

miR-29b Reverses T helper 1 cells/T helper 2 cells Imbalance and Alleviates Airway Eosinophils Recruitment in OVA-Induced Murine Asthma by Targeting Inducible Co-Stimulator

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Keywords

Asthma · Inducible co-stimulator · miR-29b ·
Anti-inflammatory · Treatment

Abstract

Asthma is a complex chronic disease and the pathogenesis is still not entirely clear. In this study, we aimed to clarify the role and mechanism of miR-29b in the development of asthma. We observed that miR-29b levels were decreased in the lung and spleen of OVA-induced asthmatic mice. Reverse transcription-quantitative polymerase chain reaction and flow cytometry demonstrated that the inducible co-stimulator (ICOS) expression at mRNA and protein levels was elevated in the lung of asthmatic mice, and miR-29b expression in the lung of asthmatic mice was negatively associated with ICOS mRNA levels by Pearson Correlation analysis. Additionally, flow cytometry showed that the percentage of CD4⁺ICOS⁺ T cells in the lung and spleen was regulated by miR-29b, and dual luciferase reporter assay confirmed ICOS was a target gene of miR-29b. Furthermore, miR-29b overexpression in asthmatic mice was induced with miR-29b agomir by intranasal administration; miR-29b alleviated total inflammatory cell infiltration and CCL24 levels, decreased IL-5 levels in

bronchoalveolar lavage fluid and serum, and upregulated IFN- γ expression in serum. This study demonstrates that miR-29b targets ICOS, thereby reverses the imbalance of T helper 1 cells (Th1)/Th2 responses and decreases eosinophils recruitment in the airway, which are key features of allergic airway inflammation. Therefore, miR-29b might be an attractive candidate target for asthma treatment.

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Introduction

Asthma is a common chronic respiratory disease and can affect persons of all ages. There were currently about 300 million asthmatic patients reported in 2017 around the world, and the prevalence is still increasing every year [1]. Asthma is characterized by increased airway inflammation and remodeling of airways and dysregulated type 2 immune responses. This results from sustained exposure to aeroallergens, which lead to elevated concentrations of type 2 cytokines including IL-4, IL-5, and IL-13,

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alongside allergen-specific immunoglobulin E (IgE), eosinophilia, and airway hyperresponsiveness [2].

The pathogenesis of asthma has not been fully understood, but T helper 2 cells (Th2) and the Th2 cytokines have been traditionally thought of as the main drivers of the disease. Inducible co-stimulator (ICOS), a relatively newly defined member of the CD28 family, is constitutively expressed in low levels by naive T cells but is significantly increased after T-cell activation [3, 4]. It was reported CD28 is critical during priming responses but does not contribute to recall responses once effector Th2 cells have been generated. In contrast, ICOS plays the predominant role in regulating Th2 effector cell activation [5]. It was reported that ICOS ligand knockout (ICOSL KO) mice exhibited significantly less lung eosinophilic infiltration, histopathology, mucus production after OVA (ovalbumin) sensitization and challenge, as compared with wild type mice. Moreover, CD4⁺ T cells isolated from ICOSL KO mice produced less Th2 cytokines (IL-4, IL-5 and IL-13) but more Th1 (IFN- γ) and IL-17 than wild type mice [6]. Taken together, ICOS plays a key role in asthma. However, the molecular mechanism for regulating ICOS in asthma has not been very clear.

Recently, there is increasing evidence supporting that miRNAs are involved in the asthma pathogenesis. miRNAs are a class of small, non-coding RNAs that regulate post-transcriptional gene expression via mRNA degradation or translation suppression [7]. miRNAs are of great importance in regulating several pulmonary diseases, such as chronic obstructive pulmonary disease, interstitial lung disease and asthma [8]. In human smooth muscle actin cells, miR-708 directly targets CD38, thereby regulating JNK MAPK and PI3K/AKT signaling, which might control airway inflammation [9]. In addition, miR-155 was reported to involve in airway eosinophils recruitment and in the regulation of Th2 responses in allergic airway inflammation [10]. These findings suggest that many miRNAs are involved in the development of asthma.

miR-29 family includes miR-29a/b/c. The 3 family members are generated from 2 alleles containing a bicistronic transcriptional unit, miR-29ab1 and miR-29b2c, in both the human and mouse genomes [11]. Mature miRNA sequences of miR-29a/b/c are well conserved, contain identical seed sequences, and are likely to target the same transcripts [12]. Besides, miR-29c expression was significantly decreased in the lung tissues of doxycycline-induced IL-13 transgenic mice compared with mice that received no doxycycline, suggesting that miR-29c was regulated directly or indirectly by IL-13, which may in-

volve in the development of asthma [13]. Specifically, miR-29b expression has been linked to pulmonary cancers, fibrosis, and bronchopulmonary dysplasia [14]. Pulmonary miRNA profiling in murine allergic airway inflammation showed decreased miR-29b expression [15]. Taken together, miR-29 family might play a central role in the development of asthma. Furthermore, bioinformatic analysis indicates that ICOS might be the target gene of miR-29b. As such, we attempt to explore the role of miR-29b in regulation of ICOS gene in asthma in this study, which has never been reported yet.

In this study, we established murine asthma model and detected miR-29b and ICOS expression in the lung tissues and spleens. miR-29b mimics and inhibitors transfection assay and flow cytometry showed ICOS was negatively regulated by miR-29b, which was confirmed by dual luciferase reporter assay. Meanwhile, miR-29b overexpression in asthmatic mice by nasal administration revealed that miR-29b alleviated eosinophil's recruitment in the airway and alter the balance of Th1/Th2 responses by suppressing ICOS expression. Therefore, miRNA-29b might be a new therapeutic target for managing asthma.

Materials and Methods

Mice

BALB/c female mice aged 6–8 weeks were raised under specific pathogen-free conditions and were from Chongqing Medical University.

Asthma Model and Treatment

To induce allergic asthma, mice were intraperitoneally sensitized with 100 μ g OVA (Sigma-Aldrich, St. Louis, MO, USA) absorbed to 1 mg of aluminum hydroxide (Thermo scientific, Waltham, MA, USA) in 200 μ L of sterile phosphate-buffered saline (PBS). After sensitization on days 1 and 8, mice were challenged with 5% OVA inhalation for 30 min every day from day 14 to 21. For miR-29b treatment, 100 μ g miR-29b agomir (GenePharma, Shanghai, China) in 30 μ L PBS was administered by intranasal inhalation starting on days 17, 19, and 21. The control group was treated in the same way with PBS. Mice were sacrificed 24 h after the last OVA challenge.

ELISA

IL-5, IFN- γ , IgE levels in bronchoalveolar lavage fluid (BALF) and serum were determined by commercial ELISA kits (Biolegend, San Diego, CA, USA). IL-13 was measured by ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Reverse Transcription-Quantitative Polymerase Chain Reaction

Total RNA including miRNA was extracted from lung tissue using HiPure Universal miRNA Kits (Magen, Shanghai, China). The cDNA was obtained from total RNA using a PrimeScriptTM

Table 1. Primers in this study

Primer	Sequence (5'–3')
mmu-miR-29b	RT: GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACAACACT F: CCGCGCGTAGCACCATTGAAATC R: ATCCAGTGCAGGGTCCGAGG
U6	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT
pGL6-miR-ICOS-3'UTR	F: CAGGGTACCTATGTATATAAAATTCATAATGAATATATTTGCCTATATTCTCCCTACAAGAATAT R: GCGAAGCTTGCTCCAGGCTGATGGGGAC
Muc5ac	F: AATGACTCAATCTGCGTGCCTTCC R: CAGGTTAGCGTGGCTTCCTTACAG
CCL11	F: TCACTGTGTAGACCAGGCTGACC R: ATTCTGGCTTGGCATGGTAGCAC
CCL24	F: CAGAGACGCCGTGGCAATAGC R: TCACTCAGCACAGGAGACACCAG
ICOS	F: GAAGCCGTACTTCTGCCGTGTC R: CTTGGTGAGTTCGCAGAGGACTTC
ICOS, inducible co-stimulator.	

RT reagent Kit (Takara, Shiga, Japan) according to the manufacturer's protocol. Reverse Transcription-Quantitative Polymerase Chain Reaction was performed using the SYBR Premix Ex Taq kit (Takara, Shiga, Japan). All mRNA expressions were normalized to GAPDH gene expression and miRNA was normalized to U6 gene expression. The primers used in this study were shown in Table 1.

Histopathological Analysis of Lung

The lung tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin to examine lung inflammation, or Periodic Acid-Schiff to observe hyperplasia and mucus secretion of airway mucosa goblet cells under the microscope.

Flow Cytometry

Single-cell suspension was performed as described below. The lung tissues and spleen tissues were removed from mice, the lung tissues were minced and incubated for 30 min at 37 °C with RPMI 1640 and Collagenase IV (1 mg/mL, Sigma-Aldrich). The spleen tissues were grinded and filtered through a sieve. Then red blood cells were lysed with Red Blood Cell Lysis Buffer. After washing 3 times with PBS, cells were incubated for 30 min at 4 °C with FITC-CD4 (BD Pharmingen, San Jose, CA, USA) and PE-ICOS (Biolegend, San Diego, CA, USA). Cells were washed 3 times with PBS and then measured with flow cytometry.

Isolation and Culture of Spleen CD4⁺ T cells

Spleen CD4⁺ naive T cells were isolated using a CD4⁺ T-cell isolation kit (Miltenyi Biotec, Auburn, AL, USA) and cultured in 1,640 medium supplemented with 10% serum and penicillin-streptomycin liquid (Invitrogen, Carlsbad, CA, USA) in a 5% CO₂ incubator at 37 °C. CD4⁺ naive T cells were transfected with mimics NC,

Table 2. The RNA oligo used in this study

RNA oligo	Sequence (5'–3')
Mimics NC	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT
miR-29b mimics	UAGCACCAUUUGAAAUCAGUGUU CACUGAUUUCAAUUGGUGCUAUU
Inhibitors NC	CAGUACUUUUGUGUAGUACAA
miR-29b inhibitors	AACACUGAUUUCAAUUGGUGCUA
miR-29b agomir	UAGCACCAUUUGAAAUCAGUGUU CACUGAUUUCAAUUGGUGCUAUU
NC implies a scrambled sequence control.	

miR-29b mimics, inhibitors NC, miR-29b inhibitors (GenePharma, Shanghai, China) using Lipofectamine 2000 as recommended by manufacture's guidelines. After 24 h transfection, murine CD4⁺ T cells were activated in presence of anti-CD3 (2 µg/mL) (eBioscience, San Diego, CA, USA) and anti-CD28 (1 µg/mL; eBioscience, San Diego, CA, USA) for additional 72 h then used for flow cytometry. The RNA oligo used in this study were shown in Table 2.

MicroRNA Target Prediction

MiRNA databases and target prediction tools miRBase (<http://microrna.sanger.ac.uk/>), DIANA (<http://www.microrna.gr/microT-CDS>), and TargetScan (<http://www.targetscan.org/index>).

html) were used for the identification of the potential targets of miR-29b. We focused on targets predicted by at least 2 prediction databases and found there was an miR-29b seed matching the ICOS 3'UTR region.

Dual Luciferase Reporter Assay

The target gene 3'UTR was cloned into multiple cloning sites downstream of the pGL6-miR (Beyotime, Wuhan, China) luciferase gene expression vector. The ICOS 3'UTR-WT reporter plasmid was constructed containing the miR-29b binding site, and the ICOS 3'UTR-Mut reporter plasmid was constructed containing the mutation site to which miR-29b can't bind. The HEK-293T cell line was obtained from Dr. Tingmei Chen, Chongqing Medical University. Cells (0.5×10^5) were seeded into 24-well plate per well. In 16–24 h, following with miRNA mimics (500 ng), pGL6-miR ICOS 3'UTR WT/Mut (500 ng), pRL-SV40 (10 ng) were co-transfected into each well by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 72 h, the cell lysis was collected, luciferase and Renilla activity was measured by Dual-Luciferase Reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Statistical analysis was performed (Prism software version 5.0; GraphPad Software, San Diego, CA, USA) using Student *t* test or the Wilcoxon-Mann-Whitney test. Results are shown as the mean $\pm$ SEM. *p* values <0.05 were considered significant.

Results

The Murine Asthma Model Is Successfully Established

We established an OVA-induced murine asthma model. As compared with the NC group, the total inflammatory cell infiltration (Fig. 1a), eosinophilic counting (Fig. 1b, c), IgE (Fig. 1d), IL-5 (Fig. 1e) and IFN- γ (Fig. 1f) levels in BALF were increased in the OVA group, which suggested that the murine asthma model was successfully established.

miR-29b Expression Is Decreased in Lung and Spleen of OVA-Induced Murine Asthma

In order to explore the role of miR-29b in asthma, we detected the expression of miR-29b in lung by qRT-PCR and found that miR-29b was significantly decreased compared with that of the control group (Fig. 2a). Furthermore, miR-29b expression in the spleen of asthmatic mice was decreased (Fig. 2b). These results indicate that miR-29b might play a role in asthma.

miR-29b Is Negatively Correlated with ICOS Expression in OVA-Induced Murine Asthma

The TargetScan and DIANA soft predict that ICOS may be a target of miR-29b. Next, we detected ICOS mRNA expression in lung by qRT-PCR and the results

showed that the levels of ICOS were increased in the OVA group compared with that of the control group (Fig. 3a). Pearson correlation analysis showed that miR-29b expression was negatively correlated with ICOS mRNA in lung tissues of asthmatic mice (Fig. 3b). Airway inflammation is an important feature of asthma, and T lymphocytes are central to the induction and regulation of the allergen-induced inflammation. ICOS⁺ T cells are required for the development of local airway inflammation and IgE production [16]. Thus, we analyzed CD4⁺ICOS⁺ T cells in lung and spleen by flow cytometry. The cell gating strategy was used as follows (Fig. 3c): The percentage of CD4⁺ICOS⁺ T cells was significantly higher in lung tissues of mice in the OVA group than that in the control group, as were these cells in spleen (Fig. 3d). Together, allergen exposure induces both local and systemic CD4⁺ICOS⁺ T-cells responses. In addition, we also analyzed the mean fluorescence intensity (MFI) of ICOS expression on CD4⁺ T cells between the groups. The MFI of ICOS expression on CD4⁺ T cells were higher in lung (Fig. 3e) and spleen (Fig. 3f) from asthmatic mice than saline mice, which was consistent with a relative number of CD4⁺ICOS⁺ T cells in Figure 3d. These suggest that miR-29b is negatively correlated with ICOS expression in OVA-induced murine asthma.

miR-29b Negatively Regulates the Expression of ICOS in vitro

To gain insight into the role of miR-29b in regulation of ICOS expression, we isolated spleen-naïve CD4⁺ T cells, then we transfected with miR-29b mimics or inhibitors into the spleen-naïve CD4⁺ T cells and investigated the percentage of CD4⁺ICOS⁺ T cells following 72 h culture with anti-CD3 and anti-CD28 stimulation. As compared with the control group, the percentage of CD4⁺ICOS⁺ T cells was markedly decreased in the miR-29b overexpression group (Fig. 4a, b), whereas it was significantly increased in the miR-29b inhibitors group (Fig. 4c, d). These investigations suggest that miR-29b can negatively regulate the expression of ICOS in vitro.

ICOS Is a Molecular Target of miR-29b

The bioinformatics analysis found a putative binding site between ICOS and miR-29b by using 3 authoritative databases, TargetScan, mirWalk, and DIANA (Fig. 5a). To determine whether ICOS was a molecular target of miR-29b, we constructed a wild luciferase reporter vector containing ICOS 3'UTR, as well as a mutant control vector harboring an miR-29b identical sequence in the

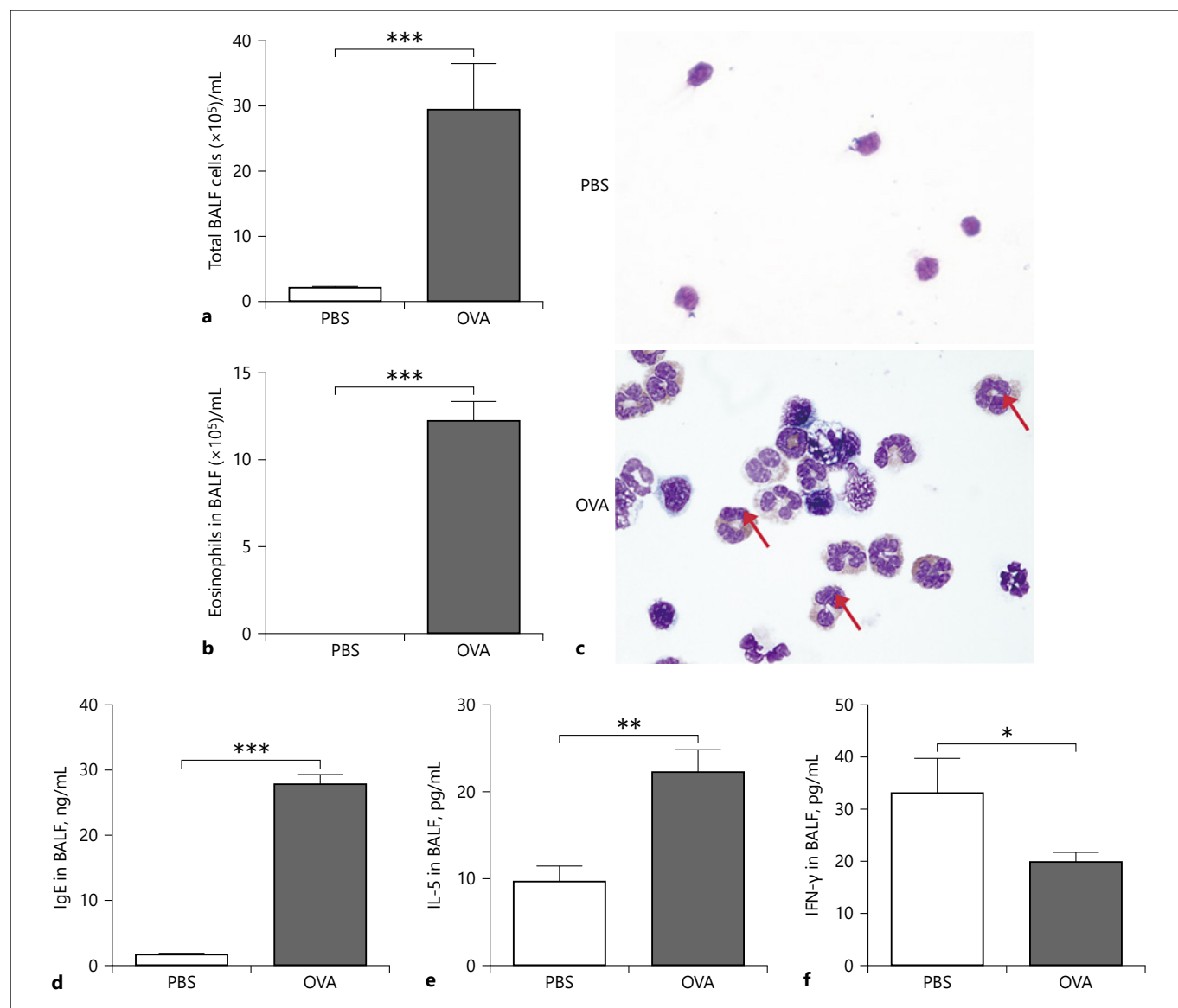


Fig. 1. The murine asthma model is successfully established. **a, b** The number of total inflammatory cells and eosinophils counts in BALF. **c** The inflammatory cell types are classified by Swiss staining. The IgE (**d**), IL-5 (**e**) and IFN- γ (**f**) levels in BALF were detected by ELISA. Statistical analysis was performed by Wilcoxon-

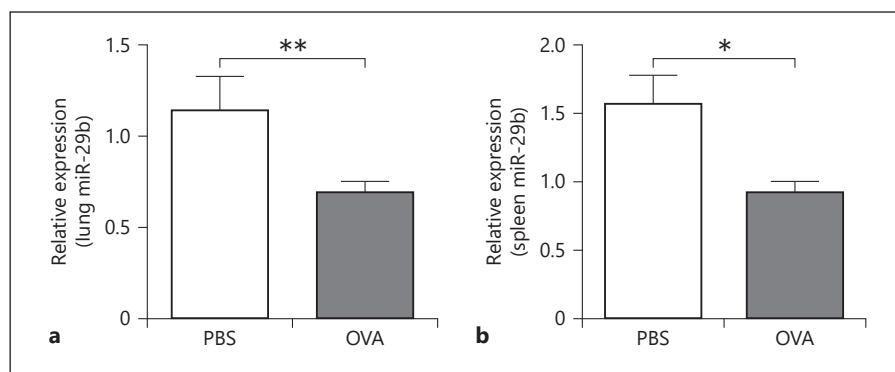
Mann-Whitney test. Data are presented as mean $\pm$ SEM, 6–8 mice/each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, not significant; PBS, phosphate-buffered saline; BALF, bronchoalveolar lavage fluid.

3'UTR region (Fig. 5a). As compared with the ICOS-WT/NC group or ICOS-Mut/miR-29b mimics group, ICOS-WT 3'UTR luciferase activity was inhibited in the miR-29b mimics group (Fig. 5b), while ICOS-Mut 3'UTR luciferase activity was not changed. These results suggest that miR-29b could specifically target the ICOS-3'UTR region and downregulate ICOS expression at the post-transcriptional level.

Intranasal Delivery of miR-29b Agomir Alleviates Airway Eosinophil's Recruitment

To define the role of miR-29b in the development of characteristic allergic asthma features, miR-29b overexpression in asthmatic mice was induced with miR-29b agomir by intranasal administration. The number of total inflammatory cells and eosinophil's in BALF was reduced significantly in the OVA+ miR-29b agomir group compared with that of the OVA group (Fig. 6a, b). It was pre-

Fig. 2. Downregulation of miR-29b expression in lung and spleen of OVA-induced asthmatic mice. miR-29b expression in lung (a) and spleen (b) was determined by quantitative PCR in the asthmatic group and the control group. Statistical analysis was performed by Wilcoxon-Mann-Whitney test. Data are presented as mean $\pm$ SEM, 6–8 mice/each group. * $p < 0.05$, ** $p < 0.01$. PBS, phosphate-buffered saline.



viously shown that eotaxin-1/CCL11 and eotaxin-2/CCL24 played pivotal roles in regulating eosinophil recruitment locally in the airways in response to allergen challenge [10]. Thus, we measured mRNA levels of these chemokines in the lung. CCL11 and CCL24 mRNA levels were significantly raised in the OVA group compared to the levels in the control group. CCL24 expression was lower in the OVA+ miR-29b agomir group than that in the OVA group (Fig. 6c, d), while CCL11 showed no differences between the 2 groups. These results imply that miR-29b-induced decreases in eosinophils might be mediated by CCL24. Allergen-specific antibodies, especially IgE, are a key feature of asthma. Results showed that miR-29b agomir administration did not decrease total serum and BALF IgE levels (Fig. 6e, f). Besides, Lung tissue HE staining in the OVA group showed thickened bronchial mucosa, lumen stenosis, increased inflammatory cells infiltration in lung tissues, and broken alveolar walls compared to control group, while the inflammation was decreased in the OVA+ miR-29b agomir group compared to the OVA group (Fig. 6h). In addition, we also detected muc5ac mRNA levels in lung tissues and found there was no difference in muc5ac levels between the OVA+ miR-29b agomir group and the OVA group (Fig. 6g). As compared to the control group, lung tissue PAS staining showed obvious goblet cell hyperplasia and accumulated airway mucus secretions in the OVA group and the OVA+ miR-29b agomir group, but there were no obvious differences between the 2 groups, which was consistent with the levels of muc5ac (Fig. 6i). Taken together, miR-

29b alleviates airway eosinophils recruitment through reducing CCL24, but has no effect on muc5ac or IgE expression.

miR-29b Regulates the Balance of Th1/Th2 Responses through ICOS

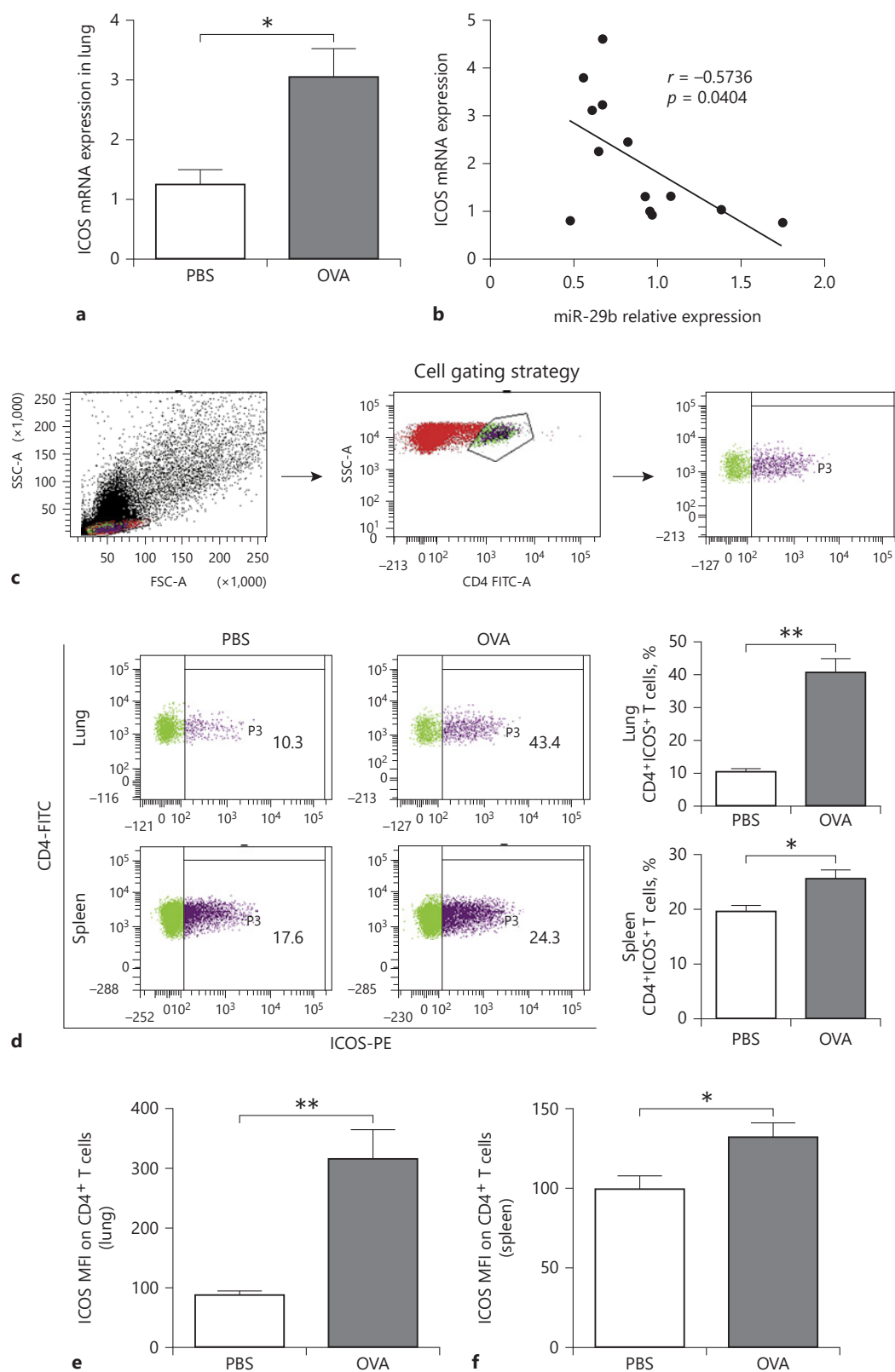
It was well known that the imbalance in Th1/Th2 helper cell responses and their effector cytokines, IFN- γ , IL-4, IL-5, and IL-13, played important roles in the pathophysiology of allergic inflammation [17, 18]. To investigate whether miR-29b affects Th1/Th2 imbalance, we next detected these cytokines in BALF and serum. IL-5 levels in BALF and serum were higher in the OVA group than corresponding levels in the control group, whereas the increases were inhibited in the OVA+ miR-29b agomir group (Fig. 7a, c). Besides, IFN- γ expression was decreased in BALF and serum in the OVA group compared to control group (Fig. 7b, d). OVA sensitization-induced decreases in IFN- γ levels were reversed by miR-29b agomir in serum, but not in BALF (Fig. 7b, d). These results indicate that miR-29b involves in the regulation of Th1/Th2 balance.

Due to the involvement of ICOS in Th1/Th2 balance in asthma, we presumed that miR-29b might regulate Th1/Th2 balance through ICOS. Thus, we detected the percentage of CD4⁺ICOS⁺ T cells in lung and spleen with flow cytometry. The cell gating strategy was used as follows (Fig. 7e): The percentage of CD4⁺ICOS⁺ T cells in lung and spleen was lower in the OVA+ miR-29b agomir group than that in the OVA group (Fig. 7f). Moreover, the

Fig. 3. miR-29b is negatively correlated with ICOS expression in OVA-induced asthmatic mice. a ICOS mRNA expression in lung was determined by quantitative PCR. b The correlation between miR-29b expression and ICOS mRNA levels in lung was analyzed by Pearson correlation analysis. c The cell gating strategy for CD4⁺ICOS⁺ T cells. d The percentage of CD4⁺ICOS⁺ T cells in lung and spleen from PBS mice and OVA-treated mice was de-

tected by flow cytometry. The MFI of ICOS expression on CD4⁺T cells in lung (e) and spleen (f) was analyzed. Statistical analysis was performed using the Wilcoxon-Mann-Whitney test. Data are presented as mean $\pm$ SEM, 6–8 mice/each group. * $p < 0.05$, ** $p < 0.01$. MFI, mean fluorescence intensity; PBS, phosphate-buffered saline; ICOS, inducible co-stimulator.

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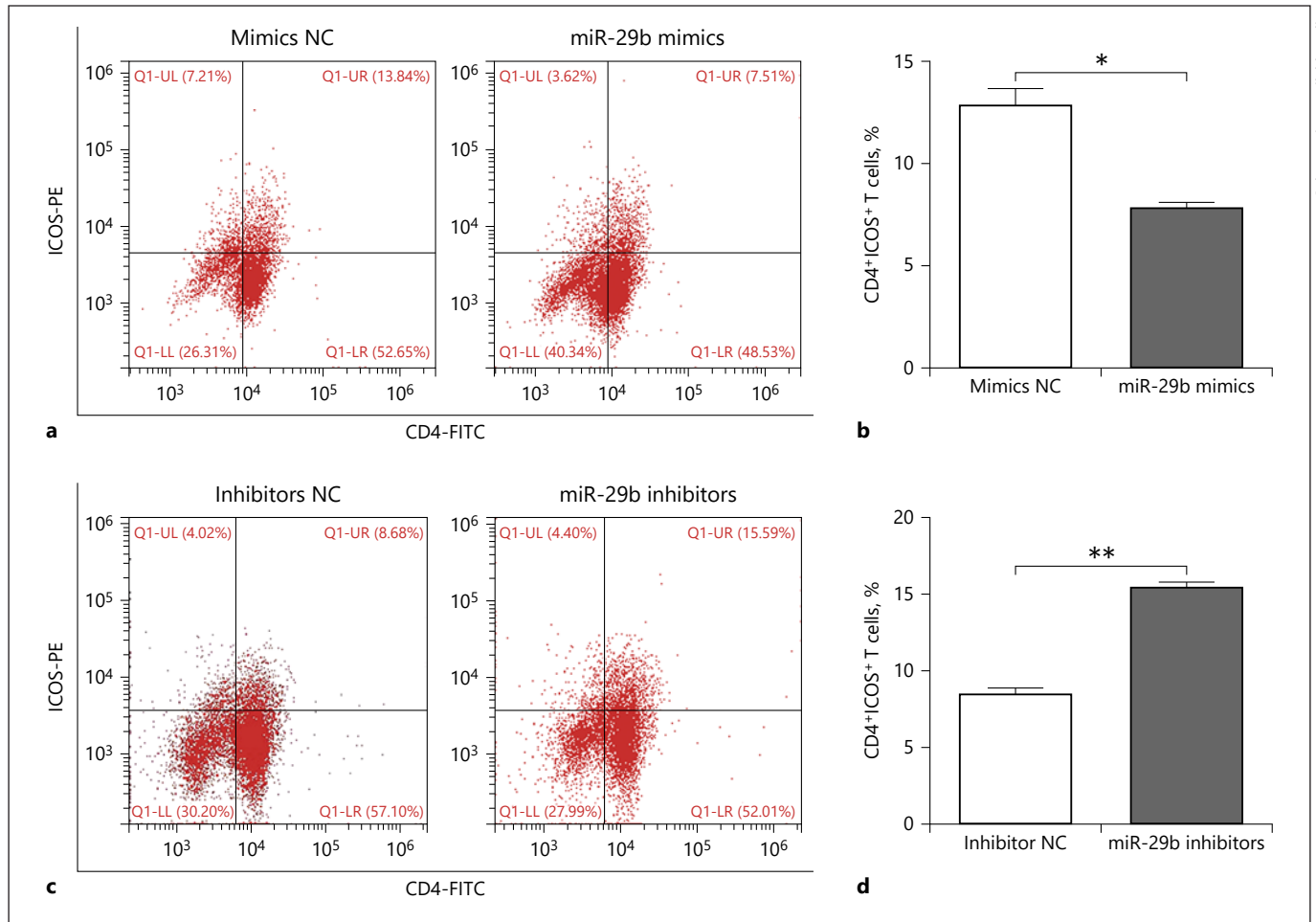


Fig. 4. miR-29b negatively regulates the expression of ICOS in vitro. **a, c** Spleen CD4⁺ naive T cells were transfected with mimics NC, miR-29b mimics, inhibitors NC, miR-29b inhibitors using Lipofectamine 2000. After 24 h transfection, murine CD4⁺ T cells were activated in presence of anti-CD3 (2 µg/mL) and anti-CD28 (1 µg/mL) for additional 72 h, and then the percentage of

CD4⁺ICOS⁺ T cells was detected with flow cytometry. **b, d** Quantification of the percentage of CD4⁺ICOS⁺ T cells. The data are representative of 3 independent experiments. Statistical analysis was performed using Student *t* test, * *p* < 0.05, ** *p* < 0.01. ICOS, inducible co-stimulator.

MFI of ICOS expression on CD4⁺ T cells in lung (Fig. 7g) and spleen (Fig. 7h) was lower in OVA+ miR-29b agomir than that in the OVA group, which was consistent with a relative number of CD4⁺ICOS⁺ T cells in Figure 7f. These findings suggest that miR-29b involves in the regulation of Th1/Th2 balance responses through ICOS.

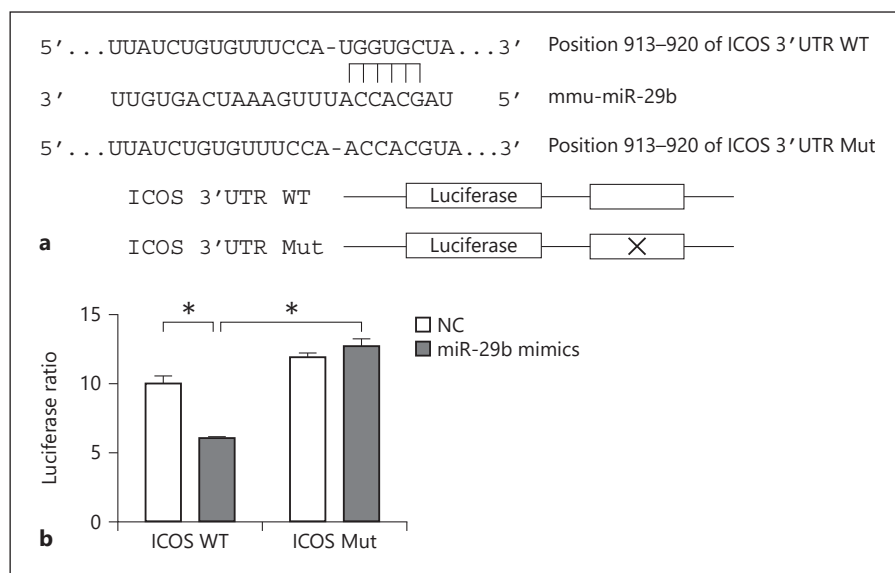
Discussion

miRNAs are involved in many biological and pathological processes including inflammation through the modulation of their target genes, and they play a role in the pathogenesis of allergic inflammatory diseases in-

cluding asthma [19]. In this study, we characterized that miR-29b downregulated the expression of ICOS and thereby affected the Th1/Th2 balance and eosinophils recruitment in asthma for the first time.

ICOS-ICOS ligand axis was previously demonstrated to be involved in the allergic airway hyperresponsiveness and airway eosinophilia [6]. We observed that the ICOS expression was significantly elevated at transcriptional and post-transcriptional levels in the lung tissues of asthmatic mice. Flow cytometry showed that the percentage of CD4⁺ICOS⁺ T cells was increased in the lung and spleen in asthmatic mice. These results indicate that ICOS is involved in the pathogenesis of asthma and this result is consistent with that of the previous study.

Fig. 5. ICOS is a target of miR-29b. **a** Predicted miR-29b binding site within the ICOS 3'UTR. **b** 293T cells were transfected with ICOS-3'UTR-WT/Mut, and dual-luciferase reporter assay was performed at 72 h post transfection. The data are representative of 3 independent experiments. Data are presented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$. ICOS, inducible co-stimulator.



Our results showed that decreased miR-29b and elevated ICOS expression in the lung tissues of OVA-induced the murine asthma model. At the same time, Pearson correlation analysis showed that miR-29b expression was negatively correlated with ICOS mRNA in lung tissues of asthmatic mice. The percentage of CD4⁺ICOS⁺ T cells in spleen was decreased after transfection with miR-29b mimics, while miR-29b inhibitors made it increased. Moreover, a binding site of miR-29b was predicted in ICOS 3'UTR and experimentally validated by dual luciferase reporter assay. These data confirm that ICOS is a target of miR-29b. We treated asthmatic mice with miR-29b agomir by nasal administration, and the result showed that the percentage of CD4⁺ICOS⁺ T cells was decreased in the lung and spleen compared with that of the OVA group. This result indicates the regulation of ICOS by miR-29b in the in vivo model of asthma.

The total inflammatory cells and eosinophils in BALF were reduced significantly in the OVA+ miR-29b agomir group compared with that of the OVA group. These results are consistent with the results of the previous study that the number of lymphocytes and eosinophils were significantly reduced in the BALF of OVA-sensitized mice after treatment with a neutralizing mAb or ICOS-Ig [5]. Moreover, CCL24 expression was lower in lung tissues in the OVA+ miR-29b agomir group than that in the OVA group. These results imply that miR-29b-induced decreases in eosinophils in asthmatic mice are mediated by CCL24 and probably through targeting ICOS.

ICOS is related to the CD28 superfamily and is highly expressed on activated T cells [3]. And its expression is

crucial for the survival and function of T cells, Th2 cells differentiation, and for lung inflammatory responses [20]. CD4⁺ T cells isolated from spleen in OVA-sensitized TCR-transgenic mice were cultured and stimulated with OVA peptide in vitro. ICOS blockade with a neutralizing mAb reduced expression of IL-4 and IL-10 and augmented secretion of IFN- γ [21]. Although ICOS regulated Th2 cytokine production, it was also preferentially expressed on Th2 versus Th1 cells, which supported the identification of the gene encoding murine ICOS as a Th2-overexpressed gene [22]. These data indicated that Th2 cytokines were directly regulated by ICOS. In our model, IL-5 levels were increased in BALF and serum in the OVA group compared to the levels in the control group, whereas the increases were inhibited in the OVA+ miR-29b agomir group. Besides, OVA-induced decreases in IFN- γ levels were reversed by miR-29b agomir in serum. These results indicate that the decreased IL-5 levels and increased IFN- γ levels in the OVA+ miR-29b agomir group are probably caused by miR-29b targeting ICOS.

Tfh (T follicular helper cells) are a distinct CD4⁺ T cell subset identified by the expression of CXCR5, PD1, Bcl-6, and ICOS and are predominantly located within the B-cell follicles of secondary lymphoid organs (SLOs), such as lymph nodes and the spleen [23]. ICOS defined a subset of T effector cells that were required for B cells infiltration and local IgE production in lung tissues on allergen airway exposure [16]. CD4⁺ICOS⁺ T cells were present within the B-cell follicles when mice were immunized and challenged, which raised the possibility that ICOS interacted with B7RP-1 to promote B cell class switch to IgE.

However, although ICOS played a critical role in IgE production, miR-29b agomir administration did not decrease the total IgE levels in serum and BALF in our model. It might be because the time required for IgE production was long enough, but we detected IgE expression

only 5 days after treatment with miR-29b agomir, and so we did not detect the difference.

Previously, it has been shown that miR-29c could regulate the expression of matrix metalloproteinase-2 in primary rat aorta smooth muscle cells. Meanwhile, matrix

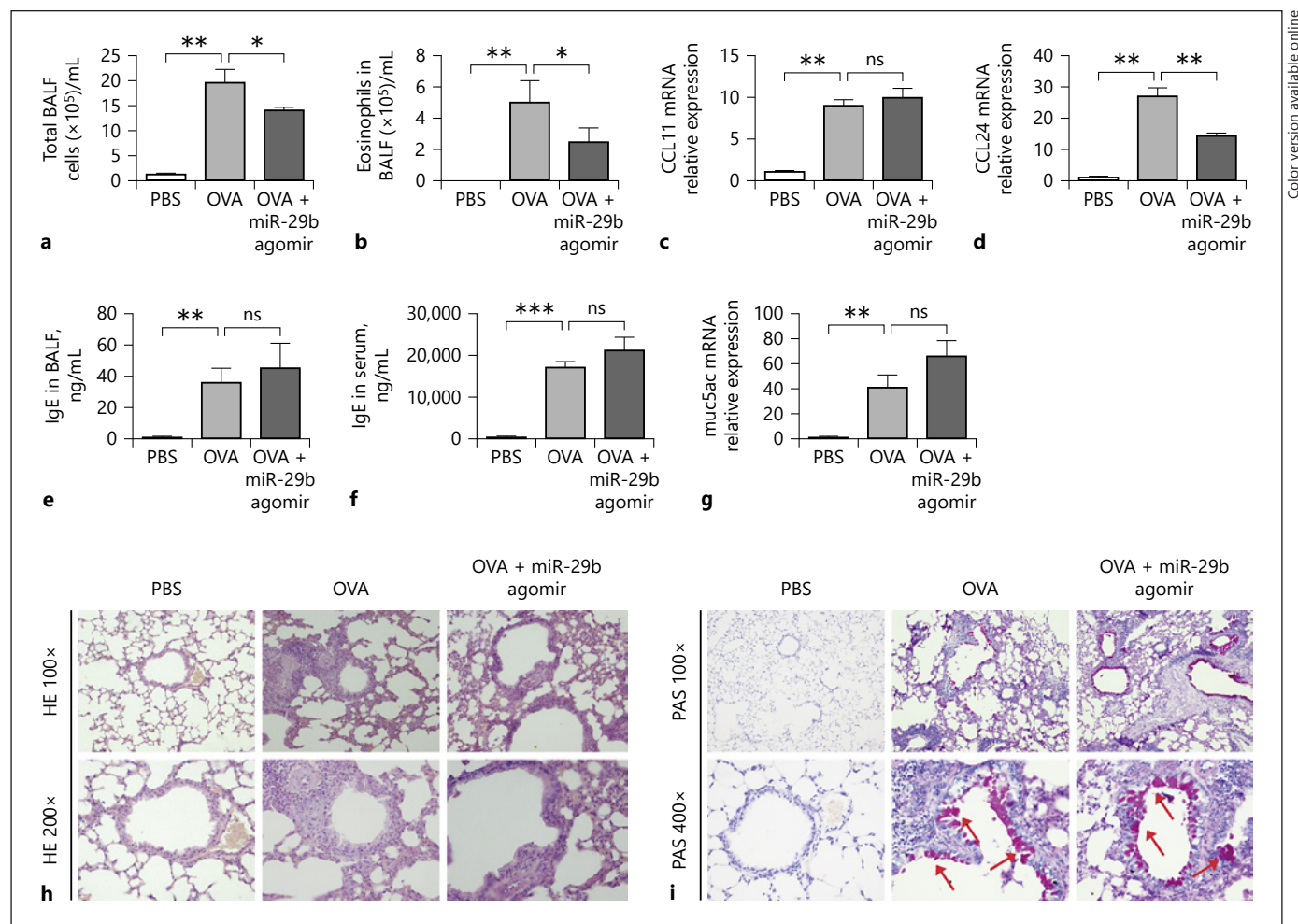


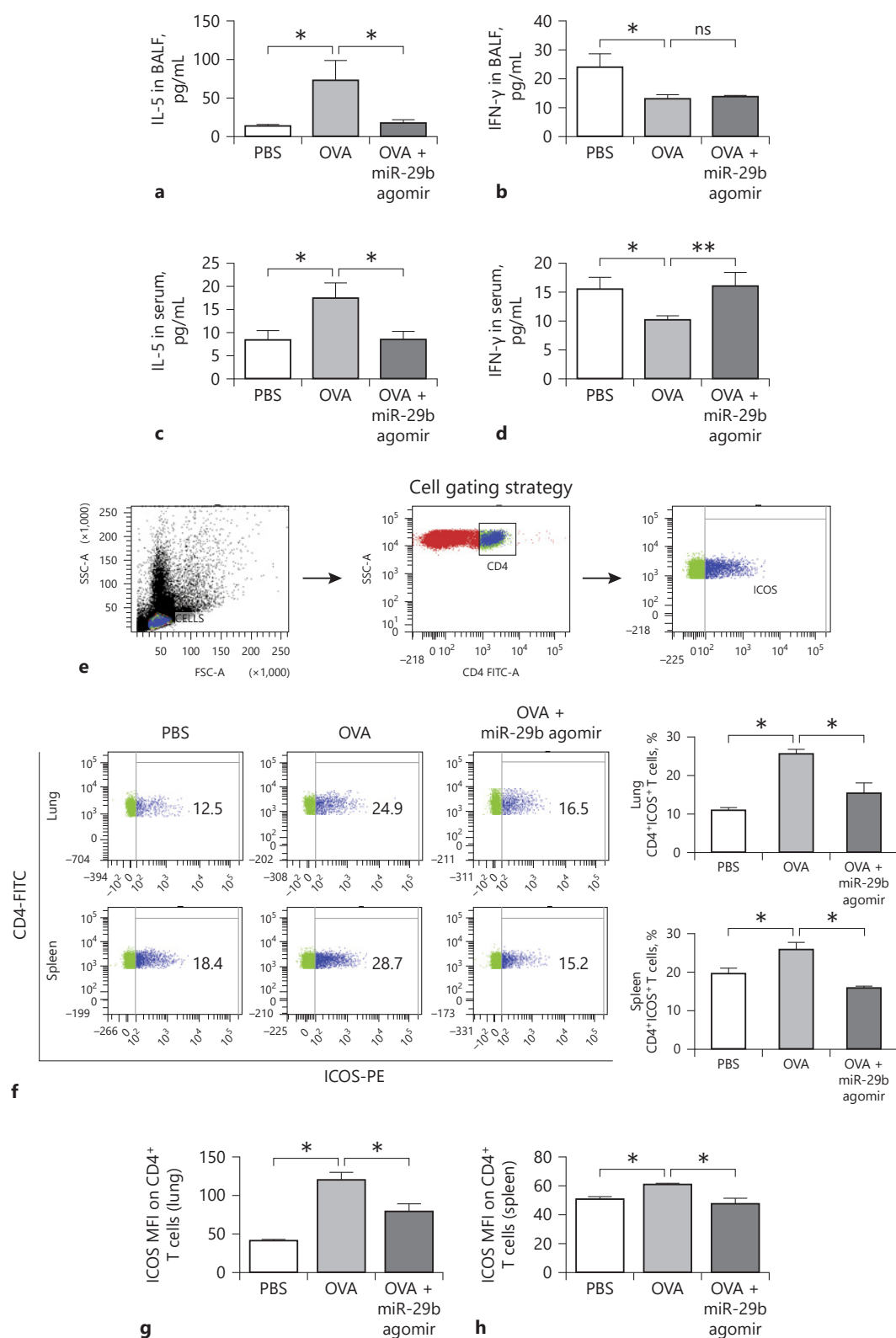
Fig. 6. Intranasal delivery of miR-29b agomir alleviates airway eosinophils recruitment. miR-29b overexpression in asthmatic mice was induced with miR-29b agomir by intranasal administration. **a**, **b** The number of total inflammatory cells and eosinophils counts in BALF. **c** CCL11 **d** CCL24 mRNA expression was determined by q-PCR in the PBS group, OVA group, and OVA+ miR-29b agomir group. **e**, **f** IgE levels were detected in BALF and serum. **g** Muc5ac

mRNA expression was determined by q-PCR. **h**, **i** Lung sections stained with H&E, or PAS were examined under a microscope. Statistical analysis was performed with Wilcoxon-Mann-Whitney test. Data are presented as mean $\pm$ SEM, 6–8 mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, not significant; PBS, phosphate-buffered saline; BALF, bronchoalveolar lavage fluid; IgE, immunoglobulin E; PAS, periodic acid-Schiff; H&E, hematoxylin and eosin.

Fig. 7. miR-29b regulates the balance of Th1/Th2 responses through ICOS. **a**, **b** The IL-5 and IFN- γ levels in BALF were detected by ELISA in PBS group, OVA group and OVA+ miR-29b agomir group. **c**, **d** The IL-5 and IFN- γ levels in serum were detected by ELISA. **e** The cell gating strategy of CD4⁺ICOS⁺ T cells. **f** The percentage of CD4⁺ICOS⁺ T cells in lung and spleen was detected by flow cytometry. The MFI of ICOS expression on

CD4⁺T cells in lung **g** and spleen **h** was analyzed. Statistical analysis was performed by Wilcoxon-Mann-Whitney test. Data are presented as mean $\pm$ SEM, 6–8 mice/group. * $p < 0.05$, ** $p < 0.01$. ns, not significant; PBS, phosphate-buffered saline; BALF, bronchoalveolar lavage fluid; ICOS, inducible co-stimulator; MFI, mean fluorescence intensity.

(For figure see next page.)



metalloproteinase-2 is one of main mediators in the pathogenesis of airway remodeling in asthma [24]. Interestingly, glucocorticoids, a classical treatment of asthma, can increase miR-29c expression in a time-dependent manner [25]. miR-29b activated NF- κ B signaling in KRAS-induced non-small cell lung cancers [26]. Additionally, NF- κ B induction was increased in allergen-sensitized murine lung tissues and airways of human asthma [27]. Therefore, it is worth studying signal pathways through which miR-29b affects the development of asthma by targeting ICOS, and whether other molecules, besides ICOS, targeted by miR-29b involves in pathogenesis of asthma.

In summary, our findings suggest that the miR-29b can suppress ICOS expression, and thereby alter the balance of Th1/Th2 responses and reduce eosinophils recruitment in the airway, which are hallmarks of asthma. These findings indicate that miR-29b might serve as a potential therapeutic target in the treatment of allergic asthma.

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Statement of Ethics

The protocols of the animal studies were reviewed and approved by the Chongqing Medical University Institutional Animal Care and Use Committee (Assurance Number: SYXK-2012–0001).

Disclosure Statement

The authors declare that they have no conflicts of interest to disclose.

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Author Contributions

W.X. and J.Y. designed the studies and wrote the paper; J.Y. and S.S. performed animal and cell experiments; T.Y. and J.Y. analyzed data; Y.Q., G.W., X.Z., and Y.Y. provided advice on experiments. All authors read and approved of the final manuscript.

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