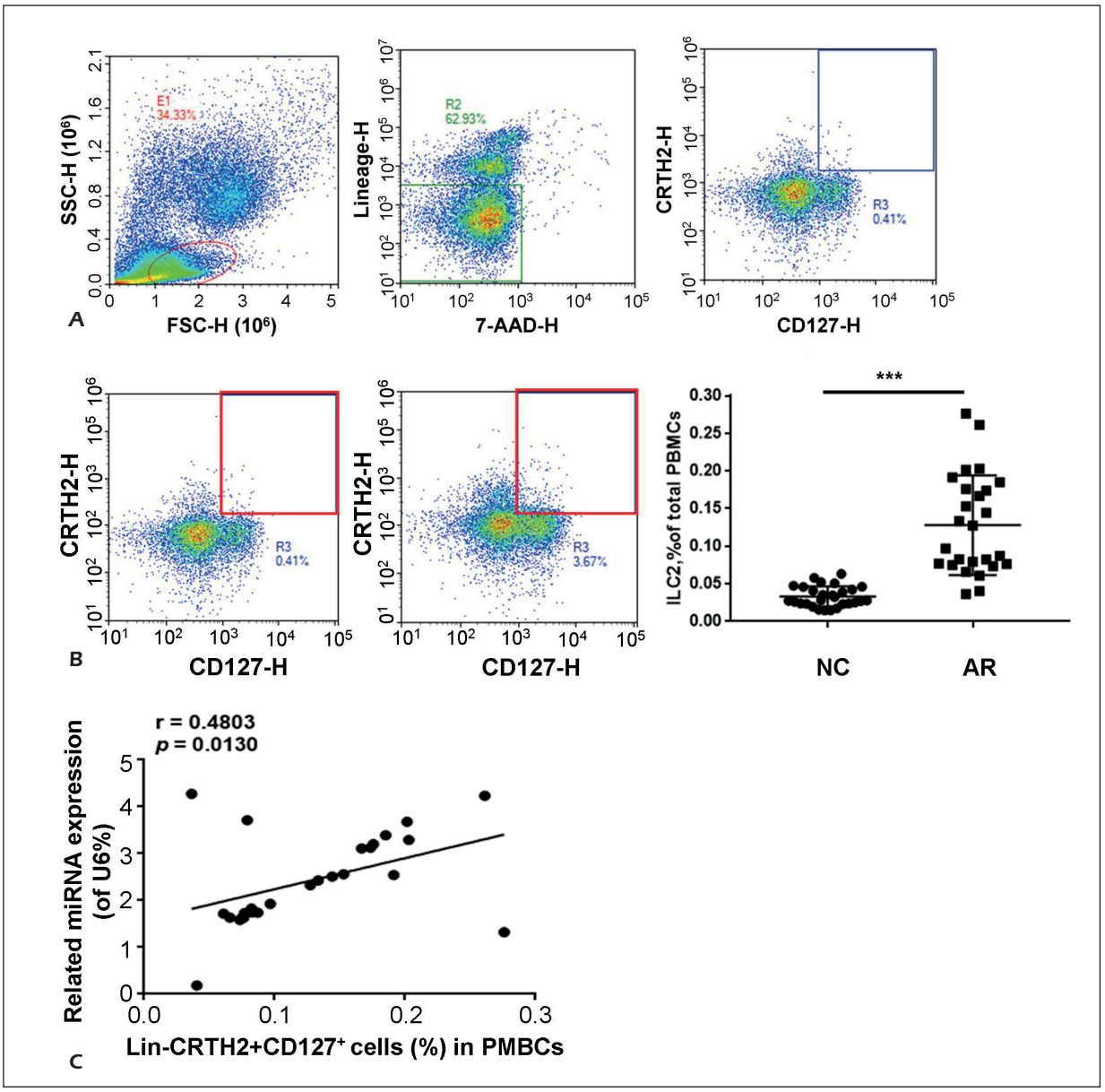

Figure 3. Determination for the ILC2s levels in peripheral blood during AR inflammation. **A**, ILC2s are enriched in AR patients. ILC2s were identified as lineage- CRTH2+CD127+ lymphocytes. Lineage negative (CD3, CD14, CD16, CD19, CD20, and CD56) cells were gated and further assessed for co-expression of CD127 and CRTH2. **B**, ILC2 levels in PBMCs of AR patients (n=26) and healthy controls (n=28). The proportion of ILC2s was expressed as a percentage of total PBMCs. Each symbol represents a separate donor. The black horizontal bars indicate the median for each group. Nonparametric test: ****$p<0.0001$; Unpaired t-test for two-group comparisons. **C**, Correlation between the frequencies of ILC2s and miR-155 expression. Each dot represents a separate donor (Spearman's method. $r=0.4803$, $p=0.0130$). FSC, Forward scatter; SSC, Side scatter.

0.002548%) were very low. However, the percentages of ILC2s in PBMCs in AR patients (Figure 3B, 0.1279% ± 0.01298%, $p<0.0001$) were significantly higher compared to those in healthy controls. Notably, for AR patients, there was a positive linear correlation between the proportion of ILC2s in PBMCs and miR-155 expression (Figure 3C, $r=0.4803$, $p=0.0130$).

Increased Expression of MiR-155 in the Nasal Mucosa of AR Mice

We adopted the method of intranasal challenges with recombinant murine IL-33 to establish an AR model. Briefly, 24 mice were randomly assigned to two groups: control group (n=6) and model group (n=18). The detailed AR mice modeling method was shown in Figure 1. The mice

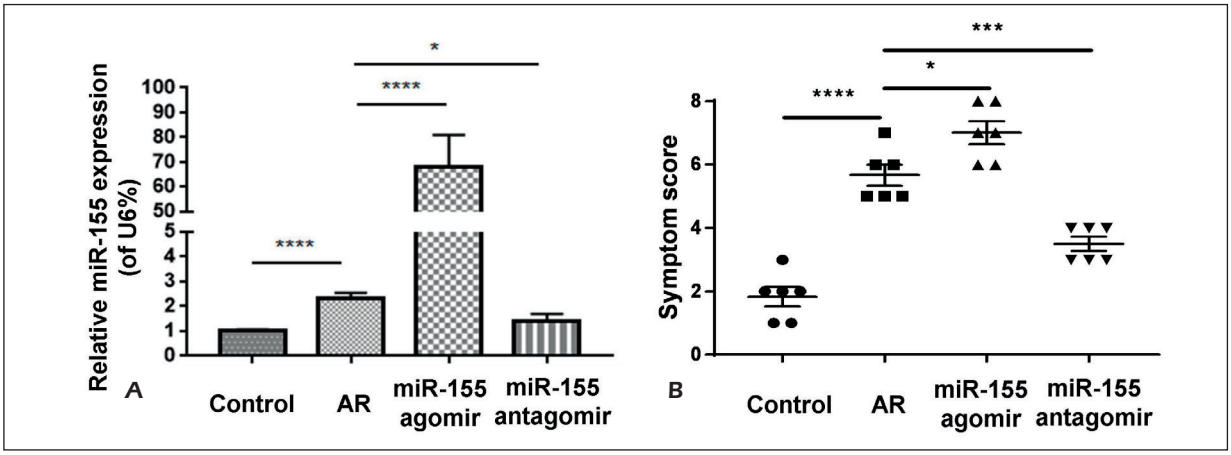

Figure 4. Expression of miR-155 in nasal mucosa and its effects on allergic symptoms in AR mice. **A**, Expression of miR-155 in nasal mucosa of AR mice following intranasal administration with miR-155 agomir or miR-155 antagomir. AR mice were induced as described in the methods section, miR-155 agomir or miR-155 antagomir were then intranasally administrated to mice after IL-33 challenge for another 7 days. The expression of miR-155 in nasal mucosa was determined by quantitative Real Time PCR. The results were expressed as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; n=6 in each group. **B**, Frequencies of rubbing and sneezing were recorded within 30 min of the final IL-33 challenge on day 12, and symptom scores were calculated as described in methods section. The results were expressed as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; n=6 in each group.

were briefly anesthetized with isoflurane before intranasal challenges. The samples were collected 24 h after the final challenge.

As shown in Figure 4A, miR-155 expression was significantly up-regulated in AR mice (2.339 ± 0.2056, p<0.0001) compared to that in controls (1.017 ± 0.05233). Moreover, miR-155 expression was significantly increased in miR-155 agomir-treated AR mice (68.1 ± 12.9, p<0.0001) and decreased in miR-155 antagomir-treated AR mice (1.429 ± 0.2721, p<0.05), both compared to AR mice, indicating a successful gene transduction in the nasal mucosa of these mice.

Over-Expression of MiR-155 Promoted Allergic Symptoms in AR Mice

We tested the nasal allergic symptoms of AR mice after the last challenge on day 12, including the number of nasal rubbing and sneezing. The symptom score increased significantly in AR mice (5.667 ± 0.3333, p<0.0001) compared with controls (1.833 ± 0.3073). Scored higher after treated with miR-155 agomir (Figure 4B, 7 ± 0.3651, p<0.05), whereas the symptom score decreased in miR-155 antagomir-treated mice (Figure 4B, 3.5 ± 0.2236, p<0.001) as compared with AR mice. Furthermore, we observed the role of miR-155 in the pathological alterations of the nasal mucosa of AR mice by HE staining. IL-33 challenged mice induced significantly pathological changes in the nasal mucosa, including hyperemia, edema, necrosis, and aberrant structure.

All of these pathological alterations were worsened after miR-155 agomir administration and ameliorated after administration of miR-155 antagomir (Figure 5).

MiR-155 Up-Regulation Increased the Levels of Cytokines in AR Mice

To explore the crucial role of miR-155 in Th2 immunity, we examined the protein expression of Th2 cytokines (IL-4, IL-5, IL-9, and IL-13) in the mice nasal mucosa of each group by Western blot assay (Figure 6A). Protein expressions of these cytokines were explosively raised in miR-155 agomir treated AR mice compared to AR mice alone, whereas lessened in miR-155 antagomir mice (Figure 6 B-E, p<0.05). Also, to further confirm the effect of miR-155 on pro-inflammatory cytokines, we measured the levels of Th2 cytokines in serum by ELISA. Not surprisingly, the levels of IL-4, IL-5, IL-9, and IL-13 were remarkably up-regulated in AR mice as compared with controls and administration of miR-155 agomir further increases the levels of these cytokines. However, miR-155 antagomir administration was associated with reduced levels of these cytokines (Figure 6 F-I, p<0.05). Consistent with the above results, the mRNA expression of IL-4, IL-5, IL-9, and IL-13 in the nasal mucosa of AR mice also increased, which were significantly attenuated by miR-155 antagomir administration. Similarly, miR-155 agomir increased the levels of these cytokines compared to AR mice (Figure 6 J-M, p<0.05).

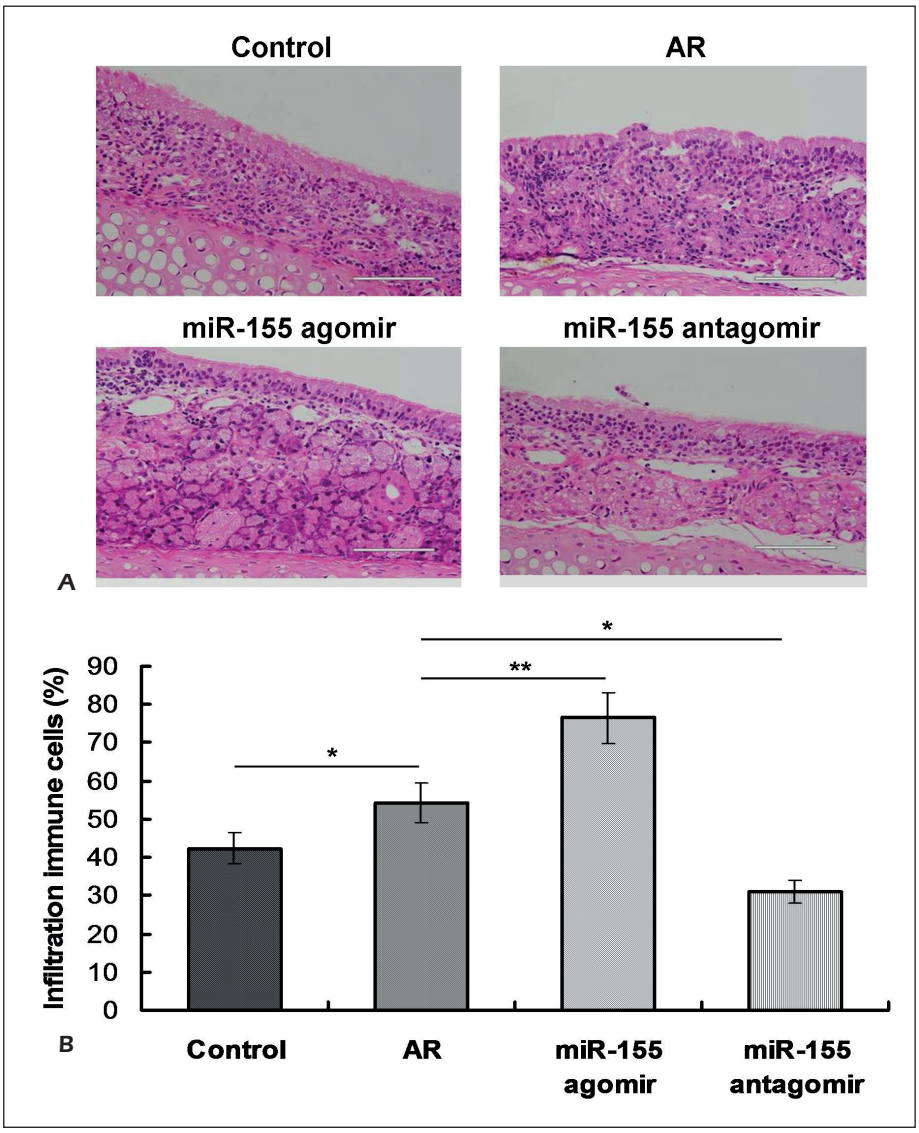

Figure 5. MiR-155 promotes immune cells infiltration in nasal mucosa of AR mice. **A**, Pathological alterations in nasal mucosa from each group were determined by using hematoxylin and eosin (HE) staining for histopathological examination. **B**, Statistical analysis for the HE staining cells. Representative images were shown (magnification ×100). *p<0.05; **p<0.01; n=6 in each group.

MiR-155 was Required for the Increase of ILC2s Expansion in Mice

To determine the regulation of miR-155 on ILC2s in mice nasal mucosa, we assessed the levels of ILC2s in the nasal mucosa of control mice, AR mice, miR-155 agomir mice, and miR-155 antagomir mice using flow cytometry. Strategies selected to identify nasal mucosal ILC2s are shown in Figure 7A. Lymphocytes were detected from mononuclear cells. Lineage-negative cells were further assessed for expression of CD45, KLRG1, CD90.2, and ILC2s were identified as lineage negative $CD45^{+}CD90.2^{+}KLRG1^{+}$ lymphocytes. The cell lineage cocktail consisted of antibodies to CD3e, CD11b, CD45R, Ly-76, Ly-6G, and Ly-6C. As expected, ILC2s were significantly enriched in AR mice (Figure 7A, 3.347% ± 0.2341%, p<0.0001) compared with controls (1.767% ± 0.08739%), demonstrating that intranasally administrated mice with IL-33 to expand ILC2s *in vivo*. Over-expression of miR-155 remarkably up-regulated the level of ILC2s (Figure 7B, 6.053% ± 0.4474%, p<0.001), whereas fewer ILC2s in the miR-155 antagomir mice (Figure 7B, 2.403% ± 0.2249%, p<0.05), both compared to AR mice.

Discussion

ILC2s are regulated by the transcription factor GATA-binding factor 3 (GATA3) and retinoic acid receptor-related orphan receptor α (RORα), secreting Th2 type cytokines (such as IL-5 and IL-13) under the stimulation of IL-25, IL-33, and

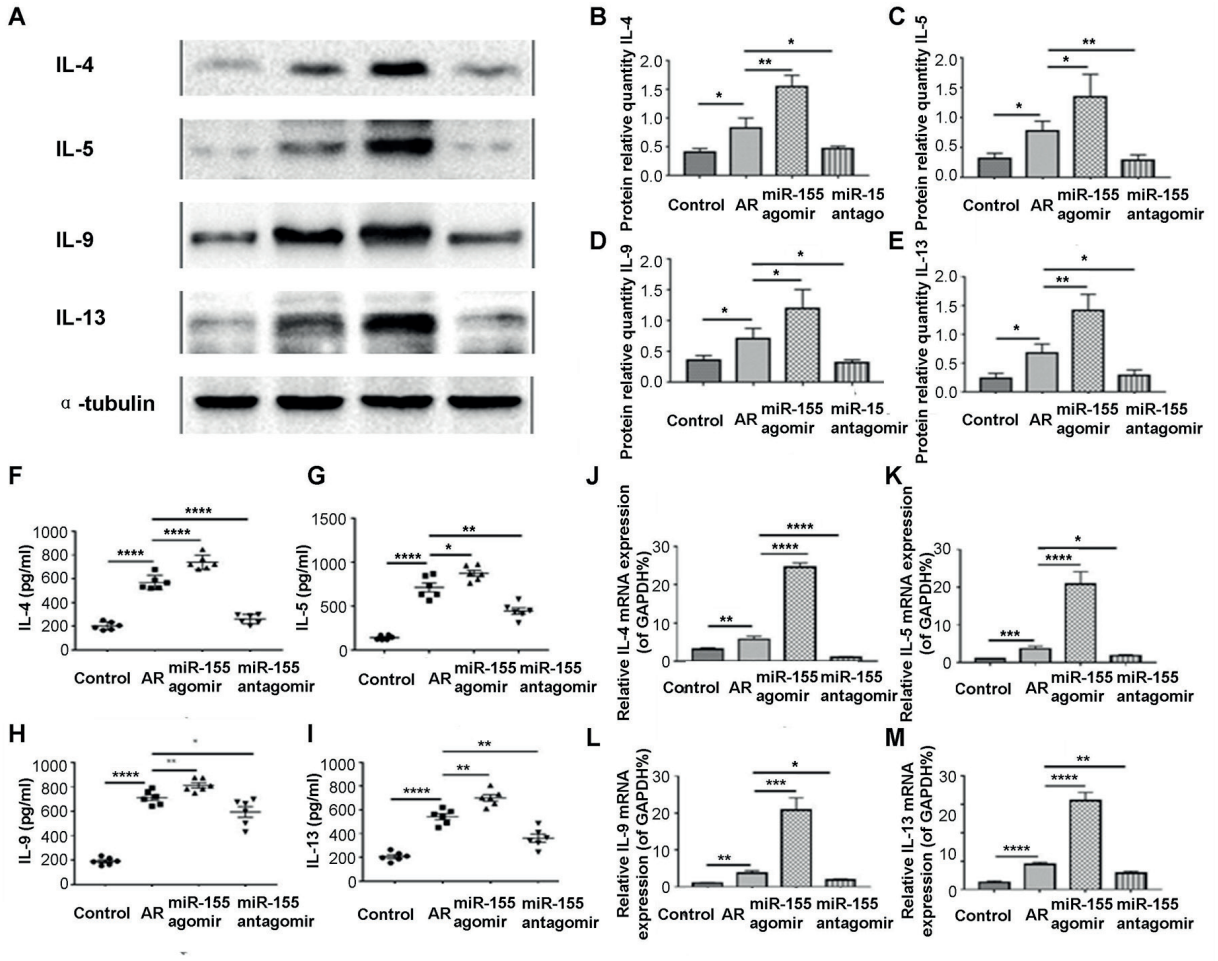

Figure 6. Up-regulation of MiR-155 raises the cytokines levels in AR mice. **A**, Western blot images. **B**-**E**, Nasal mucosal samples were lysed and analyzed for IL-4 (**B**), IL-5 (**C**), IL-9 (**D**), and IL-13 (**E**) levels using Western Blot assay. **F**-**I**, Levels of IL-4 (**F**), IL-5 (**F**), IL-9 (**H**), and IL-13 (**I**) in serum from each group were examined using ELISA. **J**-**M**, mRNA expressions of the above genes in nasal mucosa were determined by quantitative Real Time PCR. Data were presented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; n=6 in each group.

Thymic Stromal Lymphopoietin (TSLP) to participate in the Th2 immune response[18]. With the development of research on ILC2s, our understanding of the principles of immune regulation has changed, and it has also shown how innate immunity can profoundly affect the development of airway inflammation. ILC2s are the main cells involved in the early stage of acute allergic airway inflammation which provide an opportunity to select key molecular targets for early intervention for type 2 immune-mediated diseases such as asthma and allergic rhinitis[19]. A study evaluated ILC2s levels in asthmatic subjects, patients with asthma and AR, and healthy individuals. Patients with asthma and AR, as well as patients with asthma, were found to have higher ILC2s levels than healthy subjects. It was also found that ILC2s was positively correlated with the percentage of eosinophils and the severity of inflammation in patients with asthma and AR[20]. By examining the function of these cells, it was found that ILC2s plays a role in promoting adaptive immunity in addition to its definitive role in innate immunity. Thus, this new function of ILC2s needs to be considered when studying the pathophysiology of allergic airway diseases such as AR[21]. In this work, to investigate the function of ILC2s in AR patients, the proportion of ILC2s was measured by flow cytometry. The results showed that the proportion of ILC2s in AR patients was significantly higher

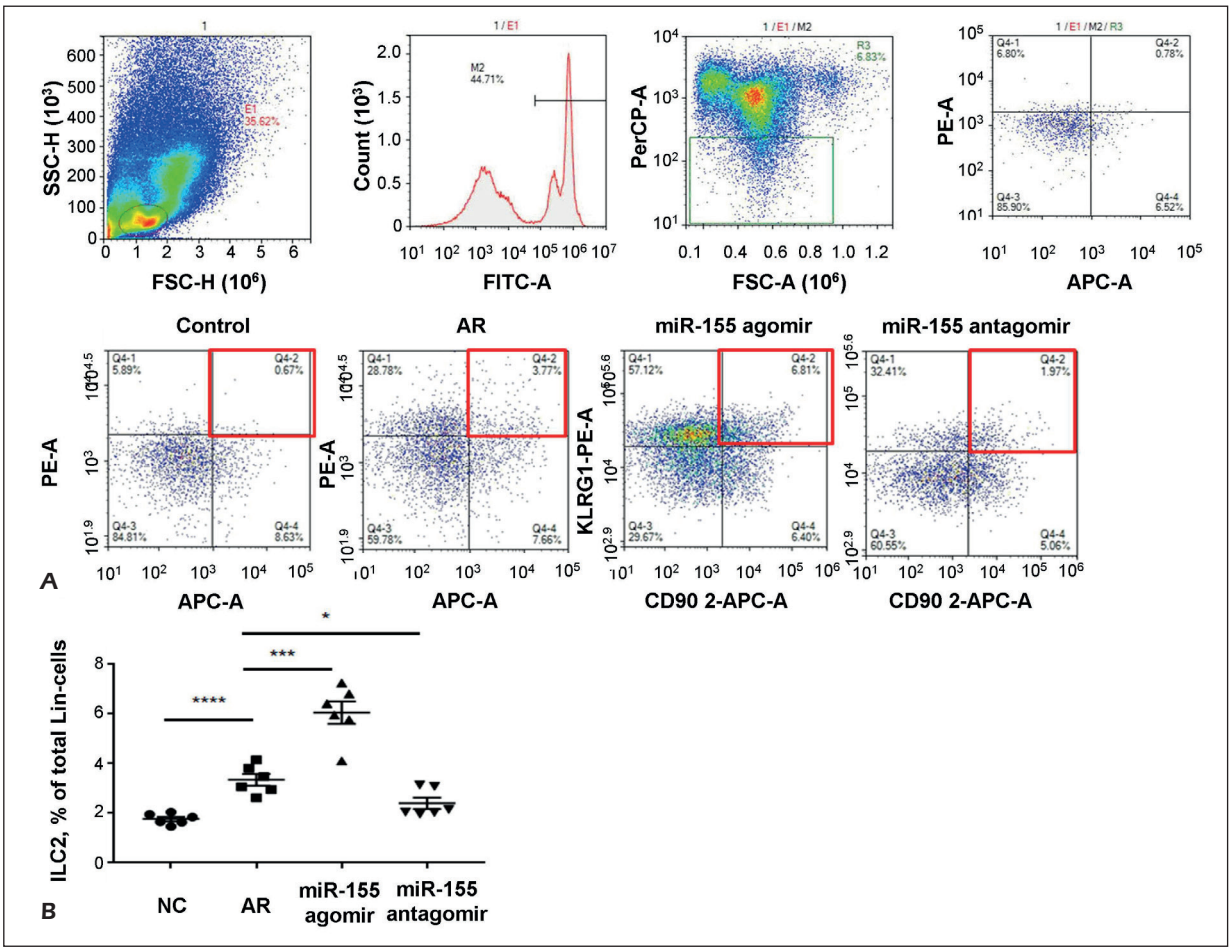

Figure 7. Functions of MiR-155 in the ILC2s expansion in mice. **A**, MiR-155 is required for the increase in ILC2s expansion. ILC2s were identified as lin- CD45+ CD90.2+ KLRG1+ lymphocytes. Lineage negative (CD3e, CD11b, CD45R, Ly-76, Ly-6G, and Ly-6C) cells were selected and further assessed for coexpression of CD45, CD90.2, and KLRG1. **B**, ILC2s levels in control mice, AR mice, miR-155 agomir mice, and miR-155 antagomir mice. The proportion of ILC2s was expressed as a percentage of total Lin-cells. ILC2s were significantly enriched in AR mice (3.347% ± 0.2341%, $p<0.0001$) compared with controls (1.767% ± 0.08739%). Compared to AR mice, over-expression of miR-155 remarkably up-regulated the level of ILC2s (6.053% ± 0.4474%, $p<0.001$), whereas fewer ILC2s in the miR-155 antagomir mice (2.403% ± 0.2249%, $p<0.05$). Each symbol represents a separate mouse. The horizontal bars indicate the median for each group. Data were presented as mean ± SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$; n=6 in each group.

than in healthy control subjects, further suggesting that ILC2s play an important role in maintaining the AR response and the expression levels of ILC2s are a potential prognostic biomarker and therapeutic target in AR. MiR-155 was thought to play a role in the function of the human immune system[22]. In fact, as an important immuno-modulator, miR-155 is mainly involved in the development and immune response of immune cells. Previous researches[23] have shown that miR-155 is becoming a critical regulatory molecule involved in the pathogenesis of various diseases, including Th2 inflammation. Suojalehto et al[24] analyzed inflammatory cells, cytokines, and microRNAs in the nasal biopsy of 159 young adult subjects, and observed that subjects with current AR symptoms had increased levels of miR-155. It has been reported that miR-155 expression is elevated in the nasal mucosa of AR patients, suggesting circulating miR-155 may be a potential biomarker in AR patients[25]. To further investigate the role of miR-155 in AR patients, we use real time-PCR to detect the level of miR-155. Consistent with the above results, we have found that miR-155 expression was increased in the nasal mucosa of AR patients, further showing that miR-155 may positively regulate the pro-allergic properties of AR. More importantly, for AR patients, there was

a positive linear correlation between the proportion of ILC2s (Spearman's method, r=0.4803, p=0.0130) and miR-155 expression. These findings imply the pivotal actors of ILC2s in AR, and miR-155 may be involved in the regulation of ILC2s function in the pathogenesis of AR. In fact, previous studies[26,27] in mice have shown that not only is ILC2s important for eliciting a type 2 immune response, but that ILC2s-induced cytokines (IL-25, IL-33) also contribute to the development of AR. IL-25 and IL-33 are epithelial-derived cytokines, which collectively drive Th2 polarisation through synergistic and complementary mechanisms. In response to IL-25 and IL-33, ILC2 produces large amounts of Th2 cytokines[28]. In our study, to further investigate the role of IL-25 and IL-33 in AR patients, we use qRT-PCR to detect the level of IL-25 and IL-33. Here, we found that IL-25 and IL-33 mRNA levels in AR patients increased compared with healthy people, indicating that ILC2s are positively correlated with the expression of IL-25 and IL-33 in AR. Compared to IL-25, IL-33 is the main inducer of the mouse model of allergic inflammation[29]. IL-33 is a member of the IL-1 family, expressing in human endothelial cells. By binding to the ST2 receptor on airway endothelial cells, playing an important role in anti-parasitic and allergen responses[30]. Neill et al[31] found that administration of IL-33 to mice caused accumulation of lineage negative cells (currently unified to name it ILC2) in the spleen and bone marrow. The mouse model of airway allergic inflammation has shown that IL-33 could induce increased expression of miR-155 in ILC2s, and miR-155 mediates the regulation of ILC2s promoting Th2 function[32]. Therefore, we adopted the method of intranasal challenges with recombinant murine IL-33 to establish an AR model. As expected, the expression of miR-155 in the nasal mucosa of AR model mice increased, suggesting that miR-155 may positively regulate the pro-allergenic properties of AR. To avoid systemic complications that may be caused by tail vein injection, we intranasally administered with miR-155 agomir and miR-155 antagomir in AR mice to investigate the directed effects of miR-155 on the nasal mucosa. After administration with miR-155 agomir, miR-155 expression was significantly increased, whereas, after administration with miR-155 antagomir, miR-155 expression was decreased, both compared to the AR model mice. In addition, the symptom of rubbing and sneezing were much higher in miR-155 agomir mice than in AR model mice, whereas miR-155 antagomir mice had the much lower behavior score, indicating that intranasal administration of miR-155 can promote allergy symptoms of AR. As mentioned above, AR is a chronic inflammatory disease mainly based on Th2 inflammatory response. Th2 cytokines such as IL-4, IL-5, and IL-13 play an important role in recruiting eosinophils, regulating the differentiation of T cells from the Th2 type and the IgE isotype switching in B cells[33]. Although ILC2s has no T cell surface marker, the functions similarly to Th2 cells, also produce a large number of Th2 cytokines (IL-4, IL-5, and IL-13) and a small amount of cytokines such as IL-9 after allergen stimulation[34]. In this study, we examined the mRNA and protein expression levels of IL-4, IL-5, IL-9, and IL-13. The results showed that the levels of IL-4, IL-5, IL-9, and IL-13 were significantly increased in miR-155 agomir mice, while intranasal administration with miR-155 antagomir inhibited the expression of the aforementioned Th2 cytokine. The pathophysiology associated with Th2 inflammatory response is regulated by cytokines. Therefore, we observed pathological alterations in the nasal mucosa of AR mice, including hyperemia, edema, necrosis, and aberrant structure. All of these pathological alterations were worsened after miR-155 agomir administration and ameliorated after administration of miR-155 antagomir. Consistent with these results, after intranasal administration with miR-155 agomir, the ratio of ILC2s in the nasal mucosa of AR mice was significantly increased. However, intranasal administration with miR-155 antagomir markedly inhibited ILC2s infiltration in nasal mucosa of AR mice. ILC2s were shown to be involved in the coordination of Th2-type immune responses through the overexpression of Th2 cytokines in AR mice models. Our results suggest that miR-155 is required for the increase in ILC2s expansion response to IL-33 in mice.

Conclusions

We demonstrated that miR-155 and ILC2s were overexpressed in AR compared with controls. These results suggest that the higher ratio of ILC2s is correlated closely with the expression of miR-155, while the high expression of ILC2s promotes AR inflammatory response by releasing large amounts of Th2 cytokines (IL-4, IL-5, IL-9, and IL-13). In any event, these findings suggest that the expression of miR-155 and ILC2s may be a potential therapeutic target for AR.

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Conflict of Interests

The Authors declared that they have no conflict of interests.

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