

# Mechanism of miR-181a-5p in Regulatory T/T-Helper 17 Immune Imbalance and Asthma Development in Mice with Allergic Rhinitis

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## Keywords

Allergic rhinitis · Treg/Th17 immune imbalance · Bronchial asthma · miR-181a-5p · HMGB1/RAGE signaling axis

## Abstract

**Introduction:** Allergic rhinitis (AR) is an immune disorder and also a risk factor of asthma. microRNAs (miRNAs) are implicated in autoimmune diseases, including RA. This study investigated effect of miR-181a-5p on regulatory T (Treg)/T-helper (Th) 17 immune imbalance in AR. **Methods:** A murine model of AR was established and treated with lentivirus modified miR-181a-5p. The allergic symptoms of mice were examined. The contents of Th17-related cytokines (interferon [IFN]- $\gamma$  and interleukin [IL]-6), Treg-related cytokine (IL-10), and Treg-specific nuclear transcription factor (Foxp3) in nasal mucosa and lung tissues were determined. The proportion of Treg and Th17 cells was analyzed by flow cytometry. The level of ovalbumin-specific immunoglobulin E in the serum, and the contents of IL-4, IL-5, and IL-13 in bronchoalveolar lavage fluid and IFN- $\gamma$ , IL-6, and IL-10 in nasal lavage fluid were measured. The targeting relationship between miR-181a-5p and high mobility group box chromosomal protein 1 (HMGB1) was verified. HMGB1 and receptor for advanced glycation end products (RAGE) expression in

RA were determined, and the interaction between HMGB1 and RAGE was detected. **Results:** miR-181a-5p expression was reduced in AR mice. miR-181a-5p overexpression attenuated allergic behaviors, alleviated Treg/Th17 imbalance, and delayed asthma development. HMGB1 and RAGE were elevated in AR mice. miR-181a-5p targeted HMGB1, and HMGB1 bound to RAGE, while miR-181a-5p overexpression reduced the binding between them. Activating HMGB1/RAGE reversed the protective effect of miR-181a-5p overexpression on AR and induced the development of asthma. **Conclusion:** miR-181a-5p overexpression reduced the binding of HMGB1 and RAGE by inhibiting HMGB1, thus alleviating Treg/Th17 immune imbalance and blocking AR from developing into asthma.

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## Introduction

Allergic rhinitis (AR) is a type I hypersensitivity disorder mediated by the allergen-specific immunoglobulin E (IgE), which constitutes one of the most prevalent immunological diseases [1]. AR is typically manifested as nasal

Edited by: H.-U. Simon, Bern.

itching, rhinorrhea, sneezing, and nasal congestion, but may also involve ocular symptoms and cough [2]. The pathogenesis of AR is complex, in which the imbalance of innate and adaptive immunity and environmental factors may play a major role [3]. Currently, AR can be temporarily relieved by inhaled corticosteroids, antihistamines, and leukotriene receptor antagonists, but the symptoms will relapse soon after withdrawal [4]. Although AR itself is not life-threatening, it causes many complications and represents a salient risk factor for bronchial asthma, thus reducing the quality of life and the productivity of study or work [5]. Hence, it is necessary to further understand the pathogenesis of AR and then formulate potent therapeutic strategies to prevent the development of AR into asthma.

It is well established that T-helper (Th)1/Th2 imbalance has a critical role in the pathogenesis of AR, characterized by Th2 cell-mediated inflammatory response [6]. Recently, the critical role of regulatory T (Treg)/Th17 cell-mediated immunity imbalance has also been identified in AR patients [7–9]. Treg cell is a subset of T lymphocytes implicated in allergic conditions, which can modulate the pathophysiological immune response, promote autoimmune tolerance, and maintain immune balance [10]. Th17 cell is another subset of T lymphocytes that contributes to pro-inflammatory inflammation and immune pathology [11]. AR patients present increased Th17 cells and decreased Treg cells in the peripheral blood, suggesting the involvement of Th17/Treg imbalance in the pathogenesis of AR [11]. Th17/Treg cells together with their representative cytokines interleukin (IL)-17A/IL-10 have significant correlation with asthma, and they are functionally antagonistic [12]. Hence, controlling Treg/Th17 balance in AR patients appears to be a promising therapeutic approach.

The role of epigenetic alterations, especially microRNA (miRNA) regulation, in allergic diseases has been emphasized [13]. miRNAs are small noncoding RNA molecules (about 18–22 nucleotides in length) that function as antisense RNAs to silence target genes at the post-transcription level [14]. miRNAs participate in diverse immunological and inflammatory diseases, including allergic inflammation, and some specific miRNA profiles have been revealed in AR and asthma [15]. Treg-derived miR-181a has been supposed as a modulator of AR progression in children [16]. miR-181a-5p is an essential regulator of T lymphocyte differentiation and activation, participating in immune modulation [17]. For example, miR-181a-5p ameliorates inflammatory response in pulmonary arterial hypertension induced by monocrotaline via target-

ing endocan [18]. Nevertheless, the role of miR-181a-5p in Treg/Th17 imbalance and AR development is still unclear. Pro-inflammatory factors mediate the development and persistence of rhinitis including AR [19]. High mobility group box chromosomal protein 1 (HMGB1), one of the target genes, acts as a promoter of inflammatory mediators. It has been reported that HMGB1 can increase the nasal secretion of AR patients [20], and HMGB1 is highly expressed in AR [21, 22]. Receptor for advanced glycation end (RAGE) is a receptor of HMGB1. After RAGE knockout, the severity of AR induced by dust mite was significantly downregulated [23]. Therefore, we chose HMGB1 as the downstream target of miR-181a-5p in this study and studied the pro-inflammatory signal axis of HMGB1/RAGE. This study sought to explore the effect of miR-181a-5p on Treg/Th17 immune imbalance and the development of AR into bronchial asthma, hoping to confer a novel theoretical basis and promising target for AR treatment.

## Materials and Methods

### *Ethics Statement*

All the animal experiments were implemented on the Guide for the Care and Use of Laboratory Animals [24] and were approved by the Institutional Animal Care and Use Committee of The Affiliated Hospital of Guangdong Medical University (approval No.: AE202001016). Extensive efforts were made to minimize the suffering of the included animals.

### *Establishment of a Murine Model of AR*

Male BALB/c mice (aged 6–8 weeks and weighing 18–21 g) purchased from Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) (SYXK [Beijing] 2017-0033) were raised in the animal room at 20–26°C with 40–60% humidity and maintained under a 12 h light/dark cycle. The mice had free access to food and water. Then, the mice were randomly grouped. The mice were intraperitoneally injected with 1 mg aluminum hydroxide (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and 300 µL normal saline containing 100 µg ovalbumin (OVA) on the 0, 7th, and 14th day to induce the first sensitization. From the 21st to 35th day, the mice were intranasally sensitized by 40 µL normal saline containing 400 µg OVA (20 µL each nostril) for secondary immunization to establish AR mice [25, 26]. The mice in the sham group were intraperitoneally injected with 300 µL normal saline on the 0, 7th, and 14th day, and intranasally injected with 40 µL normal saline from the 21st to 35th day (20 µL each nostril). The lentivirus over-expression vectors containing miR-181a-5p and HMGB1 and their negative controls were constructed by Genechem (Shanghai, China). Within 3 h after AR induction from the 21st to 35th day, lentivirus ( $2 \times 10^6$  IFUs) was intranasally administered to AR mice.

### *Symptom Score*

Briefly, 30 min after the last OVA induction, the behaviors of the mice were examined, including the frequencies of nasal rub-

bing and sneezing within a given time period, to assess nasal hypersensitivity-like symptoms. Video surveillance equipment recorded all symptoms within 10 min. Behavior analysis was conducted by professionals in a double-blind way.

#### Detection of Eotaxin and RANTES Concentration

The nasal lavage fluid (NALF) was collected after irrigation and centrifuged at 8,000 g and 4°C for 15 min to obtain supernatant. The concentration of eotaxin (DY420; R&D Systems, Minneapolis, MN, USA) and RANTES (DY478; R&D Systems) was determined using DuoSet mouse enzyme-linked immunosorbent assay (ELISA) kits. Additionally, the contents of IL-6 (ab222503; Abcam, Cambridge, MA, USA), IL-10 (ab255729; Abcam), and interferon (IFN)- $\gamma$  (ab252363; Abcam) in NALF were detected using ELISA kits.

#### Detection of OVA-Specific IgE Concentration in the Serum

To assess allergic reactions, the level of OVA-specific IgE in the serum was measured using an ELISA kit (CUSABIO, Wuhan, China). Briefly, the blood sample was collected from the orbital vein cluster of anesthetized mice at the time of death. The serum was harvested from the blood sample after 30 min, centrifuged at 2,000 g and 4°C for 10 min, and stored at -80°C. Then, the serum was added to the 96-well plates pre-coated with purified IgE mAb and cultured at 37°C for 2 h. Next, anti-mouse IgE-conjugated horseradish peroxidase was added to the plates for 1-h incubation at 37°C. Afterward, the plates were incubated with 3,3',5,5'-tetramethylbenzidine (Moss Inc., Belfast, ME, USA) for 1 h, and the reaction was terminated by adding H<sub>2</sub>SO<sub>4</sub>. The optical density at the wavelength of 450 nm was measured using a luminometer (AutoLumat Plus; Berthold Technology, Calmbacher, Germany).

#### Isolation and Differential Counting of Cells in Bronchoalveolar Lavage Fluid

Briefly, 24 h after the last OVA challenge, mice in each group were euthanized by intraperitoneal injection of 100 mg/kg pentobarbital sodium. BALF was performed on the right lung of mice after tracheotomy and intubation. Phosphate-buffered saline (1.5 mL) was slowly injected into the lung 3 times, and then BALF was retrieved. BALF was centrifuged at 100 g and 4°C for 10 min. The supernatant was frozen at -80°C for ELISA detection of cytokines. The precipitated cells after centrifugation were collected. After the red blood cells were removed using the red blood cell lysis solution, the cell sediment was re-suspended with 1 mL phosphate-buffered saline, and the total number of leukocytes was counted using a hemocytometer. Next, 0.1 mL cell suspension was taken for eosinophils (EOS) counting and the percentage of EOS was calculated (percentage of EOS in the total number of cells). Additionally, the contents of IL-4 (ab242230; Abcam), IL-5 (ab204523; Abcam), and IL-13 (ab219634; Abcam) were determined using ELISA kits.

#### Flow Cytometry

To quantify Th17 and Treg cells, the nasal mucosa was cut into small pieces, incubated at 37°C with 2.5 mm EDTA, and stirred for 20 min to remove the cells. The samples were detached using the collagenase type VIII (Sigma-Aldrich) at 37°C for 20 min. T cells were isolated by Pan T-cell separation kit II (11465d; Thermo Fisher, Rockford, IL, USA). The isolated T cells were stained with anti-CD4 antibody (PE coupling, ab252151; Abcam), anti-Foxp3 antibody (FITC coupling, ab210230; Abcam), and anti-IL-17A anti-

**Table 1.** PCR primer sequence

| Name          | Sequence (5'-3')                                        |
|---------------|---------------------------------------------------------|
| miR-181a-5p   | F: TCGGCAGGAACATTCAACGCTGT<br>R: CCAACTGGTGTCTGTGGA     |
| HMGB1         | F: ATGGGCAAAGGAGATCTTAA<br>R: TCATCTTCTTCTTCATCTTCG     |
| RAGE          | F: ATGCCAGCGGGGACAGCA<br>R: TTACGGTCCCCCGGCACCAT        |
| IFN- $\gamma$ | F: ATGAACGCTACACACTGCATC<br>R: TCAGCAGCGACTCCTTTTCCGC   |
| IL-6          | F: ATGAAGTTCCTCTCTGCAAGAG<br>R: TTAGCCACTCTTCTGTGACTCC  |
| IL-10         | F: ATGCCTGGCTCAGCACTGCTAT<br>R: GAGCTTATTAATACTACTCTTAC |
| Foxp3         | F: ATGCCCCAACCTAGGCCAGC<br>R: TCAAGGGCAGGGATTGGAGCAC    |
| IL-4          | F: ATGGGTCTCAACCCCCAGCTAG<br>R: CTACGAGTAATCCATTTGCATG  |
| IL-5          | F: ATGAGAAGGATGCTTCTGCAC<br>R: TCAGCCTCCATTGCCCACTCT    |
| IL-13         | F: ATGGCGCTCTGGGTGACTGCA<br>R: TTAGAAGGGGCGCTGGCGAAAC   |
| GAPDH         | F: ATGCTGCCCTTACCCCGGGGT<br>R: TTACTCCTTGGAGGCCATGTAGGC |
| U6            | F: GCTCGCTTCGGCAGCACATATACT<br>R: GGAACGCTTCACGAATTTGCC |

HMGB1, high mobility group box chromosomal protein 1; RAGE, anti-receptor for advanced glycation end products; IFN, interferon; IL, interleukin

body (FITC coupling, 11-7177-81; Thermo Fisher). The cell sorting analysis was performed by FACSCalpur flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

#### Dual-Luciferase Assay

Dual-luciferase reporter gene assay was performed to detect the direct binding of miR-181a-5p and HMGB1 (Gene ID: 15289; GenBank). According to the binding site of miR-181a-5p and HMGB1 obtained from Starbase database, the wild-type and mutant-type reporter plasmids of HMGB1 containing a wild or mutant miR-181a-5p binding site were designed and synthesized by GenePharma (Shanghai, China) and then amplified and cloned into the pMIR-Report™ vector (Ribobio Co., Guangzhou, China). The constructed vectors were transfected with miR-181a-5p mimic or its negative control into 293T cells (American Type Culture Collection, Manassas, VA, USA) using Lipofectamine 2000 (Invitrogen Inc., Carlsbad, CA, USA). After 48 h of incubation, the luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Madison, WI, USA).

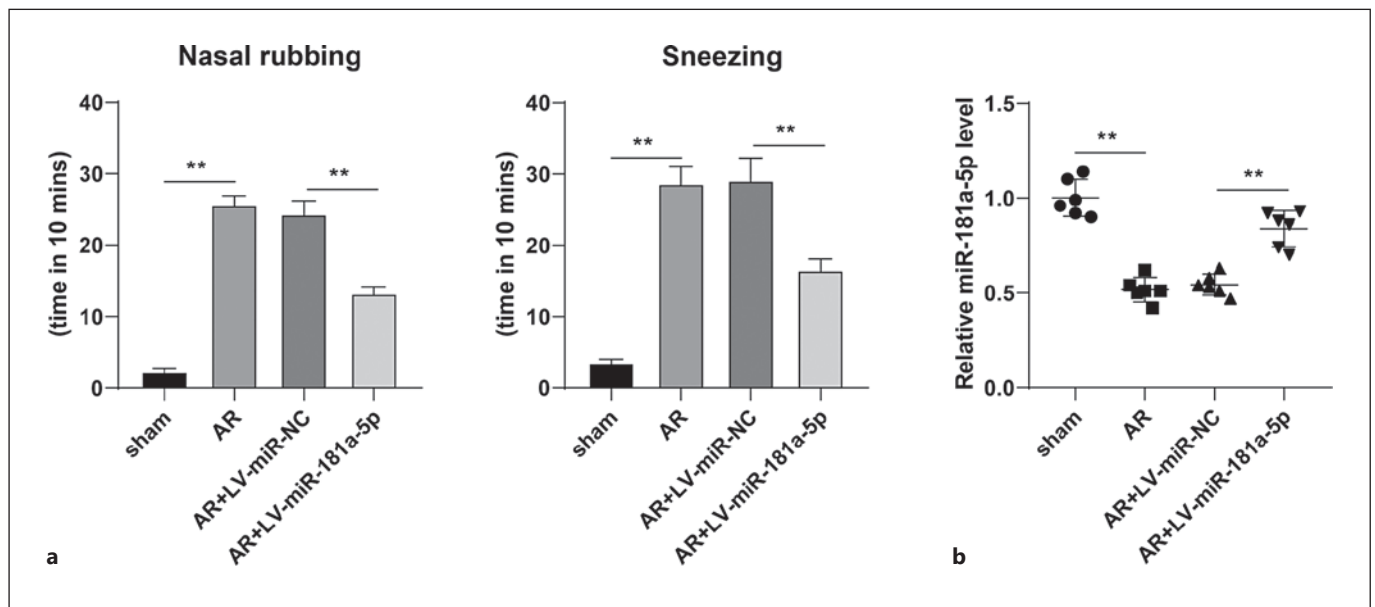


**Fig. 1.** miR-181a-5p overexpression contributed to the recovery of AR in mice. A murine model of AR was established using OVA and aluminum hydroxide, and AR mice were treated with LV-miR-181a-5p, with LV-miR-NC as control. **a** The frequencies of nasal rubbing and sneezing were observed. **b** miR-181a-5p expression in nasal mucosa tissues was determined using RT-qPCR.  $N =$

6. Data in **a** are presented as mean  $\pm$  standard deviation and analyzed using one-way ANOVA, followed by Tukey's multiple comparisons tests,  $**p < 0.01$ . AR, allergic rhinitis; LV-miR-181a-5p, lentivirus vector of miR-181a-5p; LV-miR-NC, negative control of lentivirus vector; ANOVA, analysis of variance; RT-qPCR, reverse-transcription quantitative polymerase chain reaction.

#### Co-Immunoprecipitation

Immunoprecipitation lysis buffer (Millipore, Billerica, MA, USA) containing protease inhibitor was supplemented into the harvested tissues for lysis at 4°C for 30 min. After centrifugation at 12,000 g for 30 min, the protein concentration was determined using the bicinchoninic acid (Beyotime, Shanghai, China) assay. Then, RAGE antibody (ab216329; Abcam), IgG (ab133470; Abcam), and protein A/G beads were added into the protein sample and incubated at 4°C overnight with gentle vortexing. Afterward, protein A/G beads were centrifuged at 3,000 g and 4°C and washed 3–4 times with 1 mL lysis buffer. Finally, protein A/G beads were added with 5× sodium dodecyl sulfate loading buffer and boiled for 10 min to elute protein, followed by SDS-PAGE. The protein was detected using anti-HMGB1 antibody and visualized using Western blot.

#### Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

The total RNA was extracted from nasal mucosa tissues and lung tissues using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific). PCR was performed using FastStart Universal SYBR Green Master Mix (Rox) (Roche Applied Science, Indianapolis, IN, USA) and SLAN<sup>®</sup> Real-Time PCR Detection System (Hongshi Medical Technology Co., Shanghai, China): pre-denaturation at 95°C for 10 min, and then 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 60 s, and finally extension at 60°C for 5 min. The relative expression of gene was calculated using the  $2^{-\Delta\Delta Ct}$  method [27], with GAPDH or U6 as the internal reference. The primers are shown in Table 1.

#### Western Blot

The total protein was extracted from mouse nasal mucosa tissues using radio-immunoprecipitation assay buffer (Beyotime). Equal amount of protein sample was separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk at 25°C for 2 h and incubated with the primary antibodies HMGB1 (1:500, PA5-32249; Thermo Fisher), RAGE (1:1,000, ab216329; Abcam), and GAPDH (1:10,000, ab181602; Abcam) at 4°C overnight. Next, the membranes were washed with TBST 5 times and then incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000, ab205718; Abcam) for 2 h. The immunoreactivity of membranes was detected using the enhanced chemiluminescence kit (Millipore). The relative protein content was expressed as the gray value of the corresponding protein band/the gray value of GAPDH protein band. The gray value of each band was quantified using Image J software (National Institutes of Health, Bethesda, MA, USA).

#### Statistical Analysis

Data analysis and map plotting were performed using the SPSS 21.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). Kolmogorov-Smirnov test was used to verify the normal distribution and homogeneity of variance. Data are expressed as mean  $\pm$  standard deviation. One-way or 2-way analysis of variance was employed for the comparisons among multiple groups, followed by Tukey's multiple comparisons tests or Sidak's multiple comparisons tests. A value of  $p < 0.05$  indicated significant difference.

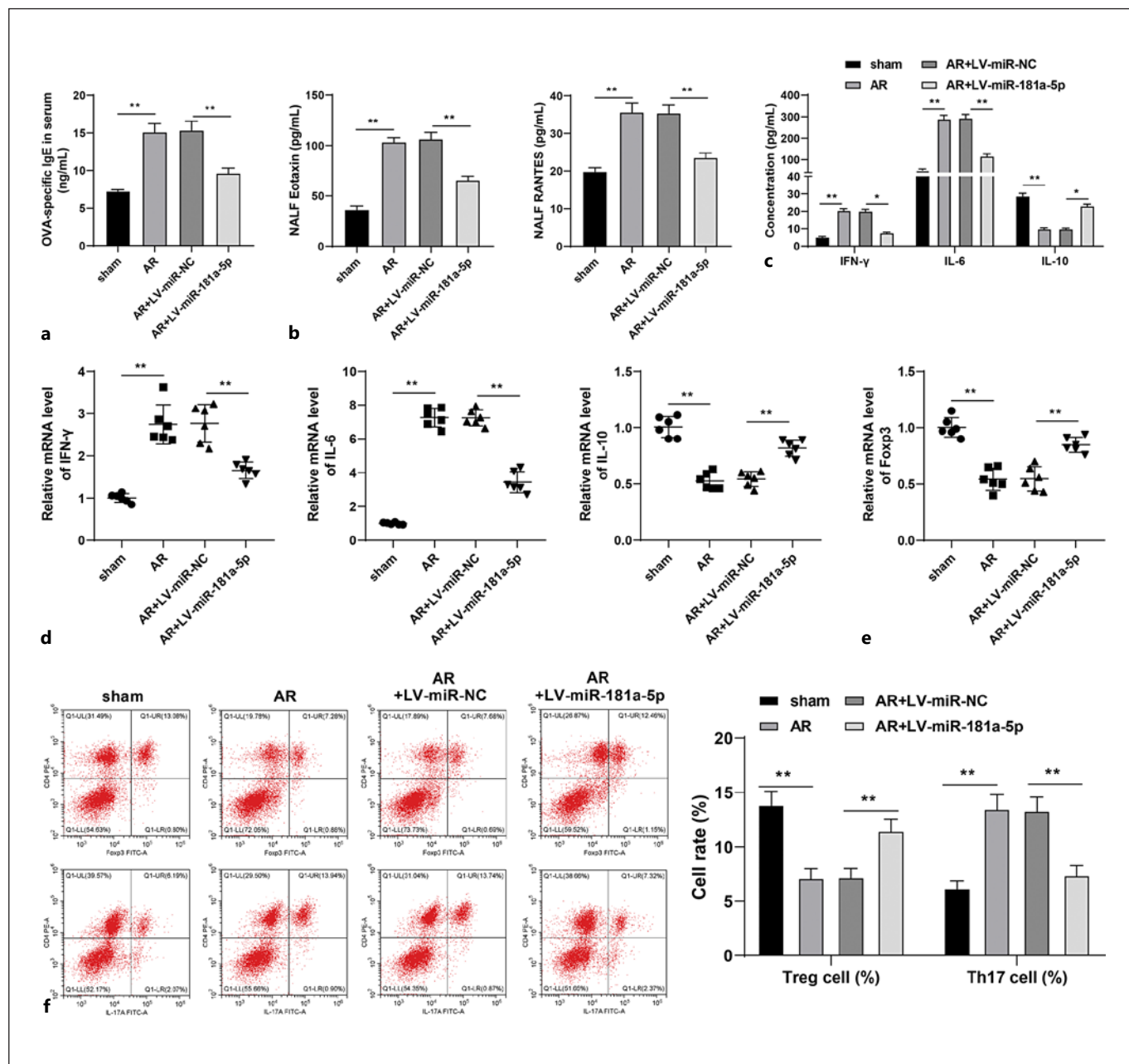


**Fig. 2.** miR-181a-5p overexpression protected against Treg/Th17 immune imbalance in AR mice. **a** The level of OVA-specific IgE in the serum. **b** The contents of eotaxin and RANTES in mouse NALF. **c** The contents of IFN- $\gamma$ , IL-6, and IL-10 in mouse NALF were detected using ELISA kits. **d**, **e** The levels of IFN- $\gamma$ , IL-6, IL-10, and Foxp3 mRNA expression in nasal mucosa tissues were detected using RT-qPCR. **f** The proportion of Treg and Th17 cells in nasal mucosa was analyzed by flow cytometry.  $N = 6$ . Data in **a–c** are presented as mean  $\pm$  standard deviation. Data in **d**, **e**

were analyzed using one-way ANOVA, and data in **c**, **f** were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons tests,  $*p < 0.05$ ,  $**p < 0.01$ . AR, allergic rhinitis; LV-miR-181a-5p, lentivirus vector of miR-181a-5p; LV-miR-NC, negative control of lentivirus vector; NALF, nasal lavage fluid; Treg, regulatory T; Th, T-helper; IFN, interferon; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; IgE, immunoglobulin E; RT-qPCR, reverse-transcription quantitative polymerase chain reaction.

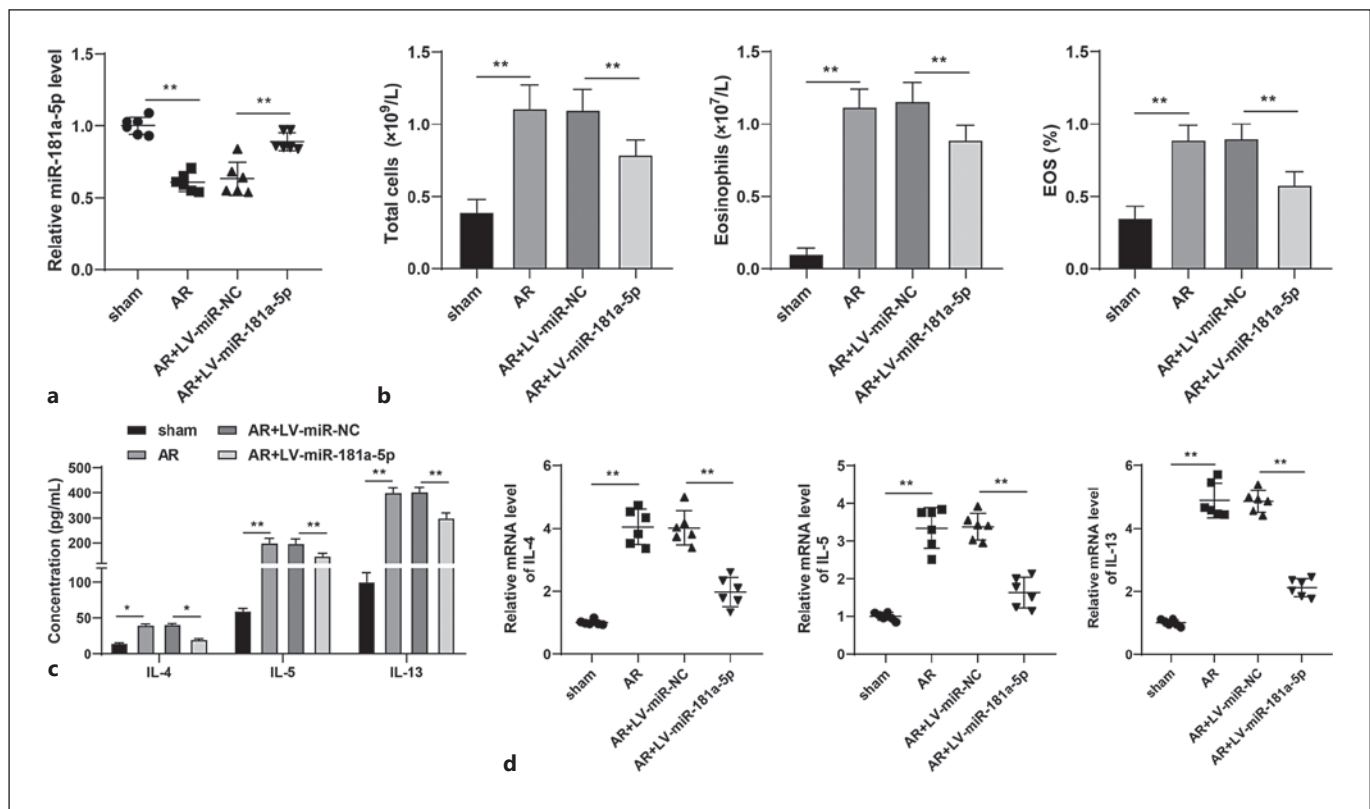


**Fig. 3.** miR-181a-5p overexpression blocked AR mice from developing asthma. **a** RT-qPCR was used to detect the expression of miR-181a-5p in lung tissue. **b** The total number of leukocytes, and the absolute count and the percentage of EOS in BALF. **c** The contents of IL-4, IL-5, and IL-13 in BALF were detected using ELISA kits. **d** The levels of IL-4, IL-5, and IL-13 mRNA expression in lung tissues were detected using RT-qPCR.  $N = 6$ . Data in **a**, **b** are presented as mean  $\pm$  standard deviation. Data in **a**, **b**, **d** were analyzed

using one-way ANOVA, and data in **c** were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons tests,  $*p < 0.05$ ,  $**p < 0.01$ . AR, allergic rhinitis; LV-miR-181a-5p, lentivirus vector of miR-181a-5p; LV-miR-NC, negative control of lentivirus vector; BALF, bronchoalveolar lavage fluid; EOS, eosinophil; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance.

## Results

### miR-181a-5p Overexpression Contributed to the Recovery of AR Mice

Increasing evidence has confirmed the involvement of miRNAs in rhinitis development [28, 29]. miR-181a-5p is involved in the regulation of AR [16, 30], but its specific mechanism in AR remains elusive. Hence, we established a murine model of AR using OVA and aluminum hydroxide, and found that the frequencies of nasal rubbing and sneezing were increased in AR mice ( $p < 0.01$ ; Fig. 1a). Then, 24 h after the last OVA challenge, the nasal mucosa tissues were collected for RT-qPCR. It was found that miR-181a-5p expression was reduced in AR mice ( $p < 0.01$ ; Fig. 1b). To determine the effect of miR-181a-5p on AR, we treated AR mice with miR-181a-5p overex-

pression lentivirus vector and found that miR-181a-5p expression in nasal mucosa tissues of mice was notably increased ( $p < 0.01$ ; Fig. 1b), while the frequencies of nasal rubbing and sneezing were decreased ( $p < 0.01$ ; Fig. 1a). These results suggested that miR-181a-5p overexpression was beneficial to the recovery of AR in mice.

### miR-181a-5p Overexpression Protected against Treg/Th17 Immune Imbalance in AR Mice

Treg/Th17 cell dysfunction may be one of the pathogenesises of AR [11, 31]. Hence, we investigated the effect of miR-181a-5p on Treg/Th17 immune imbalance. The content of OVA-specific IgE in the serum was increased after OVA induction, while miR-181a-5p overexpression decreased the content of OVA-specific IgE in the serum ( $p < 0.01$ ; Fig. 2a). Eotaxin and RANTES are chemokines-regulating

EOS [32, 33]. ELISA results showed that OVA induction increased the contents of eotaxin and RANTES in mouse NALF, while miR-181a-5p overexpression treatment reversed these trends ( $p < 0.01$ ; Fig. 2b). Moreover, IFN- $\gamma$  and IL-6 are Th17-related cytokines; IL-10 is Treg-related cytokine, and Foxp3 is Treg-specific nuclear transcription factor [34, 35]. OVA induction increased the contents of IFN- $\gamma$  and IL-6 and decreased the content of IL-10, while these trends were reversed after miR-181a-5p overexpression ( $p < 0.01$ ; Fig. 2c, d). Additionally, OVA induction down-regulated Foxp3 mRNA expression, while miR-181a-5p overexpression elevated Foxp3 mRNA expression ( $p < 0.01$ ; Fig. 2e). In addition, flow cytometry showed that the proportion of Treg cells was decreased and the proportion of Th17 cells was increased after OVA induction, which was partially reversed by overexpression of miR-181a-5p ( $p < 0.01$ , Fig. 2f). Briefly, miR-181a-5p overexpression protected AR mice from Treg/Th17 immune imbalance.

#### *miR-181a-5p Overexpression Retarded the Development of Asthma in AR Mice*

AR is also a risk factor for the development of other diseases, including bronchial asthma [36, 37], and Th17/Treg immune imbalance is closely related to the onset and severity of asthma [38, 39]. To explore the effect of miR-181a-5p on the development of asthma in AR mice, we collected the lung tissue and BALF of mice in each group. The expression of miR-181a-5p was decreased in the lung tissue of AR mice, while the expression of miR-181a-5p was increased after overexpression of miR-181a-5p ( $p < 0.01$ , Fig. 3a). In addition, the total number of leukocytes and the absolute count and the percentage of EOS in BALF of AR mice were notably increased, while the above indexes were decreased in AR mice-overexpressing miR-181a-5p ( $p < 0.01$ ; Fig. 3b). Th2 cytokines, such as IL-4, IL-5, and IL-13 can induce allergic asthma [40, 41]. ELISA and RT-qPCR results revealed that OVA induction increased the levels of IL-4, IL-5, and IL-13 in BALF and lung tissues, while miR-181a-5p overexpression treatment showed an opposite trend ( $p < 0.01$ ; Fig. 3c, d). Briefly, miR-181a-5p overexpression retarded the development of asthma in AR mice.

#### *miR-181a-5p Targeted HMGB1 and Then Reduced the Binding of HMGB1 and RAGE*

To explore the downstream mechanism of miR-181a-5p in AR, we predicted the target genes of miR-181a-5p through Starbase (<http://starbase.sysu.edu.cn/index.php>) and Targetscan ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/)) databases and took the intersection (Fig. 4a). Among them,

HMGB1 is highly expressed in AR [21, 22] and plays a role in immune diseases via the HMGB1/RAGE pro-inflammatory signaling axis [42, 43]. Our results demonstrated that HMGB1 and RAGE were elevated in AR mice, but reduced in after miR-181a-5p overexpression treatment ( $p < 0.01$ ; Fig. 4b, c). Based on the binding site of miR-181a-5p and HMGB1 predicted by database (Fig. 4d), we designed dual-luciferase assay and confirmed the targeting relationship between miR-181a-5p and HMGB1 ( $p < 0.01$ ; Fig. 4e). Also, co-immunoprecipitation assay verified that HMGB1 could bind to RAGE, while miR-181a-5p overexpression inhibited the binding of HMGB1 and RAGE (Fig. 4f). Briefly, miR-181a-5p targeted HMGB1 and then reduced the binding of HMGB1 and RAGE.

#### *HMGB1 Overexpression Attenuated the Protective Effect of miR-181a-5p on Treg/Th17 Imbalance in AR Mice by Binding to RAGE*

Next, we verified the role of the HMGB1/RAGE axis in miR-181a-5p regulating Treg/Th17 immune imbalance in AR mice. We treated the AR mice overexpressing miR-181a-5p with lentivirus vector of HMGB1 and successfully upregulated HMGB1 expression in nasal mucosa tissues ( $p < 0.01$ ; Fig. 5a, b), and the binding of HMGB1 and RAGE was also increased ( $p < 0.01$ ; Fig. 5c). HMGB1 overexpression increased the frequencies of nasal rubbing and sneezing in AR mice ( $p < 0.01$ ; Fig. 5d), elevated the content of OVA-specific IgE in the serum ( $p < 0.01$ ; Fig. 5e) and the levels of IFN- $\gamma$  and IL-6 and the proportion of Th17 cells, but decreased IL-10 level and Foxp3 mRNA expression and the proportion of Treg cells ( $p < 0.01$ ; Fig. 5f–h). Briefly, HMGB1 overexpression attenuated the protective effect of miR-181a-5p on Treg/Th17 imbalance in AR mice by binding to RAGE.

#### *HMGB1 Overexpression Reversed the Inhibitory Effect of miR-181a-5p on the Development of Asthma in AR Mice by Binding to RAGE*

Thereafter, the role of the HMGB1/RAGE axis in the inhibitory effect of miR-181a-5p on the development of AR to bronchial asthma was verified. HMGB1 overexpression upregulated the total number of leukocytes and the absolute count and the percentage of EOS in BALF ( $p < 0.01$ ; Fig. 6a). Also, the levels of IL-4, IL-5, and IL-13 in BALF and lung tissues were increased after HMGB1 overexpression ( $p < 0.01$ ; Fig. 6b, c). Briefly, HMGB1 overexpression reversed the inhibitory effect of miR-181a-5p on the development of asthma in AR mice by binding to RAGE.

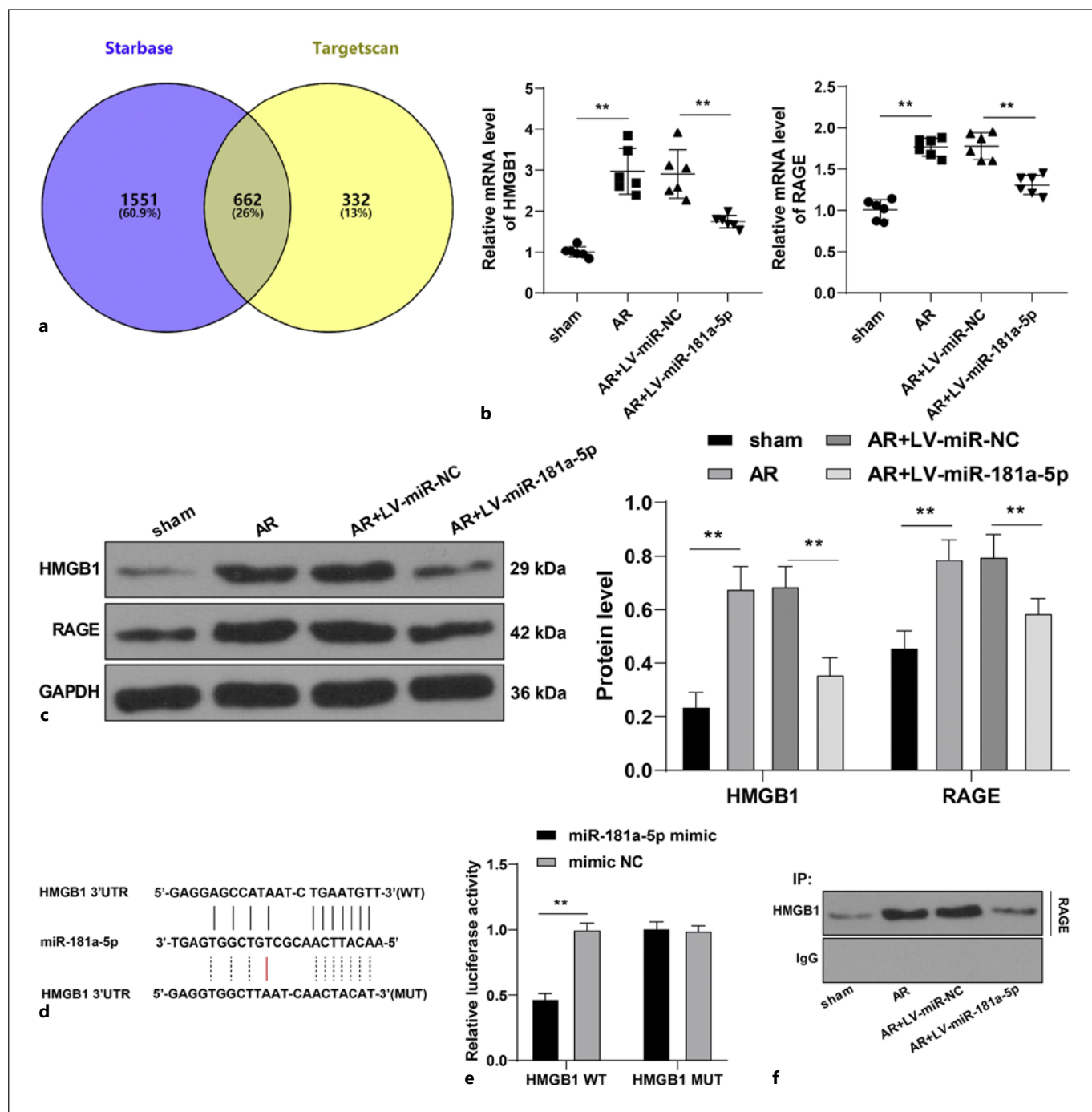
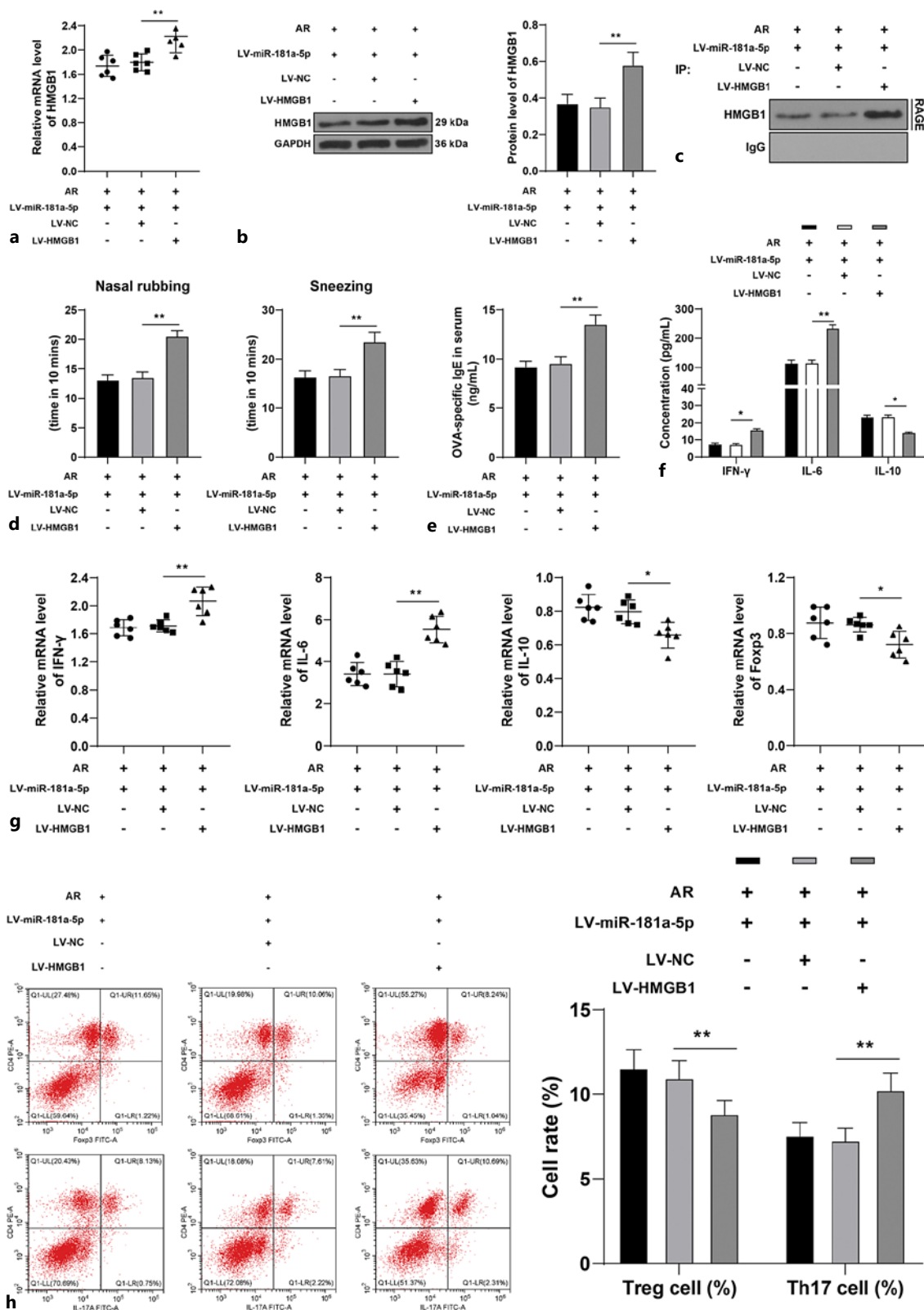


**Fig. 4.** miR-181a-5p targeted HMGB1 and then reduced the binding of HMGB1 and RAGE. **a** The downstream target genes of miR-181a-5p were predicted through the Starbase and Targetscan databases. **b, c** HMGB1 and RAGE expression in nasal mucosa tissues was determined using RT-qPCR (**b**) and Western blot (**c**). **d** The binding site of miR-181a-5p and HMGB1. **e** The binding relationship between miR-181a-5p and HMGB1 was verified using dual-luciferase assay. **f** The binding of HMGB1 and RAGE was analyzed using IP assay. *N* = 6. Data in **c–e** are presented as mean  $\pm$  standard deviation. Data in **b** were analyzed using one-way

ANOVA, and data in **c, e** were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons tests or Sidak's multiple comparisons tests,  $^{**}p < 0.01$ . AR, allergic rhinitis; LV-miR-181a-5p, lentivirus vector of miR-181a-5p; LV-miR-NC, negative control of lentivirus vector; HMGB1, high mobility group box chromosomal protein 1; RAGE, anti-receptor for advanced glycation end products; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; IP, immunoprecipitation; ANOVA, analysis of variance.



## Discussion

AR is a prevalent condition in children and adolescents that can develop into other allergic diseases, including asthma [44]. Treg/Th17 imbalance presents in AR patients, which may lead to the proceeding of AR [45]. Emerging evidence implicates the involvement of miRNAs in shaping inflammation pattern, and dysregulated miRNAs have been demonstrated in AR and asthma [46]. This study elucidated that Treg-derived miR-181a-5p alleviated AR-caused Treg/Th17 imbalance and impeded the development of AR into bronchial asthma by suppressing the HMGB1/RAGE inflammatory signaling.

miR-181a expression can be exploited as a predictor of the severity of AR and expected to be a promising target for AR treatment [47]. miR-181a is poorly expressed in AR and negatively correlated with total nasal severity scores [16]. Nevertheless, the role of miR-181a-5p in AR remained unknown. In the current study, we established a murine model of AR and found that miR-181a-5p expression was reduced in AR mice. Then, AR mice were treated with miR-181a-5p overexpression. After treatment, the frequencies of nasal rubbing and sneezing in AR mice were decreased. Also, miR-181a-5p overexpression decreased the content of OVA-specific IgE in the serum of AR mice. We were the first to demonstrate that miR-181a-5p was poorly expressed in AR and miR-181a-5p overexpression contributed to the recovery of AR in mice.

Treg/Th17 balance is essential for the maintenance of immune status [11]. Th17 cells produce pro-inflammatory cytokines, such as IFN- $\gamma$  and IL-6 to exacerbate inflammatory response and recruit neutrophils [48], while Tregs can limit allergic inflammation by generating anti-inflammatory cytokines like IL-10 or directly contacting effective immune cells [49]. Th17/Treg imbalance is impli-

cated in the pathogenesis of AR and leads to the progression of AR [31]. miR-181a can enhance Treg function [30], and reduced miR-181a is often concerned with decreased number and function of Tregs in AR [16]. Our results demonstrated that the contents of IFN- $\gamma$  and IL-6 in BALF of AR mice were notably increased and the content of IL-10 and the mRNA expression of Foxp3 (a master regulator for Treg development and function) were decreased, while miR-181a-5p overexpression could reverse the above trends, indicating that miR-181a-5p overexpression alleviated Treg/Th17 imbalance in AR mice.

AR usually precedes the development of asthma and leads to poor asthma control; the existence and type of asthma are usually affected by the duration and severity of AR [50]. Therefore, we also explored the effect of miR-181a-5p on the development of asthma in AR mice. EOS is a kind of granulated innate immune cells, and eosinophilic inflammation is typically related to the aggravation of asthma [51]. RANTES and eotaxin are critical chemoattractants for EOS [32, 33]. OVA induction increased the contents of eotaxin and RANTES in NALF, while miR-181a-5p overexpression decreased their contents. The total number of leukocytes, and the absolute count and the percentage of EOS in BALF of AR mice were notably increased, while the above indexes were decreased after miR-181a-5p overexpression. Th2 cells can serve as a sensitive indicator of aggravated symptoms in patients with asthma [52]. Th2 cell-generated cytokines, such as IL-4, IL-5, and IL-13 stimulate B cells to produce allergen-specific IgE, leading to the acute symptoms of AR [7]. miR-181a is involved in the regulation of Th2-mediated allergic immune response, and miR-181a expression is negatively correlated with serum IL-4/IL-5 in children with AR [47]. Our results showed that the levels of IL-4, IL-5, and IL-13 in BALF and lung tissues of AR mice were increased, while miR-181a-5p overexpression decreased

**Fig. 5.** HMGB1 overexpression attenuated the protective effect of miR-181a-5p on Treg/Th17 immune imbalance in AR mice by binding to RAGE. The mice in the miR-181a-5p overexpression group were treated with LV-HMGB1, with LV-NC as control. HMGB1 expression in nasal mucosa tissues was determined using RT-qPCR (**a**) and Western blot (**b**). **c** The binding of HMGB1 and RAGE was analyzed using IP assay. **d** The frequencies of nasal rubbing and sneezing were observed. **e** The level of OVA-specific IgE in the serum. **f, g** The levels of IFN- $\gamma$ , IL-6, IL-10, and Foxp3 in NALF and nasal mucosa tissues were detected using ELISA kits and RT-qPCR. **h** The proportion of Treg and Th17 cells in nasal mucosa was analyzed by flow cytometry.  $N = 6$ . Data in **b, d, e, f** are presented as mean  $\pm$  standard deviation. Data in **a, b, d, e, g**

were analyzed using one-way ANOVA, and data in **f, h** were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons test or Sidak's multiple comparisons test, \* $p < 0.05$ , \*\* $p < 0.01$ . AR, allergic rhinitis; LV-miR-181a-5p, lentivirus vector of miR-181a-5p; LV-miR-NC, negative control of lentivirus vector; LV-NC, negative control; LV-HMGB1, lentivirus vector of HMGB1; HMGB1, high mobility group box chromosomal protein 1; RAGE, anti-receptor for advanced glycation end products; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; IP, immunoprecipitation; ANOVA, analysis of variance; IFN, interferon; IL, interleukin; IgE, immunoglobulin E; ovalbumin; Treg, regulatory T; Th, T-helper.

the levels of IL-4, IL-5, and IL-13. All these results above suggested that miR-181a-5p overexpression blocked the development of asthma in AR mice.

Thereafter, we shifted to determining the downstream mechanism of miR-181a-5p in AR. We used Starbase and Targetscan databases to predict the target genes of miR-181a-5p and took the intersection. Among them, we focused on HMGB1. HMGB1, a protein that specifically binds to the linker region of nucleosomal DNA, can in-

crease the stability of nucleosome and enhance the interaction of transcription factors [53]. HMGB1 modulates the generation of many pro-inflammatory cytokines, as well as the proliferation and activation of diverse inflammatory cells [54]. HMGB1 as an endogenous danger signal participates in the pathogenesis of AR [55]. Elevated HMGB1 can be observed in the nasal brushing samples of AR patients, and HMGB1 is positively correlated with IL-4, IL-5, IL-13, or IL-17A, but negatively with IL-10

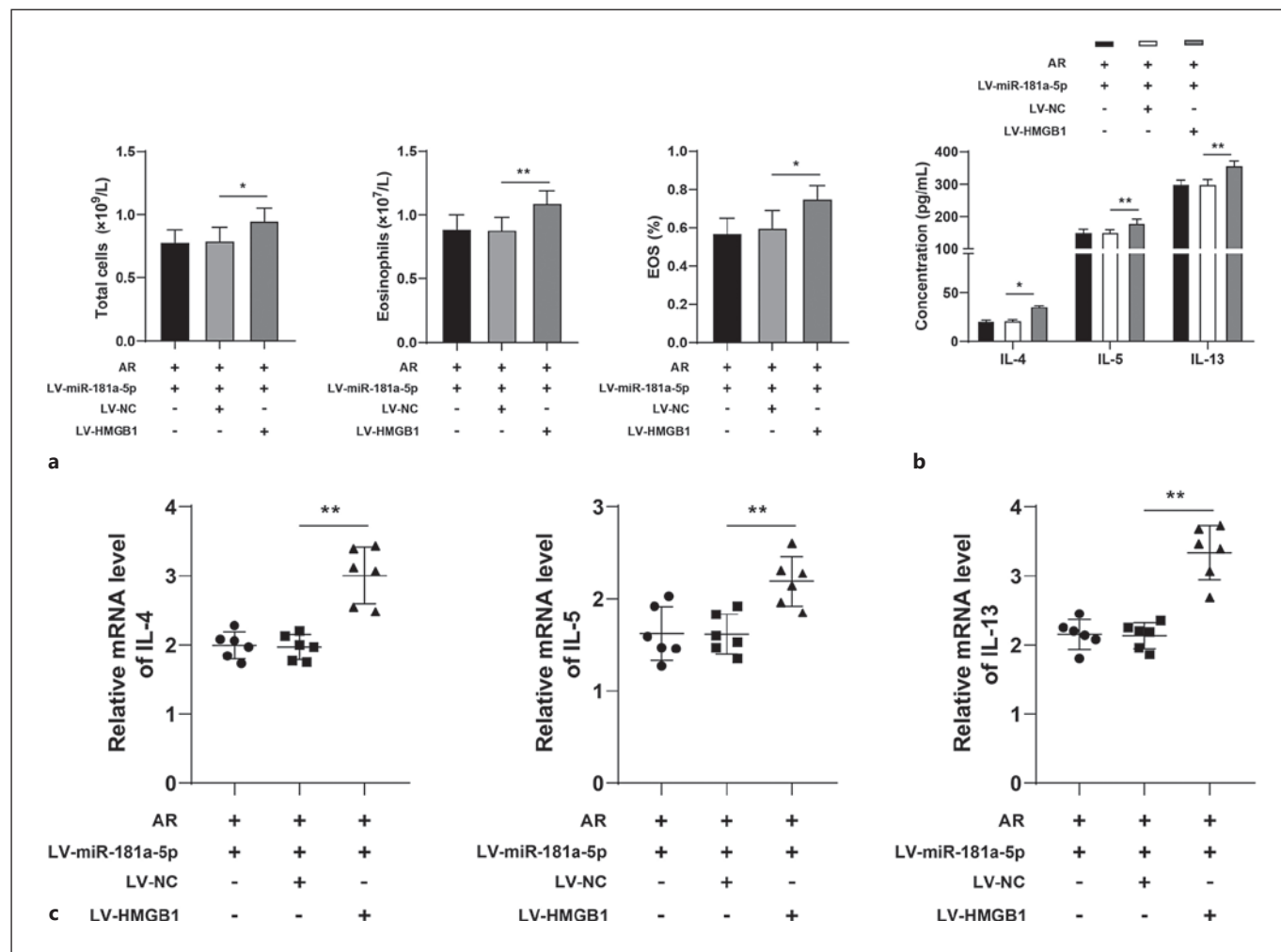


**Fig. 6.** HMGB1 overexpression reversed the inhibitory effect of miR-181a-5p on the development of asthma in AR mice by binding to RAGE. **a** The total number of leukocytes, and the absolute count and the percentage of EOS in BALF. **b** The contents of IL-4, IL-5, and IL-13 in BALF were detected using ELISA kits. **c** The levels of IL-4, IL-5, and IL-13 mRNA expression in lung tissues were detected using RT-qPCR.  $N = 6$ . Data in **a**, **b** are presented as mean  $\pm$  standard deviation. Data in **a**, **c** were analyzed using one-way ANOVA, and data in **b** were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons tests,  $*p <$

0.05,  $**p < 0.01$ . AR, allergic rhinitis; LV-miR-181a-5p, lentivirus vector of miR-181a-5p; LV-miR-NC, negative control of lentivirus vector; BALF, bronchoalveolar lavage fluid; LV-NC, negative control; LV-HMGB1, lentivirus vector of HMGB1. HMGB1, high mobility group box chromosomal protein 1; RAGE, anti-receptor for advanced glycation end products; ELISA, enzyme-linked immunosorbent assay; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; ANOVA, analysis of variance; IL, interleukin; EOS, eosinophils.

[20]. Commonly, HMGB1 initiates the inflammatory cascade in nasal inflammatory diseases through receptors, such as RAGE [54]. RAGE is a multi-ligand pattern recognition receptor involved in a variety of chronic inflammatory states; RAGE expression is low under normal physiological conditions, but it is dramatically elevated in chronic inflammation [56]. RAGE leads to the increase of many cytokines, such as IL-4 and IL-6, and these cytokines are the characteristics of innate inflammatory response [57]. Our results showed that HMGB1 and RAGE were elevated in AR mice and miR-181a-5p overexpression reduced HMGB1 and RAGE. Dual-luciferase assay and co-immunoprecipitation assay confirmed the binding relationships between miR-181a-5p and HMGB1, and between HMGB1 and RAGE. miR-181a-5p overexpression reduced the binding of HMGB1 and RAGE. Briefly, miR-181a-5p targeted HMGB1 and then reduced the binding of HMGB1 and RAGE. Subsequently, we performed functional rescue experiments to verify the role of the HMGB1/RAGE axis in AR. We treated AR mice overexpressing miR-181a-5p with HMGB1 lentivirus vector to upregulate HMGB1 expression and increase the binding of HMGB1 and RAGE. HMGB1 overexpression aggravates eosinophilic inflammation in the airway of acute AR by triggering Th2-type response and enhancing neutrophilic inflammation [58]. Knockdown of HMGB1 can decrease IL-4, IL-5, and IL-13 and increase IFN- $\gamma$  in BALF samples of asthma induced by diisooctyl phthalate [53]. Moreover, enhanced HMGB1 shifts Treg/Th17 balance to Th17 dominance in chronic hepatitis B patients, which aggregates liver injury and inflammation [59]. Consistently, our results revealed that the activation of HMGB1/RAGE reversed the protective effect of miR-181a-5p overexpression on Treg/Th17 imbalance in AR mice and promoted the development of AR into asthma. Briefly, it was indicated that miR-181a-5p overexpression alleviated Treg/Th17 imbalance in AR mice and delayed the development of AR into asthma by inhibiting the HMGB1/RAGE pro-inflammatory axis.

## Conclusion

To sum up, miR-181a-5p overexpression inhibited the HMGB1/RAGE inflammatory signaling by targeting HMGB1 expression, thereby relieving AR-caused Treg/Th17 imbalance and blocking the development of AR into asthma. However, this study merely explored the effect of miR-181a-5p on Treg/Th17 immune imbalance, and whether miR-181a-5p can regulate other functions in

AR remains to be explored. Additionally, whether other target genes of miR-181a-5p participate in this regulatory process remains elusive. In this study, we did not specifically explore what cells were infected by lentivirus and produced miR-181a-5p. According to a large number of references [60–65], which reported that nasal epithelial cells are the barrier to resist the invasion of external pathogens, which can carry out plasma, siRNA, and miRNA transduction, regulate the inflammatory response of nasal epithelial cells, and play a major role in AR. Therefore, we speculate that miR-181a-5p may be produced by nasal epithelial cells infected by lentivirus. However, considering the limitations of experimental conditions, we have not been able to explore this point through experiments. We will take this as the focus of our next research, so as to improve the regulatory mechanism of miR-181a-5p in AR. In the future, we will determine other potential mechanisms of miR-181a-5p in AR and explore the role of other target genes of miR-181a-5p.

## Statement of Ethics

All the animal experiments were implemented on the Guide for the Care and Use of Laboratory Animals [24] and were approved by the Institutional Animal Care and Use Committee of The Affiliated Hospital of Guangdong Medical University (Approval No: AE202001016). Extensive efforts were made to minimize the suffering of the included animals.

## Conflict of Interest Statement

The authors have no conflicts of interest to declare.

## Funding Sources

Not applicable.

## Author Contributions

R.H. designed the study. Y.C. and X.C. performed data analyses and interpretation. B.Y. drafted the work and revised it critically for important intellectual content. All authors approve the final version of the manuscript.

## Data Availability Statement

All the data are included in this article.

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