

miR-146a Mimics Attenuate Allergic Airway Inflammation by Impacted Group 2 Innate Lymphoid Cells in an Ovalbumin-Induced Asthma Mouse Model

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Keywords

Airway hyperresponsiveness · MicroRNA 146a · T helper 2 cell type cytokines · Group 2 innate lymphoid cells · Interleukin 33

Abstract

Background: The prevalence of allergic asthma has increased dramatically. Previous studies have found that the microRNA 146a (miR-146a) expression in asthma inhibits cell proliferation and promotes apoptosis of bronchial smooth muscle cells. We aimed to investigate the effect of miR-146a mimics on ovalbumin (OVA)-induced asthma in a mouse model. **Methods:** Inflammatory cell infiltration in bronchoalveolar lavage fluid (BALF) was measured by flow cytometry. Levels of OVA-specific immunoglobulin E (IgE) in serum and cytokines in BALF were examined by enzyme-linked immunosorbent assay. For monitoring the airway, the Penh value (% baseline) was measured using a whole-body plethysmograph. **Results:** In OVA-induced asthmatic mice, miR-146a significantly suppressed the infiltration of inflammatory cells in BALF and decreased the levels of OVA-specific IgE and T helper 2 cell type cytokines. In addition, miR-146a inhibited

the OVA-induced airway hyperresponsiveness and the group 2 innate lymphoid cell responses. Moreover, the effects of miR-146a mimics were dependent on interleukin 33 stimulation. **Conclusions:** Our results suggest that miR-146a mimics might serve as an attractive candidate for further preclinical studies as an anti-inflammatory treatment of asthma.

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Introduction

Asthma is a complex and common chronic inflammatory disease that occurs in the airways of the lungs, caused by a combination of genetic and environmental factors [1]. In 2015, approximately 358 million people worldwide suffered from asthma compared to 183 million in 1990 [2]. The incidence rate of asthma has increased significantly since the 1960s [3] and has become a global public health burden for society. Asthma is a complex

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and heterogeneous disease, including nonallergic, allergic and intrinsic forms, of which allergic asthma is the most common form [4]. The main changes in the lungs include intense leukocyte and eosinophilic infiltration, airway hyperresponsiveness (AHR), mucus hypersecretion, and overexpression of T helper 2 (Th2) cell type cytokines including interleukin (IL)-4, IL-5, and IL-13 [5]. Th2 cell type cytokines are critical in allergic asthma by promoting the productions of immunoglobulin E (IgE) and various inflammatory mediators, as well as inducing eosinopoiesis [6]. Innate lymphoid cells (ILCs), derived from common lymphoid progenitor, are a group of innate immune cells in the lymphoid lineage [7]. ILCs do not express antigen-specific B or T cell receptors, myeloid or dendritic cell (DC) markers [8]. Group 2 ILCs (ILC2, also termed natural helper cells) is valued for its important role in the pathogenesis of asthma [9]. ILC2s are abundant in the liver, lungs, skin, and gut [10]. Characteristic surface markers and receptors for chemokines, which are involved in the distribution of lymphoid cells to specific organ sites, are expressed by ILC2s [11]. Two transcription factors, ROR α and GATA3, which are stimulated by IL-7, are required for the development of ILC2s [12]. Upon stimulation with Th2 polarizing cytokines such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), mature ILC2s start to produce Th2 cell type cytokines that participate in the progression of allergic asthma [13].

MicroRNA (miRNA or miR) is a subtype of 18–22 nt nonencoding RNA, which functions in the regulation of cell proliferation, differentiation, and apoptosis [14]. It has been found that multiple miRNAs are associated with the occurrence and development of allergic asthma [15, 16]. Members of the miR-146 family, consisting of miR-146a and miR-146b, were found to be elevated in a murine model of asthma [17]. Previous studies have found that miR-146a and miR-146b serve as negative regulators of inflammatory cytokines in various cell types, including endothelial cells [18], fibroblasts [19], monocytes [20], and airway smooth muscle [21]. In addition, recent studies suggested that the expression level of miR-146a was significantly increased in the plasma of children with asthma [22]. Moreover, enhanced miR-146a expression inhibited proliferation and promoted apoptosis of bronchial smooth muscle cells [22]. However, current research on the pathogenic mechanisms of miR-146a still remains at the cellular level *in vitro* and needs to be further elucidated. Our present study aimed to determine the effects of miR-146a on ovalbumin (OVA)-induced asthma in a mouse model.

Methods

Animals

Specific pathogen-free female BALB/c mice (6–8 weeks old; 18–20 g) were used. Mice were housed in plastic boxes with a 12-h light/12-h dark cycle (light from 6:00 a.m. to 6:00 p.m.). Room temperature (19–23 °C, RT) and humidity (55 $\pm$ 10%) were governed to be constant. Food and water were supplied *ad libitum*. On days 1, 7, and 14, the mice were sensitized by intraperitoneal injection with 20 μ g of OVA emulsified with 1 mg aluminum hydroxide in 200 μ L phosphate-buffered saline (PBS) (pH 7.4) as an adjuvant. On days 22, 23, 24, and 25, the mice were challenged by intranasal inhalations with 100 μ g of OVA in 20 μ L PBS once a day. miR-146a mimics (miRBase ID mmu-miR-146a-5p; Invitrogen) were intranasally administered at 1 mg/kg body weight every day from day 22 to day 25, dexamethasone (DEX) was intraperitoneally given as a positive control and anti-IL-17RB/anti-ST2 was intraperitoneally given every day from day 22 to day 25. For the normal control group, mice were given an equal volume of PBS. Anti-IL-17RB neutralizing mAb (R&D Systems, Inc.) and anti-ST2 mAb (Centocor) were used at 2 μ g/mL. Mice were sacrificed at 24 h after the last challenge. This study was approved by the ethics committee of The First Affiliated Hospital of Nanjing Medical University.

Flow Cytometry

Cells were surface stained with fluorescently labeled antibodies to CD11c, SiglecF, Gr1, CD11b, CD4, TcR β , CD3, CD19, CD8 α , CD49b, KJ1-26, ICOS, CD25, CD44, and ST2 or the relevant isotype controls. Cell types were characterized as CD11c⁺SiglecF⁺ (alveolar macrophages), SiglecF⁺CD11c[−]CD11b⁺Gr1^{lo} (eosinophils), Gr1^{hi}CD11b^{hi}CD11c[−]SiglecF[−] (neutrophils) and CD4⁺CD11b[−] (T helper cells). ILC2s were characterized as lineage[−](CD3/CD19/CD4/CD5/CD8 α /TcR β /CD49b/CD11b/CD11c) and ICOS⁺ according to a previously published method [23]. For intracellular cytokine staining, cells were stimulated for 4 h at 37 °C with 500 ng/mL phorbol myristate acetate, 1 mg/mL ionomycin, and 10 mg/mL brefeldin A (Sigma). Cells were surface stained, then permeabilized with the BD Biosciences Fixation/Permeabilisation kit (BD Biosciences) before staining for IL-5 and IL-13. Live/dead Fixable Yellow or Aqua dyes (Invitrogen) were used to exclude dead cells. Samples were analyzed by flow cytometry using Becton-Dickinson FACS-Canto or LSR-II flow cytometers (BD Biosciences, Franklin Lakes, NJ, USA).

Collection of Bronchoalveolar Lavage Fluid and Differential Cell Count

To collect bronchoalveolar lavage fluid (BALF) samples, two 0.8-mL aliquots of cold PBS were used for cannulating and lavaging the mice tracheas. The samples were immediately centrifuged, and then we stored the supernatants at −80 °C for cytokine assay and resuspended the cell pellets with PBS for the counting of differential cells. With the help of a hemocytometer, the total cell number was counted. The eosinophils, neutrophils, macrophages, and lymphocytes in BALF were stained with the Kwik-Diff staining set (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA), and counted by 200 cells/slide.

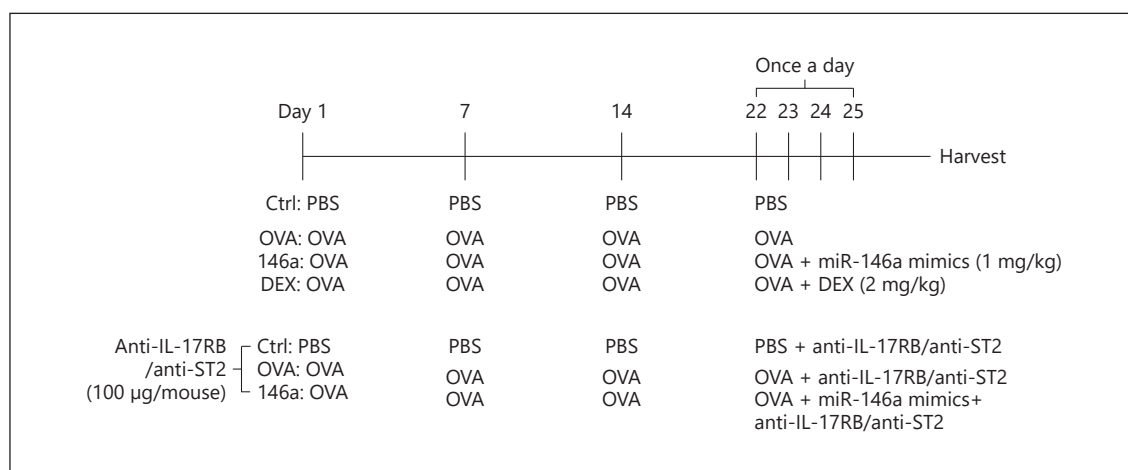


Fig. 1. Schematic diagram of the experimental protocol.

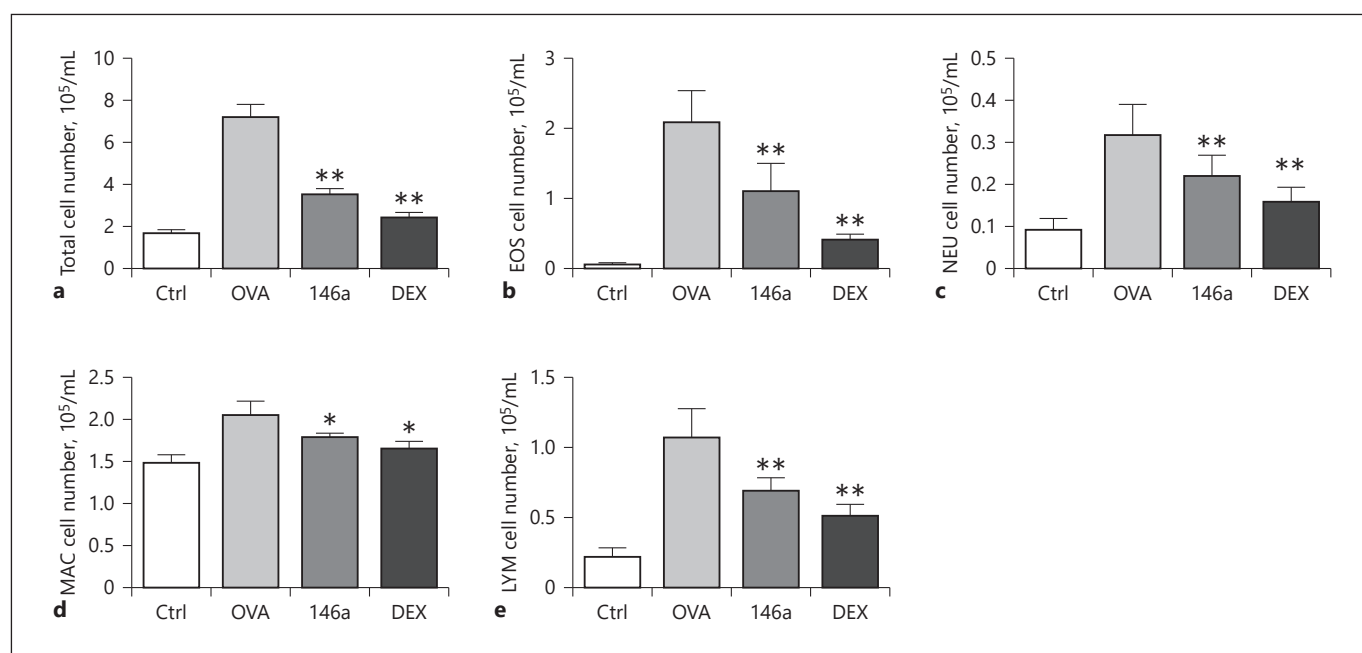


Fig. 2. Effects of miR-146a mimics on recruitment of inflammation cells in BALF. Twenty-four hours after the last challenge, cells collected from BALF were counted. Data are presented as mean $\pm$ SD values and representative of at least 3 independent experiments. $n = 7$, * $p < 0.05$; ** $p < 0.01$ versus the OVA group.

Measurement of the Levels of OVA-Specific IgE in Serum and Cytokines in BALF

The measurement of IL-4, IL-5, and IL-13 levels in BALF was guided by the manufacturer's protocols of enzyme-linked immunosorbent assay (ELISA) kits. ELISA was also used to determine the level of OVA-specific IgE. First, 96-well flat-bottom microtiter plates were coated at 4 °C overnight with 100 µg/mL OVA in NaHCO₃ buffer (pH 9.6) 100 µL, which were then washed with PBS–0.05% Tween 20 4 times. The wells were incubated for 1 h at

RT using 200 µL blocking buffer (1% BSA in PBS, pH 7.2), which was then replaced with different dilutions of serum samples and standard murine IgE into the wells and incubated for 2 h at RT. The wells were incubated with biotin-conjugated rat anti-mouse IgE for 2 h at RT and then tested for the OVA-specific immunoglobulins. The plates were incubated for 30 min with extravidin-peroxidase and for another 30 min with 3,3',5,5'-tetramethylbenzidine substrate in the dark. The reaction was terminated with 2 N H₂SO₄ followed by reading the plate at 450 nm.

Fig. 3. Effects of miR-146a mimics on the levels of OVA-specific IgE in serum (a), IL-4 (b), IL-5 (c), and IL-13 (d) in BALF. Serum and BALF were collected 24 h after the last challenge. The levels of OVA-specific IgE and these cytokines were quantified by ELISA. Data are presented as mean $\pm$ SD values and representative of at least 3 independent experiments. $n = 7$, ** $p < 0.01$ versus the OVA group.

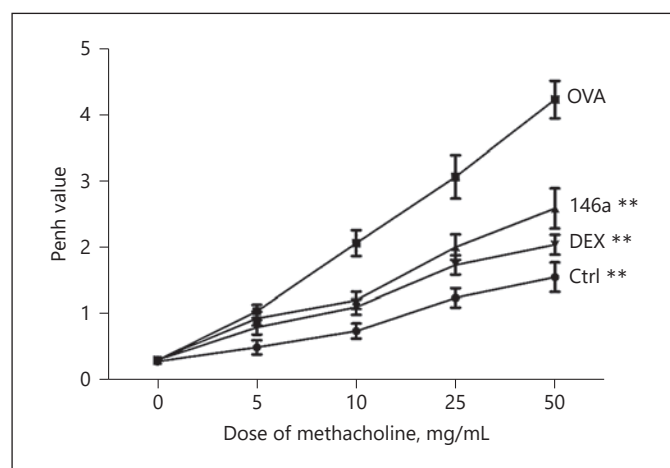
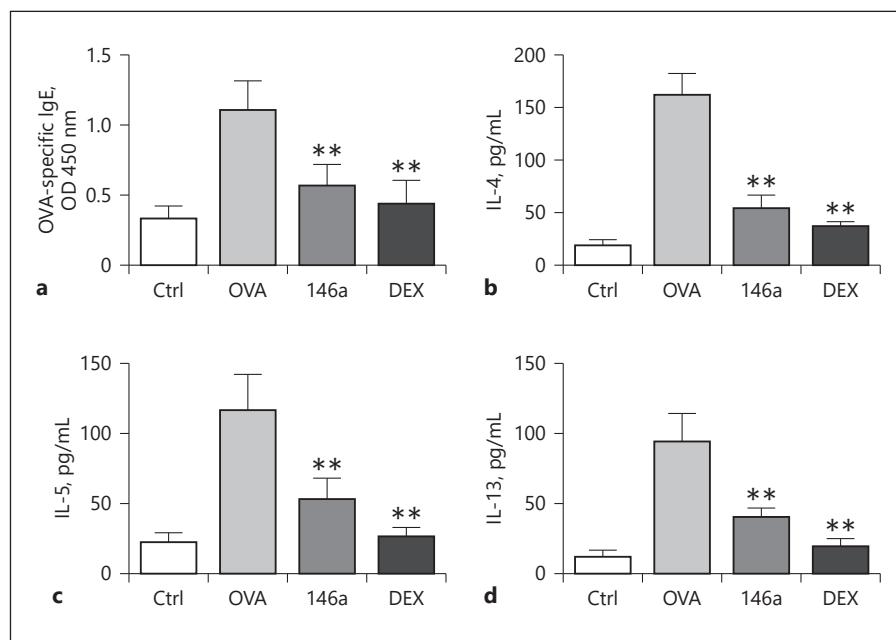


Fig. 4. miR-146a mimics alleviate the development of AHR. Twenty-four hours after the last challenge, AHR was assessed using a whole-body barometric plethysmograph. Data are presented as mean $\pm$ SD values and representative of at least 3 independent experiments. $n = 7$, ** $p < 0.01$ versus the OVA group.

Measurement of Airway Responsiveness

Using the sodium pentobarbital, mice were anesthetized on day 25 (24 h after the last challenge). The tracheas of these mice were cannulated via tracheostomy. Each mouse was exposed to nebulized PBS for 3 min with the help of an unrestrained whole-body plethysmograph. The ultrasonic nebulizer can generate nebulized methacholine with different concentrations (2.5, 5, 10, 25, and 50 mg/mL). Enhanced minute pause (Penh) is a calculated value that can reflect pulmonary resistance and can express the AHR. The

Penh values were averaged to get the absolute Penh values. The baseline Penh (after PBS challenge) is expressed as 100%. After performing calculations with each concentration of methacholine (Sigma), the results are shown as the percent increase in Penh over the baseline.

Statistical Analysis

All data were shown as the mean $\pm$ standard deviation (SD). Differences between samples were analyzed using the two-tailed Student t test or two-way ANOVA. Statistical significance was accepted at $p < 0.05$.

Results

miR-146a Suppresses the Inflammatory Cell Infiltration in BALF

As shown in Figure 1, the experimental groups were described as follows: normal control group (PBS), OVA group, miR-146a group, DEX group, anti-IL-17RB-ctrl group, anti-IL-17RB-OVA group, anti-IL-17RB-146a group, anti-ST2-ctrl group, anti-ST2-OVA group, and anti-ST2-146a group.

Intense infiltration of inflammatory cells in the lung is a primary sign of allergic asthma. To determine the effect of miR-146a on airway inflammation, total and differential inflammatory cells (eosinophils, neutrophils, macrophages, and lymphocytes) in BALF were counted 24 h after the last challenge. As shown in Figure 2a–e, OVA challenge in mice elicited a substantial infiltrate of eosin-

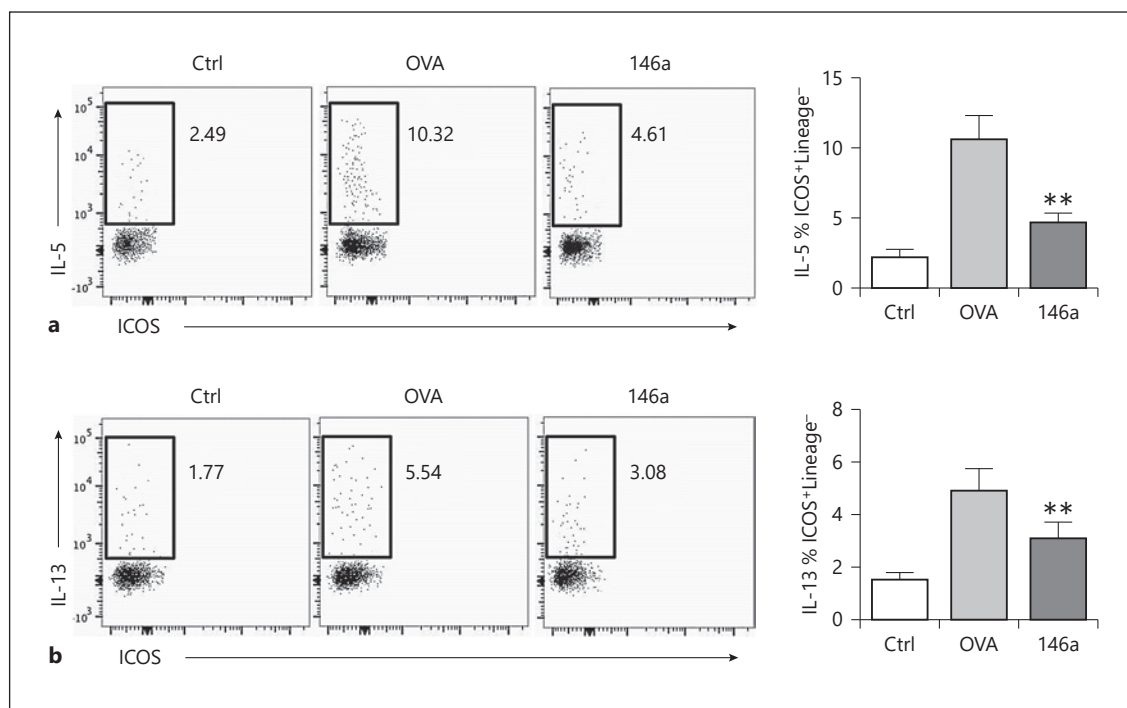


Fig. 5. miR-146a mimics suppress type 2 responsiveness in ILC2s. Twenty-four hours after the last challenge, lungs were collected and ILC2s were defined by expression of ICOS in the absence of classical lineage markers (ICOS⁺Lineage⁻). Data are presented as mean $\pm$ SD values and representative of at least 3 independent experiments. $n = 7$, ** $p < 0.01$ versus the OVA group.

ophils, neutrophils, macrophages, and lymphocytes in BALF, whereas a striking reduction in inflammation was seen when miR-146a was coadministered, with all cell types reduced to similar levels as the positive DEX group. In addition, the dose-response curve of miR-146a mimics on recruitment of eosinophils in BALF (online suppl. Fig. S1A; see www.karger.com/doi/10.1159/000491438 for all online suppl. material) and on the levels of OVA-specific IgE in serum (online suppl. Fig. S1B) also confirmed the therapeutic efficacy. These results indicated that miR-146a inhibited recruitment of inflammatory cells into the airway.

miR-146a Suppresses the Levels of OVA-Specific IgE and Th2 Cell Type Cytokines

The allergic response in OVA-induced asthma displayed all the canonical features of type 2 immune responsiveness, including the excessive productions of IgE and Th2 cell type cytokines. Thus, IgE was significantly upregulated with OVA challenge (Fig. 3a), along with a trend for increased Th2 cell type cytokines IL-4, IL-5, and IL-13 (Fig. 3b–d). Furthermore, in mice receiving miR-

146a mimics, these cytokine responses were remarkably suppressed to similar levels as the positive DEX group, indicating the inhibiting role of miR-146a in OVA-induced asthma.

miR-146a Decreases OVA-Induced AHR

Allergic asthma is clinically characterized by reversible bronchial constriction and AHR. As shown in Figure 4, mice displayed a significant increase in AHR with OVA administration, which was indicated by enhanced Penh growth. Notably, miR-146a remarkably alleviated the increased Penh value to an extent close to the positive DEX group. Thus, miR-146a might possess an inhibitory effect on OVA-induced AHR.

miR-146a Suppresses ILC2 Responses

Previous studies have found that ILC2s respond to the alarming cytokines IL-25 or IL-33 by producing large amounts of IL-5 and IL-13, which are instrumental for allergic responses in the lung [13]. Thus, we examined the ILC2 compartment, defined by expression of ICOS in the absence of classical lineage markers

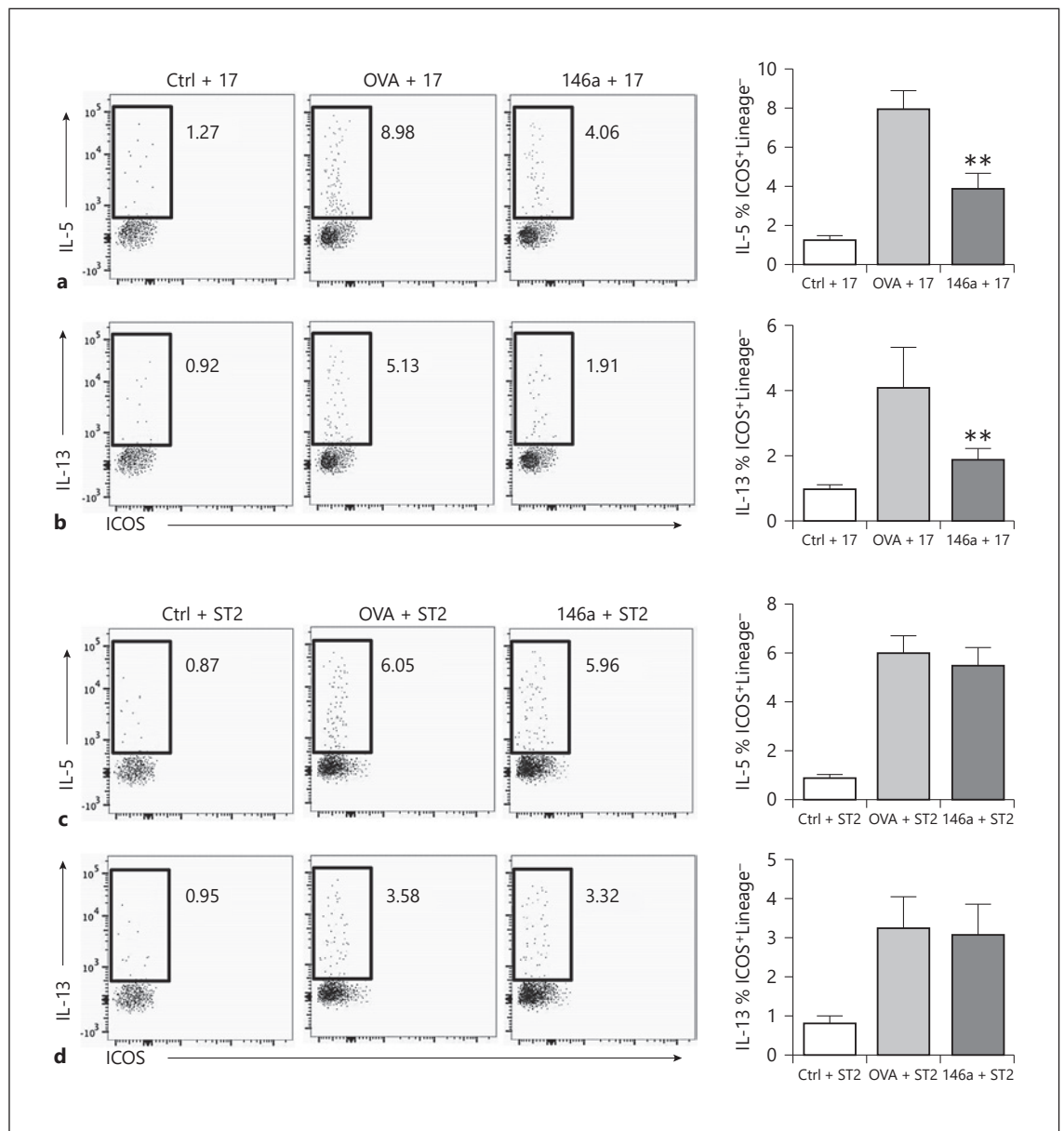


Fig. 6. Effects of miR-146a mimics are blocked by anti-ST2 in ILC2s. Twenty-four hours after the last challenge and treated with anti-IL-17RB (**a, b**) or anti-ST2 (**c, d**), lungs were collected and ILC2s were defined by expression of ICOS in the absence of classical lineage markers (ICOS⁺Lineage⁻). Data are presented as mean $\pm$ SD values and representative of at least three independent experiments. $n = 7$, ** $p < 0.01$ versus the OVA group.

(ICOS⁺Lineage⁻). As shown in Figure 5a and b, a marked increase in the productions of IL-5 and IL-13 was found within this subset of allergic mice challenged by OVA. However, we found significant decreases in lung ILC2 IL-5 and IL-13 productions when miR-146a was coadministered, showing that miR-146a might inhibit the activation of ILC2.

Effects of miR-146a Mimics Are Dependent on IL-33 Stimulation

As ILC2s are known to be activated by IL-25 and IL-33, the experiments of allergic inflammation were followed in mice treated with the antibody against IL-17RB (the IL-25-specific subunit of the receptor) or IL17RBxST2 (the IL-25 and IL-33 receptors) to block

the respective signaling pathways. As shown in Figure 6a and b, in the anti-IL-17RB group, the production levels of IL-5 and IL-13 were similar to those in the untreated group mentioned above (Fig. 5), and the suppression with miR-146a coadministration was equally profound in the presence or absence of IL-25 signaling. In contrast, no suppression of ILC2 response with miR-146a administration was evident in anti-IL17RBxST2 mice (Fig. 6c, d), suggesting that the inhibitory effect of miR-146a on OVA-induced asthma was dependent on the IL-33 signaling pathway.

Discussion

Allergic asthma, a complex inflammatory disorder of the lung, is clinically characterized by mucus hypersecretion by goblet cells, AHR, infiltration of inflammatory cells into lung tissues, and overexpression of Th2-mediated cytokines including IL-4, IL-5, and IL-13 [24]. Although various effective therapies have been developed, the incidence rate of allergic asthma and the frequency of its complications have dramatically increased over the last decade, making it one of the most common forms of bronchial inflammation of respiratory diseases [25]. Therefore, allergic asthma is becoming a serious health burden worldwide.

Previous studies discovered that the expression levels of miR-146a and miR-146b were positively correlated with the number of inflammatory cells infiltrating to BALF, which were enhanced in mouse spleen CD4⁺ T cells [26]. In addition, the expression of miR-146a was remarkably decreased following treatment with DEX, suggesting that miR-146a might be involved in the progression of AHR in allergic asthma [27]. Williams et al. [28] observed an elevated expression of miR-146a in airway biopsies of patients with mild asthma, indicating an important role of miR-146a in asthma treatment. In addition, inflammatory factors (such as Th2-mediated cytokines) could increase the expression levels of miR-146a in airway smooth muscles [29]. Elevated miR-146a levels were also found to be positively associated with the proliferation and apoptosis of bronchial smooth muscle cells [22]. However, to date, the function and the molecular mechanisms of miR-146a in the mouse asthma model have yet to be reported. The present study aimed to investigate the effect of miR-146a on OVA-induced asthma in a mouse model.

Recent studies have found that Th2 cell type cytokines are critical in the pathogenesis of allergic asthma by regu-

lating the production of IgE, the release of various inflammatory mediators, and the differentiation and activation of eosinophils and mast cells [30]. In addition, allergen-mediated cross-linking of IgE antibodies bound to the high affinity IgE receptor (FcεRI) may cause the activation and degranulation of pulmonary mast cells, leading to the IgE-dependent early airway response in asthma [31]. It has been reported that airway contraction is caused by the release of various mediators, and higher IgE production is more conducive to mast cell degranulation and airway contraction [32]. In this paper, we found that pretreatment with miR-146a decreased the level of OVA-specific IgE, which might lead to reduced recruitment of IgE antibodies to FcεRI, eventually causing the activation and degranulation of pulmonary mast cells. Furthermore, allergic asthma is thought to be driven by Th2 cell type cytokines including IL-4, IL-5, and IL-13. IL-4 promotes the expression of vascular cell adhesion molecule-1 on endothelial cells and the production of IgE by B cells [33]. As we mentioned earlier, miR-146a reduced the level of IL-4 in BALF. Thus, miR-146a might regulate the expression of vascular cell adhesion molecule-1, and the reduced IgE level might correlate with the expression of IL-4. In addition, IL-5 induces the development, recruitment and activation of eosinophils to the lung [34]. Activated eosinophils generate extensive chemokines and cytokines and are a predominant source of platelet-activating factor and lipid mediator leukotriene C4, inducing smooth muscle contraction and mucus secretion [35]. IL-13 has been found to play a crucial role in regulating the effector phase of allergic asthma by prompting mucus hyperproduction and AHR [36]. Our results revealed that pretreatment with miR-146a decreased the productions of IL-5 and IL-13 in BALF. These data suggest that miR-146a might regulate the AHR through the expression of IL-13 and mediate the smooth muscle contraction and mucus secretion in asthma through monitoring the level of IL-5, respectively.

Although various mouse models of asthma have evidently demonstrated the significance of Th2 cells in monitoring the inflammatory responses in the lung, recent studies have indicated that innate immune cells also play a crucial role in allergic asthma [37]. Multiple cytokines, including IL-25, IL-33, and TSLP, produced by lung epithelial cells could induce the production of Th2 cell type cytokines and the infiltration of eosinophilic into lung tissues [38]. It has been suggested that many asthma symptoms could be reproduced by the intranasal administration of IL-25 [39], and IL-25 blocking antibody could inhibit the OVA-induced allergic airway inflammation

[40]. Previous studies have found that natural killer T cells could be stimulated by IL-25, leading to the expression of IL-25 receptor IL-17RB [41]. On the other hand, IL-33 has been shown to function in basophils and mast cells [42]. Activated by IL-33, basophils generate IL-4 and TSLP, which also stimulate Th2 cell type responses. In addition, IL-33-blocking antibody significantly suppresses eosinophilic lung inflammation, suggesting an essential role of IL-33 in regulating allergic asthma. Besides, Th2 cell type responses could also be enhanced by TSLP through activating DCs [43]. Thus, these epithelial cell-derived cytokines are considered critical for promoting Th2 cell type responses and inducing lung inflammation. As a pivotal alarm cytokine in allergy, IL-33 could directly stimulate not only ILCs, but also osteoclasts, mast cells, macrophages, DCs, and other subsets expressing IL-33 receptor T1/ST2 [43]. We hereby show that miR-146a significantly suppresses the ILC2 allergic responses through the IL-33 signaling pathway. Despite the exclusion of IL-25 pathway, our results do not rule out inhibitory pathways other than IL-33. However, we suggest that the most likely mechanism of miR-146a suppression in this model is blockade of early IL-33 release upon allergen administration, leading to a debilitated early ILC2 response, and slighter allergic recall responses at later time points.

Conclusions

We hereby describe suppression of allergic airway responses in an OVA-induced mouse asthma model by co-administration of miR-146a mimics. Our data demonstrate that miR-146a significantly attenuates AHR and allergic airway inflammation through reducing the infiltration of inflammatory cells into lung tissues and suppressing the expressions of OVA-specific IgE and Th2 cytokines, which might correlate with its capacity to block the IL-33 signaling pathway. Thus, the ability of miR-146a to downregulate the allergic reactivity in OVA-induced mouse asthma observed in this paper might stimulate research for more specific molecular mechanisms, which may provide new routes for treating allergic diseases in human.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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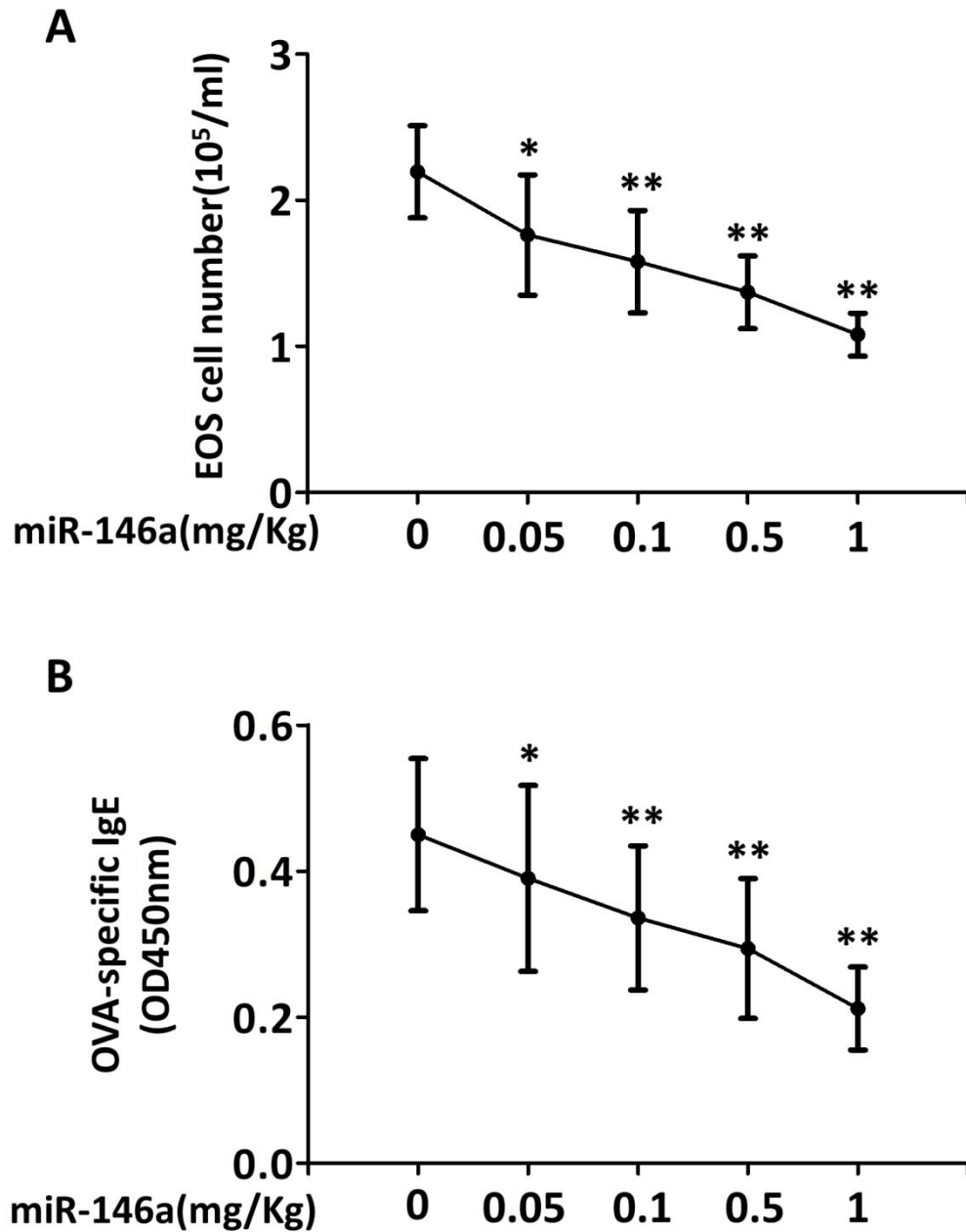


Fig. S1. The dose-response curve of miR-146a mimics in OVA mice. (A) Effect of miR-146a mimics with different doses on recruitment of eosinophils in BALF. (B) Effect of miR-146a mimics with different doses on the levels of OVA-specific IgE in serum. Serum and BALF were collected 24 h after the last challenge. The levels of OVA-specific IgE were quantified by ELISA. Data are presented as mean $\pm$ SEM values. N=5 mice, * $p < 0.05$; ** $P < 0.01$ versus the 0 mg/Kg group.