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ORIGINAL ARTICLE

Asthma and Rhinitis

microRNA-218-5p plays a protective role in eosinophilic airway inflammation via targeting δ-catenin, a novel catenin in asthma

Yuxia Liang[1,2] | Yuchen Feng[1,2] | Wenliang Wu[1,2] | Chenli Chang[1,2] | Dian Chen[1,2] | Shengchong Chen[1,2] | Guohua Zhen[1,2]

[1]Division of Respiratory and Critical Care Medicine, Department of Internal Medicine, Tongji Medical College, Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China

[2]Key Laboratory of Respiratory Diseases of Ministry of Health, Wuhan, China

Correspondence
Guohua Zhen, Division of Respiratory and Critical Care Medicine, Department of Internal Medicine, Tongji Hospital, 1095 Jiefang Avenue, Wuhan 430030, China.
Email: ghzhen@tjh.tjmu.edu.cn

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Abstract

Background: microRNA (miR)-218-5p is involved in cigarette smoke-induced airway inflammation. In our earlier asthma epithelial miRNA profiling data, miR-218-5p was the top 2 down-regulated miRNA. We hypothesize that miR-218-5p plays a role in asthma airway inflammation.

Objective: To unveil the role of miR-218-5p and its target gene in asthma airway inflammation.

Methods: We measured miR-218-5p expression in bronchial brushings of asthma patients (n = 50) and healthy controls (n = 15), and analysed the correlations between miR-218-5p expression and airway eosinophilia. We examined whether *CTNND2* was a target of miR-218-5p, and the expression of 12 catenin family members in bronchial brushings, in cultured human bronchial epithelial (HBE) cells and BEAS-2B cells. We explored the role of miR-218-5p-CTNND2 pathway using a murine model of allergic airway inflammation.

Results: Epithelial miR-218-5p expression was significantly decreased and negatively correlated with eosinophils in induced sputum and bronchial biopsies, and other type 2 biomarkers in asthma patients. We verified that *CTNND2* (encoding δ-catenin) was a target of miR-218-5p. Remarkably, CTNND2 was the most significantly up-regulated catenin compared with the other 11 catenin family members in bronchial brushings of asthma patients, IL-13-stimulated HBE and BEAS-2B cells. Moreover, epithelial CTNND2 expression positively correlated with airway eosinophilia in asthma. Airway mmu-miR-218-5p expression was also decreased, and Ctnnd2 expression was increased in a murine model of allergic airway inflammation. Intriguingly, mmu-miR-218-5p overexpression suppressed airway hyperresponsiveness, eosinophilic airway inflammation and Ctnnd2 up-regulation in the mouse model. Finally, perturbation of miR-218-5p or CTNND2 expression significantly altered chemokine CCL26 expression in the cell cultures and the mouse model.

Conclusions and Clinical Relevance: Epithelial miR-218-5p plays a protective role in eosinophilic airway inflammation via targeting CTNND2, a novel catenin in asthma, and suppressing chemokine CCL26 expression.

KEYWORDS

asthma, eosinophilia, epithelial cell, microRNA, δ-catenin

1 | INTRODUCTION

Asthma is a chronic airway disease characterized by airway hyperresponsiveness (AHR), chronic airway inflammation, mucus overproduction and submucosal fibrosis. Asthma is a heterogeneous disease with different phenotypes.[1-3] Type 2-high phenotype comprises up to 50% of asthma patients.[3] Eosinophilic airway inflammation is one of the cardinal features of type 2-high asthma.[1,3] Airway epithelial cells can trigger type 2 inflammation via expressing innate cytokines IL-25, IL-33 and thymic stromal lymphopoietin.[4-9] Airway epithelial cells also promote the trafficking of eosinophils into the airways via expressing chemokine (C-C motif) ligand (CCL) 11/eotaxin-1, CCL24/eotaxin-2 and CCL26/eotaxin-3.[10-13] However, the mechanism by which the epithelial cells contribute to eosinophilic airway inflammation remains not fully understood.

miRNAs are non-coding ~22 nucleotide RNAs regulating gene expression post-transcriptionally.[14] It was reported that airway epithelial miRNA expression profiling was dramatically altered with 217 differentially expressed miRNAs between asthma patients and controls.[15] Among the dysregulated epithelial miRNAs, miR-34/449 family members contribute to epithelial cell differentiation, and let-7 family members target IL-13 expression and regulate the type 2 response.[16,17] A recent study profiled miRNA expression in cultured airway epithelial cells from severe asthma and identified miR-19 family member that has an established role in airway epithelial cell proliferation and Th2 cell differentiation.[18-20] Our earlier study profiled miRNA expression in bronchial brushings from treatment-naïve asthma and control. We reported the epithelial miR-181b-5p and miR-221-3p were involved in eosinophilic airway inflammation via regulating the expression of pro-inflammatory and anti-inflammatory cytokines, respectively.[21,22] miR-218-5p was the top 2 down-regulated epithelial miRNA identified in our earlier study,[22] and its expression was also decreased in bronchial epithelium of smokers and patients with chronic obstructive pulmonary disease (COPD).[23] miR-218-5p was reported to be involved in cigarette smoke-induced airway inflammation.[23,24] However, the role of miR-218-5p in asthma remains unknown. We hypothesized that epithelial miR-218-5p plays a role in asthma airway inflammation.

The catenin family members play essential roles in cell junctions and Wnt-signalling pathway.[25,26] There are 12 catenin family members subdivided into α-catenin (*CTNNA1*, *CTNNA2* and *CTNNA3*), β-catenin (*CTNNB1* and *CTNNG*) and p120-catenin subfamilies (*CTNND1*, *CTNND2*, *ARVCF*, *P0071*, *PKP1*, *PKP2* and *PKP3*).[27] In asthma airway epithelium, it was reported that α-catenin expression was decreased, whereas β-catenin expression was not altered.[28] *CTNNA3* (encoding αT-catenin) was identified as a promising locus for asthma exacerbation through multiple genome-wide association studies,[29] and genetic loss of αT-catenin attenuated airway inflammation and AHR in allergen-challenged mice.[30] So far, there is no comprehensive comparison of the 12 catenin members' expression in asthma airway epithelium. Although the activation of β-catenin (encoded by *CTNNB1*) signalling has an established role in asthma airway remodelling,[31] the role of catenin family members in asthma airway inflammation remains largely unknown.

In the present study, we demonstrated that epithelial miR-218-5p expression was decreased and negatively correlated with airway eosinophilia in asthma. *CTNND2*, a target of miR-218-5p, was the most significantly up-regulated catenin of the 12 catenin family members in asthma bronchial brushings, in IL-13-stimulated HBE and BEAS-2B cells. Intriguingly, mmu-miR-218-5p overexpression suppressed airway eosinophilic inflammation and AHR in a murine model of allergic airway inflammation. Finally, perturbation of miR-218-5p or CTNND2 expression altered chemokine CCL26 expression in the cell cultures and in the mouse model.

2 | METHODS

2.1 | Subjects

We recruited 15 healthy control subjects and 50 asthma patients. All subjects were Chinese and recruited from Tongji Hospital. Asthma patients had symptoms of recurrent episodes of wheezing, breathlessness, chest tightness and coughing, and had accumulated dosage of methacholine provoking a 20% fall (PD_{20}) of forced expiratory volume in the first second (FEV_1) <2.505 mg and/or ≥12% increase in FEV_1 following inhalation of 200 μg salbutamol. Healthy control subjects had no respiratory symptoms, normal spirometric value and methacholine PD_{20} ≥ 2.505 mg. None of the subjects had ever smoked or received inhaled or oral corticosteroid or leukotriene antagonist. For each subject, we recorded demographic information, collected induced sputum, and measured spirometry and FeNO. We performed bronchoscopy with endobronchial brushing and biopsy. Biopsy techniques and methods for spirometry and FeNO measurement were described previously.[22] Written informed consent was obtained from all subjects. The ethics committee of Tongji Hospital, Huazhong University of Science and Technology, approved the study.

2.2 | Murine model of allergic airway inflammation

Eight- to 10-week-old female C57BL/6 mice (CDC) were sensitized with intraperitoneal injection of OVA solution (100 μg in 100 μL saline; Sigma-Aldrich) mixed with $Al(OH)_3$ as an adjuvant on days 0, 7 and 14. Mice were challenged with intranasal administration of OVA solution (1 mg in 50 μL saline) on days 21, 22, 23, 24 and 25. mmu-miR-218-5p agomir (5 nmol in 40 μL saline; RiboBio) or control agomir was administered intranasally 2 hours before OVA challenge on days 21, 23 and 25. Twenty-four hours after the last challenge of OVA, we measured pulmonary resistance in response to a range of concentrations of intravenous methacholine using the forced oscillation technique with the FlexiVent system (SCIREQ). Lung tissues were collected for histological analysis, quantitative PCR and immunostaining. Animal experiments were approved by the ethics committee of Tongji Hospital, Huazhong University of Science and Technology.

2.3 | Cell culture and treatment

Human bronchial epithelial (HBE) cells (ScienCell) were cultured at air–liquid interface as previously described[32] and stimulated with or without IL-13 (20 ng/mL; PeproTech). BEAS-2B cells (ATCC) were stimulated with or without IL-13 and transfected with control or miR-218-5p mimic (50 μmol/L; RiboBio), control or miR-218-5p inhibitor (100 μmol/L), scrambled control or *CTNND2* siRNA (80 μmol/L; GeneCopoeia), or empty or *CTNND2* cDNA expression vector (500 ng/mL) using Lipofectamine 3000 (Invitrogen). The sequence of the sense strand of *CTNND2* siRNA was 5′-GCAGUGAGAUCGAUAGCA ATTUUGCUAUCGAUCUCACUGCTT-3′. Forty-eight hours after IL-13 stimulation, cells were harvested for quantitative PCR and Western blotting. The cell culture media were collected for ELISA.

2.4 | Quantitative PCR

Total RNA from bronchial epithelial brushing, mouse lungs and cultured cells was isolated using TRIzol (Invitrogen) and reverse-transcribed using the PrimeScript RT reagent kit (Takara). The expression of each gene was determined using an ABI Prism 7500 PCR System (Applied Biosystems). The primers used are listed in Table S1. The gene expression was determined by the $2^{-\Delta\Delta CT}$ method.[33] The gene expression was expressed as log2 transformed and relative to the median of healthy control subjects or the mean of control group.

2.5 | In situ hybridization

miRNA-218-5p was detected in paraffin-embedded human bronchial biopsies or mouse lung tissue using biotin-labelled miR-218-5p miRNA probe (Qiagen). The sequence of the probe for both hsa- and mmu-miR-218-5p was 5′-ACATGGTTAGATCAAGCACAA-3′. The protocol was according to the manufacturer's instructions.

2.6 | Histology and immunohistochemistry

Human bronchial biopsy and mouse lung sections were stained with haematoxylin and eosin (H&E). Observers who were blinded to the clinical status of the subjects counted numbers of eosinophils/mm^2 submucosa as previously described.[34] The methods for immunohistochemistry, assessment of airway inflammation and PAS staining are described in Appendix S1.

2.7 | Luciferase activity assay

Vector harbouring wild-type, mutant *CTNND2* 3′-UTR or empty vector was co-transfected with miR-218-5p mimic or non-targeting control into BEAS-2B cells, respectively. Luciferase activity was detected using dual-luciferase reporter assay system (Promega). Normalized relative light units represent firefly luciferase activity/Renilla luciferase activity.

2.8 | Western blotting

CTNND2 protein in BEAS-2B cells was measured by Western blotting using monoclonal mouse anti-human CTNND2 antibody (1:500; Abnova). Antibody was detected using horseradish peroxidase-conjugated goat anti-mouse IgG (1:4000; Aspen) followed by ECL Western blot detection reagent (Beyotime Biotech). Densitometry was assessed using ImageJ (National Institutes of Health), and the protein levels of CTNND2 were indexed to GAPDH.

2.9 | ELISA

CCL11, CCL24 and CCL26 protein levels in BEAS-2B cell culture media were measured using commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions. All samples and standards were measured in duplicate.

2.10 | Statistical analysis

We analysed data using Prism version 7 (GraphPad Software). For normally distributed data, we calculated means ± standard deviation (SD) and used parametric tests (Student's *t* test or one-way ANOVA followed by Tukey's multiple comparison test) to compare across groups. For non-normally distributed data, we calculated medians with interquartile ranges and used non-parametric tests (Mann–Whitney test). We analysed correlation using Spearman's rank-order correlation. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | The expression of epithelial miR-218-5p is decreased and correlates with airway eosinophilia in asthma

We recruited 15 healthy controls and 50 asthma patients who were newly diagnosed and treatment-naïve. Subject characteristics are summarized in Table 1. The expression of miR-218-5p in bronchial epithelial brushings was measured using quantitative PCR. We found that epithelial miR-218-5p transcript levels were significantly decreased in asthma patients compared with healthy controls (Figure 1A). In situ hybridization in bronchial biopsies revealed that miR-218-5p was mainly expressed in bronchial epithelial cells, and its expression was decreased in asthma patients compared with controls (Figure 1B).

Eosinophilic airway inflammation is a key feature of type 2-high asthma. We analysed the correlations between epithelial miR-218-5p expression and the clinical indicators of airway eosinophilic inflammation including eosinophils in induced sputum and bronchial biopsy, and fraction of exhaled nitric oxide (FeNO). In asthma patients, epithelial miR-218-5p expression negatively correlated with the eosinophil percentage in induced sputum (Figure 1C; Table S2), eosinophils in bronchial submucosa (Figure 1D; Table S2) and FeNO (Figure 1E; Table S2). However, there were no correlations between epithelial miR-218-5p and sputum neutrophils in asthma patients

TABLE 1 Subject characteristics

	Healthy controls	Asthma patients	*P* value
Number	15	50	
Age, y	39.93 ± 11.01	40.00 ± 11.99	.985
Sex, M:F (%F)	4:11 (73.3)	12:38 (76.0)	.833
Body mass index	22.07 ± 2.82	22.52 ± 3.11	.535
FEV_1, % predicted	95.66 ± 12.18	76.98 ± 19.71	.0002
Methacholine PD_{20}, mg	2.505 ± 0	0.13 ± 0.24	<.0001
Sputum eosinophil, %	0.72 ± 0.58	12.60 ± 12.71	<.0001
Biopsy eosinophil, #/mm^2	1.07 ± 0.63	15.39 ± 15.58	<.0001
FeNO, ppb	15.58 ± 13.68	82.08 ± 58.11	<.0001

Note: Values were presented as mean ± SD.
Abbreviations: FeNO, fraction of exhaled nitric oxide; FEV_1, forced expiratory volume in the first second; PD_{20}, provocative dosage required to cause a 20% decline in FEV_1. The minimal and maximal provocative dosages were 0.01 and 2.505 mg, respectively.

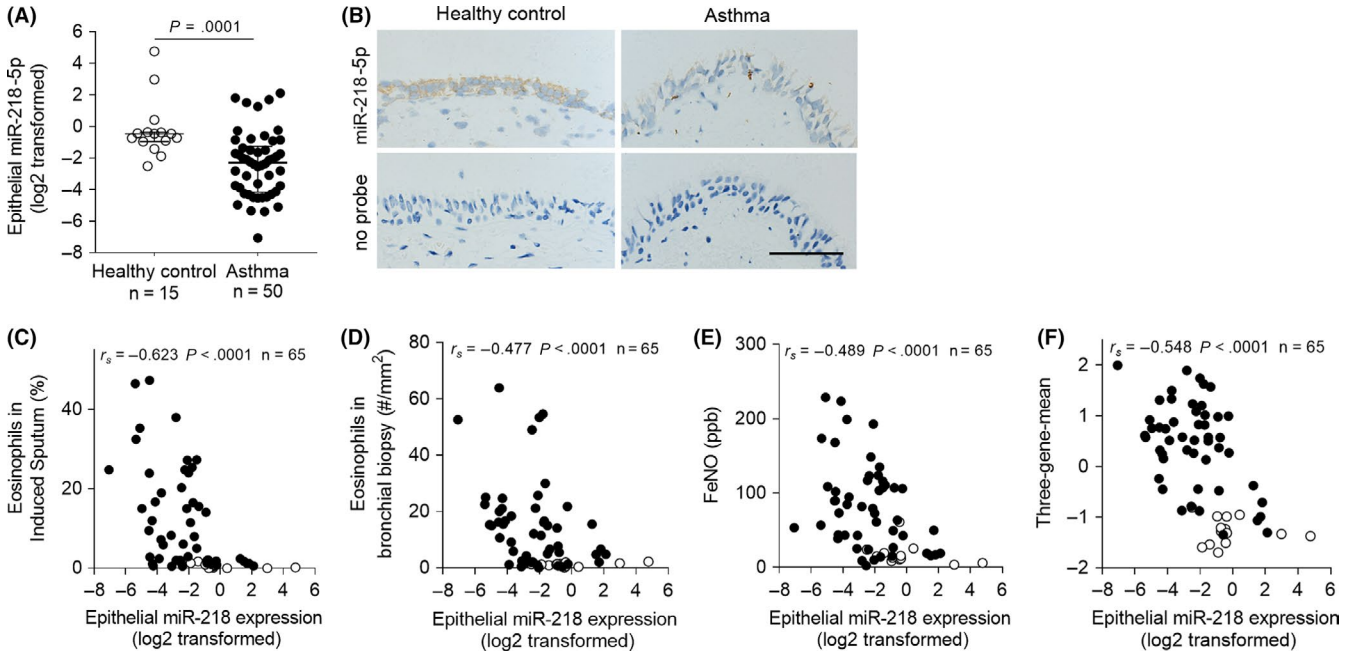


FIGURE 1 Epithelial miR-218-5p is decreased and correlates with eosinophilic airway inflammation in asthma. A, miR-218-5p transcript levels in bronchial brushings from asthma patients (n = 50) and healthy controls (n = 15) were measured by quantitative PCR. The transcript levels were expressed as log2 transformed and relative to the median value for healthy controls (two-tailed Mann–Whitney test). B, Representative images of in situ hybridization of miR-218-5p in epithelium of bronchial biopsies from asthma patients and healthy controls. Scale bar, 50 μm. C-F, Correlation assays between epithelial miR-218-5p transcript levels and eosinophil in induced sputum (C) and bronchial biopsies (D), FeNO (E) and three-gene-mean of *CLCA1*, *POSTN* and *SERPINB2* (F). Asthma patients were presented as dots (n = 50), and healthy controls as circles (n = 15). Correlation assays were performed using Spearman's rank-order correlation

(Figure S1). Our data indicate that epithelial miR-218-5p expression is down-regulated and correlates with airway eosinophilia in asthma. Moreover, epithelial miR-218-5p expression negatively correlated with the three-gene-mean of *CLCA1*, *SERPINB2* and *POSTN*, a type 2 gene signature,[1,35] in bronchial brushings (Figure 1F; Table S2).

3.2 | CTNND2 is a target of miR-218-5p

We predicted the target genes of miR-218b-5p using online algorithms miRanda, miRWalk, PicTar and TargetScan. The same targets predicted by these algorithms were selected as candidate targets (Table S3). We found that *CTNND2* (encoding δ-catenin) was one of the candidate targets. Since *CTNNA3* plays a role in asthma airway inflammation,[30] we next focused on the role of *CTNND2* in asthma. miRNA regulates gene expression via acting on the 3′-UTR of the mRNA. When miR-218-5p mimic was co-transfected with luciferase reporter vector harbouring wild-type 3′-UTR of *CTNND2* in BEAS-2B cells, the luciferase activity was significantly reduced compared with the cells co-transfected with miR-218-5p mimic and the vector harbouring mutant *CTNND2*

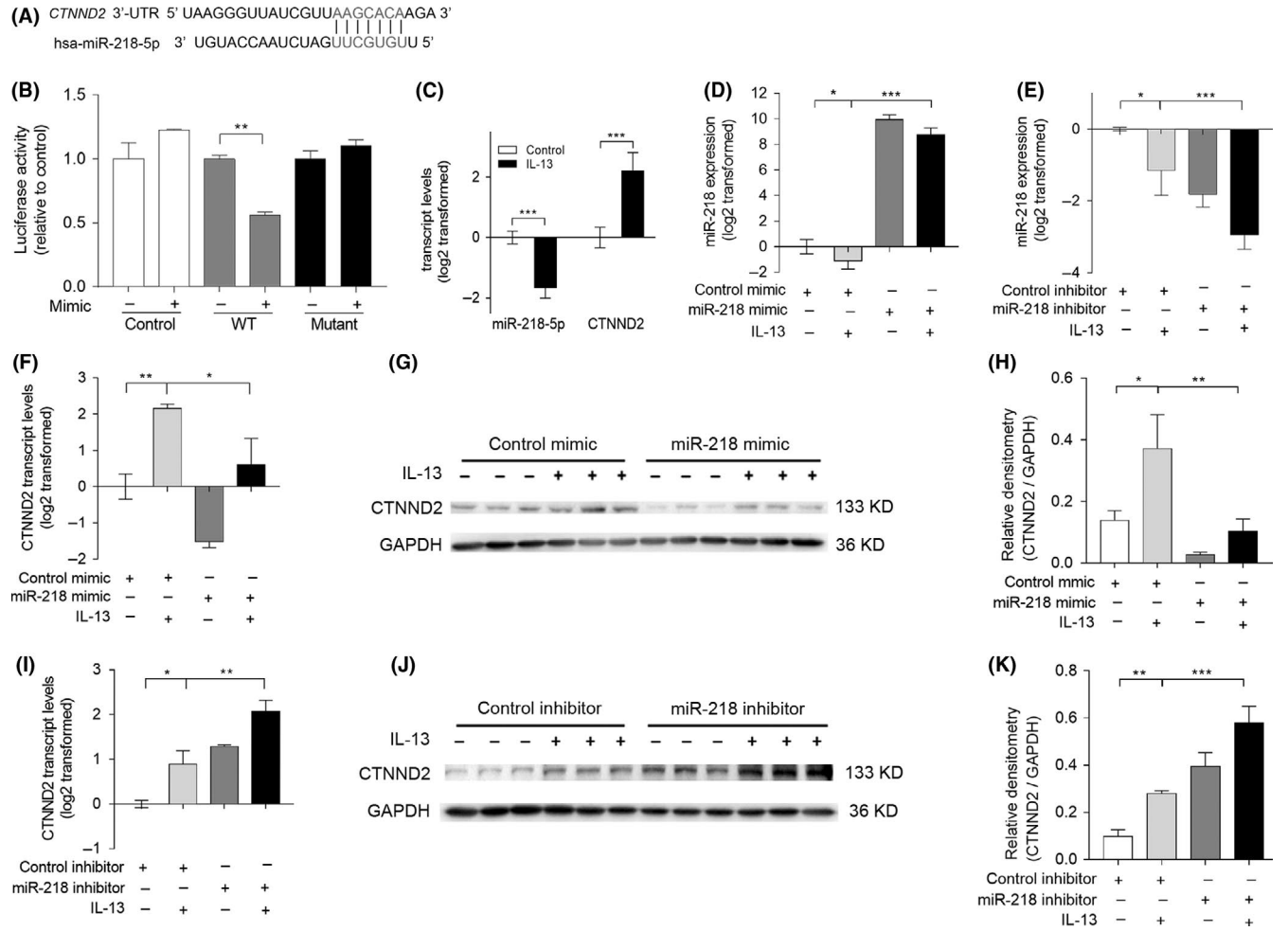


FIGURE 2 CTNND2 is a target of miR-218-5p. A, The 3′-UTR of *CTNND2* contains the region matching the seed sequence of hsa-miR-218-5p. B, 3′-UTR luciferase report assay with vector harbouring wild-type (WT), mutant *CTNND2* 3′-UTR (mutant) or empty vector (control) co-transfected with miR-218-5p mimic or control mimic. Luciferase activity was measured by dual-luciferase reporter assay system. The firefly luciferase activity was normalized to Renilla luciferase activity. C, The expression of miR-218-5p and CTNND2 in IL-13-stimulated HBE cells was measured by quantitative PCR. D, E, The expression of miR-218-5p in BEAS-2B cells after transfection with miR-218-5p mimic or inhibitor with or without IL-13 stimulation was measured by quantitative PCR. F, G, I, J, CTNND2 mRNA (F, I) and protein (G, J) expression in BEAS-2B cells after transfection with control or miR-218-5p mimic, or miR-218 inhibitor with or without IL-13 stimulation was measured by quantitative PCR and Western blotting, respectively. The transcript level was expressed as log2 transformed and relative to the mean of control group. n = 3-4 per group. H, K, Densitometry assay of the Western blotting results was analysed using ImageJ, and the protein levels of CTNND2 were indexed to GAPDH. Data are mean ± SD. **P* < .05; ***P* < .01; ****P* < .001 (one-way ANOVA followed by Tukey's multiple comparison test). The data are representative of three independent experiments

3′-UTR or control vector (Figure 2A, B). This indicates that miR-218-5p directly acts on the 3′-UTR of *CTNND2* mRNA.

We used IL-13, a type 2 cytokine, to stimulate HBE cells cultured at air–liquid interface and BEAS-2B cells. miR-218-5p transcript levels were decreased, whereas *CTNND2* transcript levels were increased in IL-13-stimulated cells compared with unstimulated cells (Figure 2C for HBE cells; Figure S2A for BEAS-2B cells). Overexpression or inhibition of miR-218-5p suppressed or enhanced baseline and IL-13-induced *CTNND2* transcripts and protein levels in BEAS-2B cells, respectively (Figure 2D-K). These results confirm that miR-218-5p targets *CTNND2*. In addition, overexpression or inhibition of miR-218-5p had no effect on the expression of *CTNNA3*, another catenin implicated in asthma airway inflammation (Figure S3).

3.3 | CTNND2 is the most significantly up-regulated catenin in asthma airway epithelium

We next examined CTNND2 expression in bronchial brushings and biopsies from control and asthma patients. *CTNND2* transcript levels were significantly higher in asthma bronchial brushings compared with controls (Figure 3A). Immunostaining revealed that CTNND2 protein was mainly expressed in the cytoplasm of asthma bronchial epithelial cells (Figure 3B, C). In support of CTNND2 as a target of miR-218-5p, epithelial *CTNND2* transcripts negatively correlated with miR-218-5p expression in asthma patients (Figure 3D; Table S4).

We compared the transcript levels of the 12 catenin family members in bronchial brushings using quantitative PCR. Remarkably,

CTNND2 was the most significantly up-regulated epithelial catenin in asthma patients (Figure 3E). The transcripts of *CTNNA3*, a catenin with an established role in asthma airway inflammation,[30] were also significantly increased in asthma. Moreover, we examined the expression of the 12 catenins in IL-13-stimulated HBE cells and BEAS-2B cells. Similar to our findings in bronchial brushings, *CTNND2* was the most significantly up-regulated catenin after IL-13 stimulation in both cell cultures (Figure 3F; Figure S2B). *CTNNA3* transcripts were also increased in IL-13-stimulated HBE cells (Figure 3F) whereas not in BEAS-2B cells (Figure S2B). These data suggest that CTNND2 may be involved in asthma pathogenesis, and type 2 cytokine such as IL-13 is responsible for epithelial CTNND2 up-regulation in asthma.

3.4 | Epithelial CTNND2 expression correlates with airway eosinophilia

Given epithelial miR-218-5p expression correlated with airway eosinophilia in asthma, we analysed the correlation between epithelial CTNND2 expression and airway eosinophilia. Epithelial CTNND2 transcript levels positively correlated with eosinophil percentage in induced sputum (Figure 3G; Table S4), eosinophils in bronchial submucosa (Figure 3H; Table S4) and FeNO (Figure 3I; Table S4). Moreover, epithelial CTNND2 transcripts correlated with the three-gene-mean (Figure 3J; Table S4). These data suggest that epithelial CTNND2 may contribute to eosinophilic airway inflammation in asthma.

3.5 | Airway overexpression of mmu-miR-218-5p suppresses Ctnnd2 expression and airway eosinophilia in a murine model of allergic airway inflammation

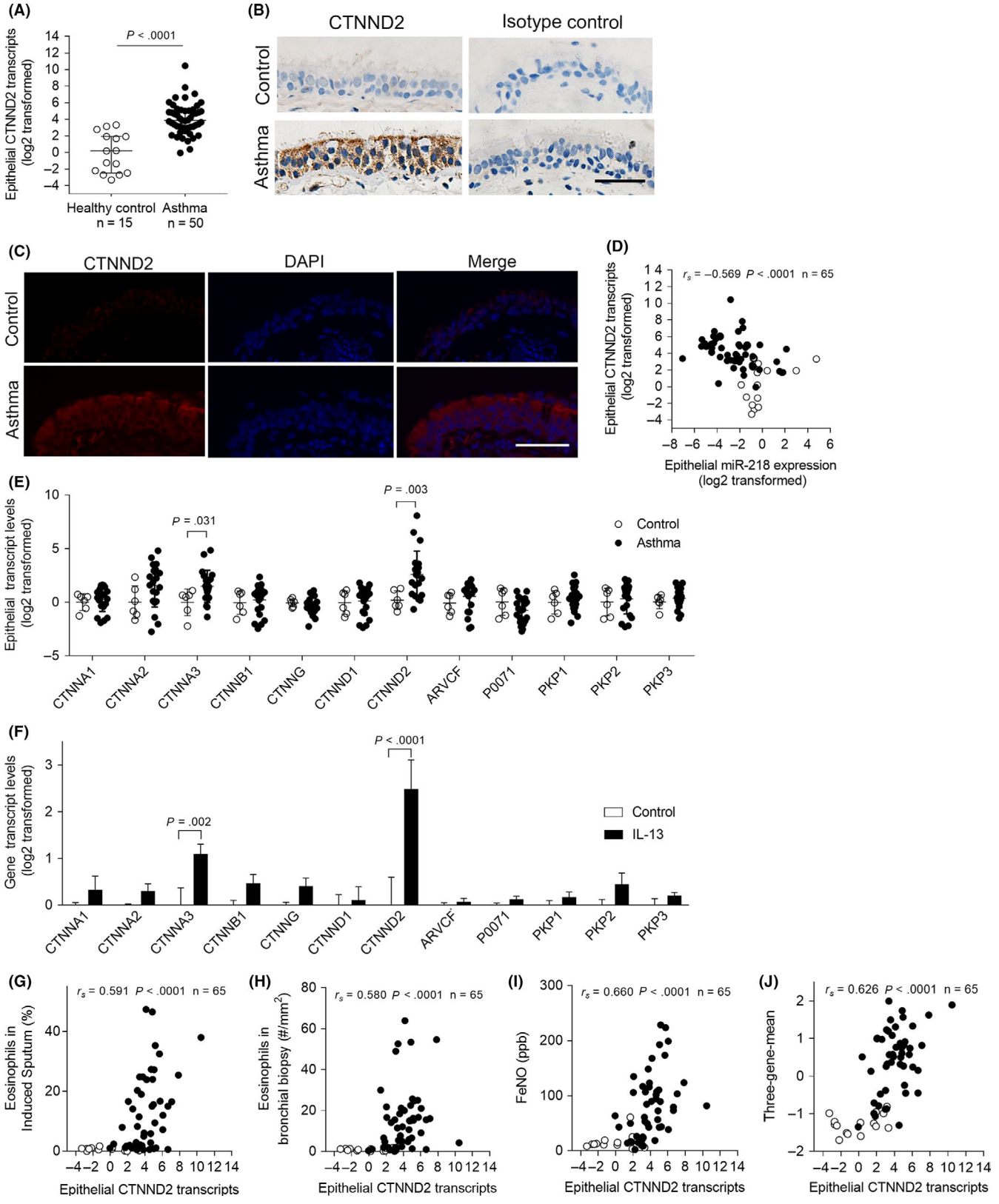
Since miR-218-5p is conserved across species, we further explored the role of miR-218-5p-CTNND2 pathway in asthma using a murine model of allergic airway inflammation. Mice were sensitized and challenged with OVA, and mmu-miR-218-5p or control agomir was administered intranasally 2 hours before OVA challenges (Figure 4A). OVA sensitization and challenge increased airway resistance to methacholine and resulted in infiltration of inflammatory cells around the airways as assessed using H&E staining, airway inflammation scoring and BALF cell counting (Figure 4B-E). Intriguingly, mmu-miR-218-5p overexpression suppressed OVA challenge-induced airway hyperresponsiveness and airway inflammation (Figure 4B-D). Specifically, mmu-miR-218-5p overexpression suppressed the increase in eosinophils in BALF after OVA challenge (Figure 4E). Our data provide direct evidence for a protective role of miR-218-5p in airway eosinophilia in the murine model of allergic airway inflammation. However, PAS staining and measurement of *Muc5ac* transcript levels revealed that mmu-miR-218-5p overexpression had no effect on OVA challenge-induced airway mucus overproduction (Figure S4).

Using in situ hybridization, immunostaining and quantitative PCR, we found that mmu-miR-218-5p expression was decreased, whereas *Ctnnd2* mRNA and protein expression was increased in the airway of mice challenged with OVA and treated with control agomir when compared to control mice (Figure 5A-D). We also observed similar changes in mmu-miR-218-5p and *Ctnnd2* expression in OVA-challenged and control mice without intervention with control or mmu-miR-218-5p agomir (Figure S5). These results were consistent with our findings in human asthma. Moreover, mmu-miR-218-5p overexpression successfully reversed mmu-miR-218-5p down-regulation and suppressed *Ctnnd2* up-regulation after OVA challenge (Figure 5A-D). This supports that mmu-miR-218-5p targets *Ctnnd2*. Together, our in vivo findings suggest that miR-218-5p-Ctnnd2 pathway plays an essential role in allergic airway inflammation.

3.6 | miR-218-5p-CTNND2 pathway regulates chemokine CCL26 expression in airway epithelial cells

To further investigate the mechanism by which miR-218-5p-CTNND2 pathway contributes to eosinophilic airway inflammation, we examined whether this pathway regulates the expression of chemokines CCL11, CCL24 and CCL26. Although CCL11 and CCL24 protein levels mildly increased in BEAS-2B cell culture media, the increase was not statistically significant (Figure 6A, B). CCL26 protein levels were markedly increased after IL-13 stimulation, whereas this increase was markedly suppressed by miR-218-5p overexpression with transfecting miR-218-5p mimic. In contrast, inhibition of miR-218-5p expression further enhanced IL-13-induced CCL26 expression (Figure 6C, D). Moreover, CTNND2 overexpression further enhanced IL-13-induced CCL26 expression (Figure 6E, F), whereas CTNND2 knockdown markedly suppressed CCL26 expression (Figure 6G, H). These results suggest that miR-218-CTNND2

FIGURE 3 Epithelial CTNND2 is the most significantly up-regulated catenin and correlates with airway eosinophilia in asthma. A, *CTNND2* transcript levels in bronchial brushings from controls (n = 15) and asthma patients (n = 50) were measured by quantitative PCR. The transcript level was expressed as log2 transformed and relative to control (two-tailed Mann–Whitney test). B, Representative images of CTNND2 immunohistochemistry in bronchial biopsies from healthy controls and asthma patients. Scale bar, 50 μm. C, Representative images of CTNND2 immunofluorescence staining (red) in bronchial biopsies from controls and asthma patients. Nuclei were stained with DAPI (blue). Scale bar, 50 μm. D, Correlation assay between epithelial *CTNND2* and miR-218-5p transcript levels in controls (n = 15) and asthma patients (n = 50). E, The transcript levels of catenin family members in bronchial brushings controls (n = 6) and asthma patients (n = 21) were measured by quantitative PCR (two-tailed Mann–Whitney test). F, The transcript levels of catenin family members in control and IL-13-stimulated HBE cells were measured by quantitative PCR. The transcript level was expressed as log2 transformed and relative to control (two-tailed Student's *t* test). G-J, Correlation assays between epithelial *CTNND2* transcript levels and eosinophils in induced sputum (G) and bronchial biopsies (H), FeNO (I) and three-gene-mean of *CLCA1*, *POSTN* and *SERPINB2* (J). Asthma patients were presented as dots and healthy controls as circles. Correlation assays were performed using Spearman's rank-order correlation



pathway regulates chemokine CCL26 expression in airway epithelial cells. Since CCL26 is a key mediator of airway eosinophilia,[12] our data partly explain the protective role of miR-218-5p-CTNND2 pathway in eosinophilic airway inflammation.

In asthma bronchial brushings, CCL26 transcript levels negatively correlated with miR-218-5p expression, whereas positively correlated with CTNND2 transcript levels (Figure 6I, J; Table S5). In the mouse model, OVA sensitization and challenge increased Ccl26

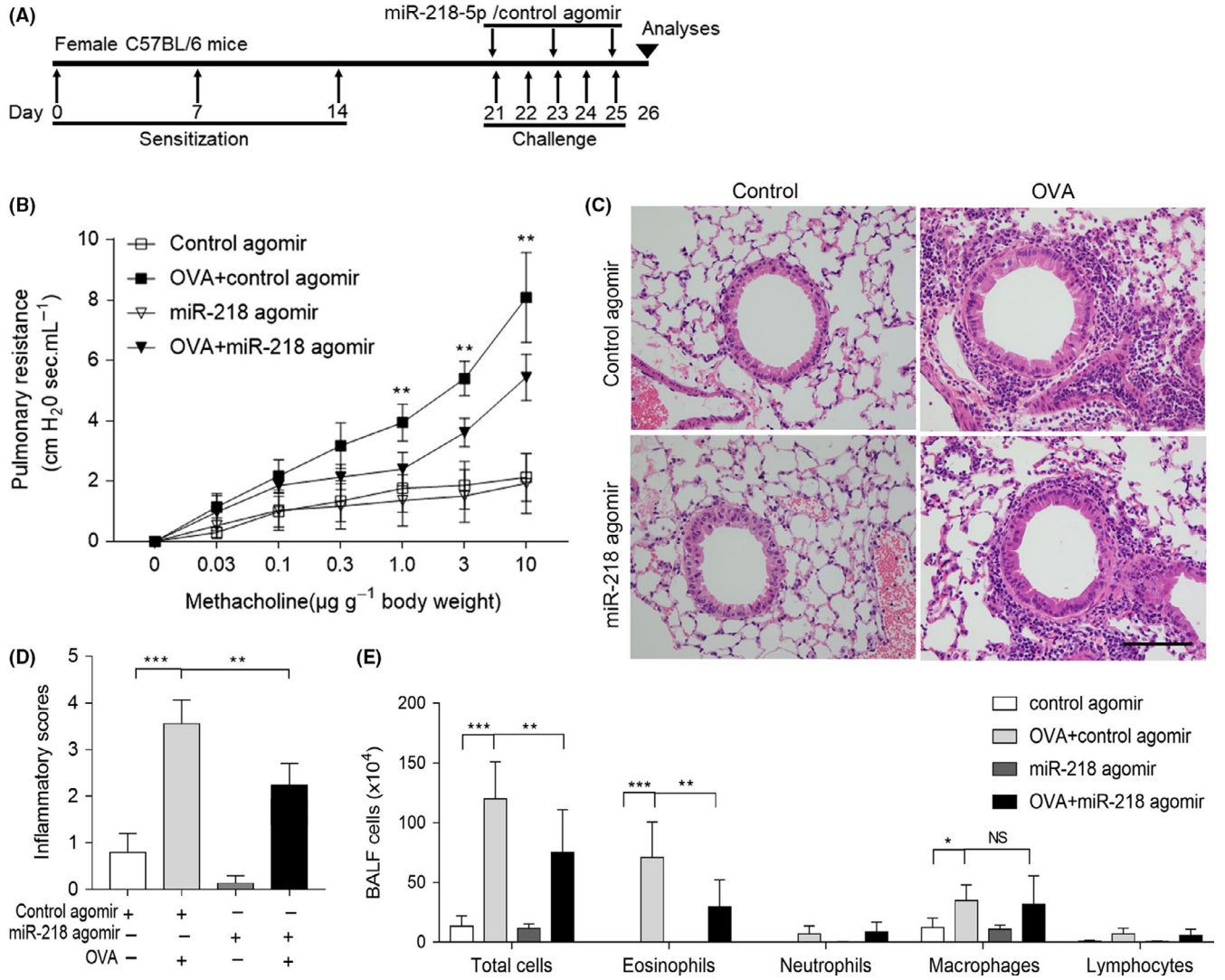


FIGURE 4 mmu-miR-218-5p overexpression suppresses AHR and airway eosinophilia in a mouse model of allergic airway inflammation. A, Experimental schedule. mmu-miR-218-5p or control agomir was administered intranasally 2 hours before OVA challenge on days 21, 23 and 25. B, Pulmonary resistance in response to different concentration of intravenous methacholine in mice that were administered intranasally with miR-218-5p agomir or control agomir after sensitization and challenge with OVA or saline. C, Representative images of H&E staining of mouse lung sections. Scale bar, 50 μm. D, Lung inflammatory scores were calculated as described in Methods. E, Cell counting for macrophages, eosinophils, lymphocytes and neutrophils in BALF. n = 6 - 10 mice per group. Data are mean ± SD. **P* < .05; ***P* < .01; ****P* < .001 (one-way ANOVA followed by Tukey's multiple comparison test). The data are representative of three independent experiments

mRNA in mouse lungs, whereas miR-218-5p overexpression suppressed OVA challenge-induced Ccl26 as well as Ctnnd2 expression (Figure 6K). These data support the role of miR-218-5p-CTNND2 pathway in chemokine CCL26 expression.

4 | DISCUSSION

In the present study, we demonstrated that epithelial miR-218-5p expression was significantly down-regulated and negatively correlated with airway eosinophilia in asthma. For the first time, we reported that *CTNND2*, a target of miR-218-5p, was the most significantly up-regulated catenin of the 12 catenin family members in bronchial brushings of asthma patients. Moreover, epithelial CTNND2 expression positively correlated with airway eosinophilia in asthma. Intriguingly, mmu-miR-218-5p overexpression attenuated AHR and airway eosinophilic inflammation in a murine model of allergic airway inflammation. The protective effect of miR-218-5p was, at least in part, due to suppressing Ctnnd2 and chemokine Ccl26 expression. In support of this, miR-218-5p-CTNND2 pathway contributed to CCL26 expression in epithelial cell culture.

In our earlier epithelial miRNA profiling data, miR-218-5p was the top 2 down-regulated epithelial miRNA in asthma.[22] Here, we confirmed that miR-218-5p expression was significantly decreased in bronchial epithelium in treatment-naïve asthma patients compared with controls. Consistently, miR-218-5p expression was decreased in both IL-13-stimulated human bronchial epithelial cells

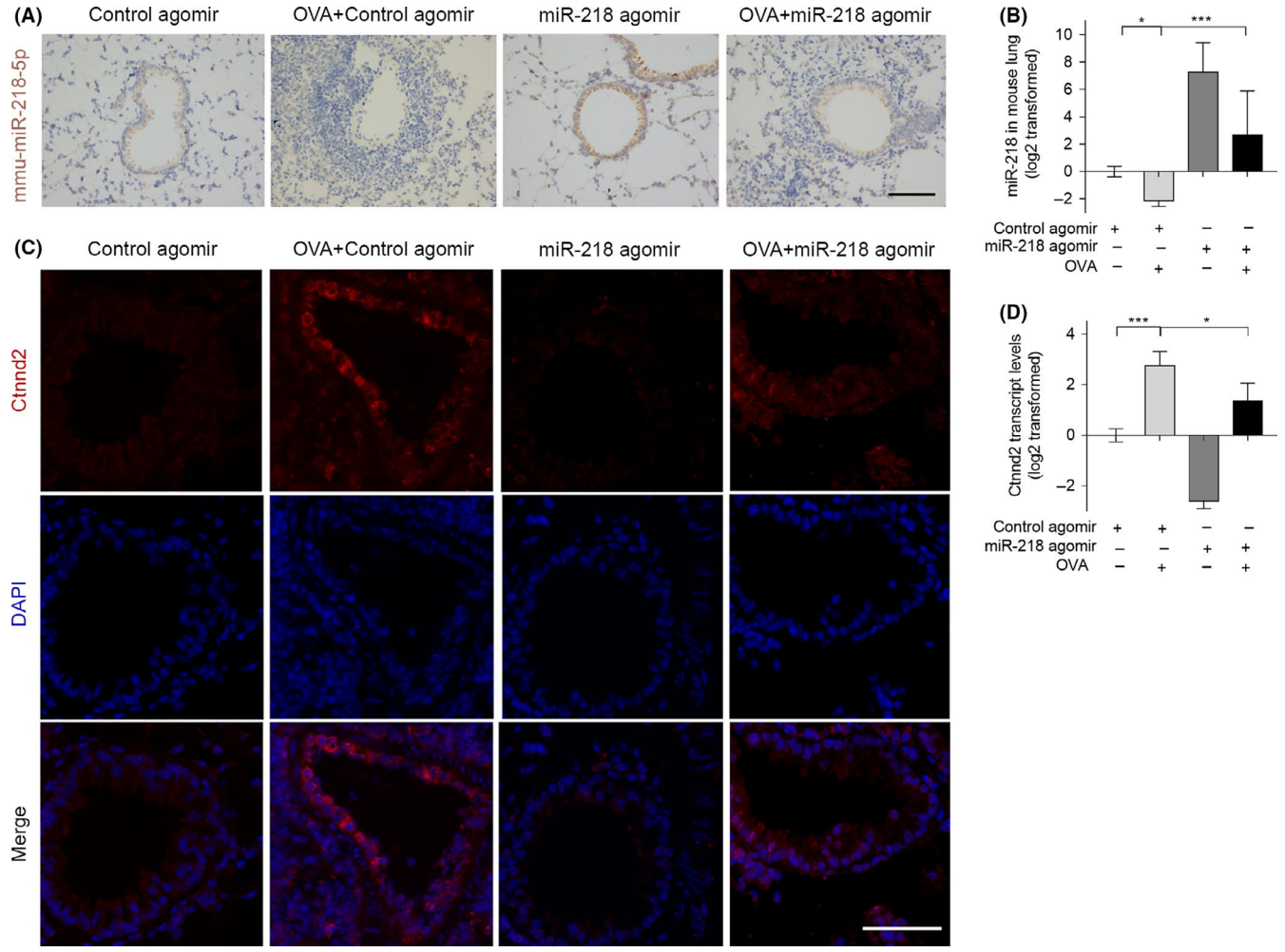


FIGURE 5 mmu-miR-218-5p overexpression suppresses Ctnnd2 up-regulation in the mouse model. A, Representative images of in situ hybridization of miR-218-5p in mouse lung sections. Scale bar, 50 μm. B, miR-218-5p transcript levels in mouse lungs were measured by quantitative PCR. C, Representative images of Ctnnd2 immunofluorescence staining in mouse lung sections. Scale bar, 50 μm. D, *Ctnnd2* transcript levels in mouse lungs were measured by quantitative PCR. The transcript level was expressed as log2 transformed and relative to the mean of control group. n = 6 - 10 mice per group. Data are mean ± SD. * $P < .05$; ***$P < .001$ (one-way ANOVA followed by Tukey's multiple comparison test). The data are representative of three independent experiments

and OVA-challenged mice airways. It was reported that miR-218-5p was down-regulated in bronchial epithelium in smokers and COPD patients, and in the lungs of mice exposed to cigarette smoke.[23,24] These data suggest that miR-218-5p is involved in both of the chronic airway diseases, COPD and asthma.

Airway eosinophilic inflammation is one of the key features of asthma. We found that epithelial miR-218-5p expression negatively correlated with airway eosinophilia in asthma patients. Epithelial miR-218-5p expression also negatively correlated the expression of type 2 signature genes. Accordingly, we showed that IL-13, a type 2 cytokine, reduced miR-218-5p expression in both primary HBE cells cultured at air–liquid interface and BEAS-2B cells. Our findings support a role of miR-218-5p in eosinophilic airway inflammation in asthma.

We next verified that *CTNND2*, a catenin never described in asthma, is a target of miR-218-5p. Although catenin was implicated in the pathogenesis of asthma,[28,30] there was no comprehensive examination of the catenin family members in asthma airway epithelium. In a head-to-head comparison of the 12 catenins' expression, we demonstrated that CTNND2 was the most significantly up-regulated catenin in bronchial brushings of asthma patients. CTNND2 expression was also markedly increased in IL-13-stimulated HBE cells and BEAS-2B cells. *CTNND2* belongs to the p120 catenin subfamily. In addition to the role in cell junction, members of this subfamily are involved in regulation of gene expression.[36,37] CTNND2 can regulate gene expression via nuclear translocation and interacting with transcription factor.[38,39] Mice lacking *CTNND2* exhibited severe cognitive and synaptic dysfunction.[40,41] Interestingly, mice deficient in miR-218-5p exhibited neuromuscular junction defects.[42]

Further, epithelial CTNND2 expression negatively correlated with miR-218-5p expression, whereas positively correlated with airway eosinophilia in asthma. This led us to hypothesize that miR-218-5p-CTNND2 pathway may play a role in eosinophilic airway inflammation in asthma. Similar to the human data, airway

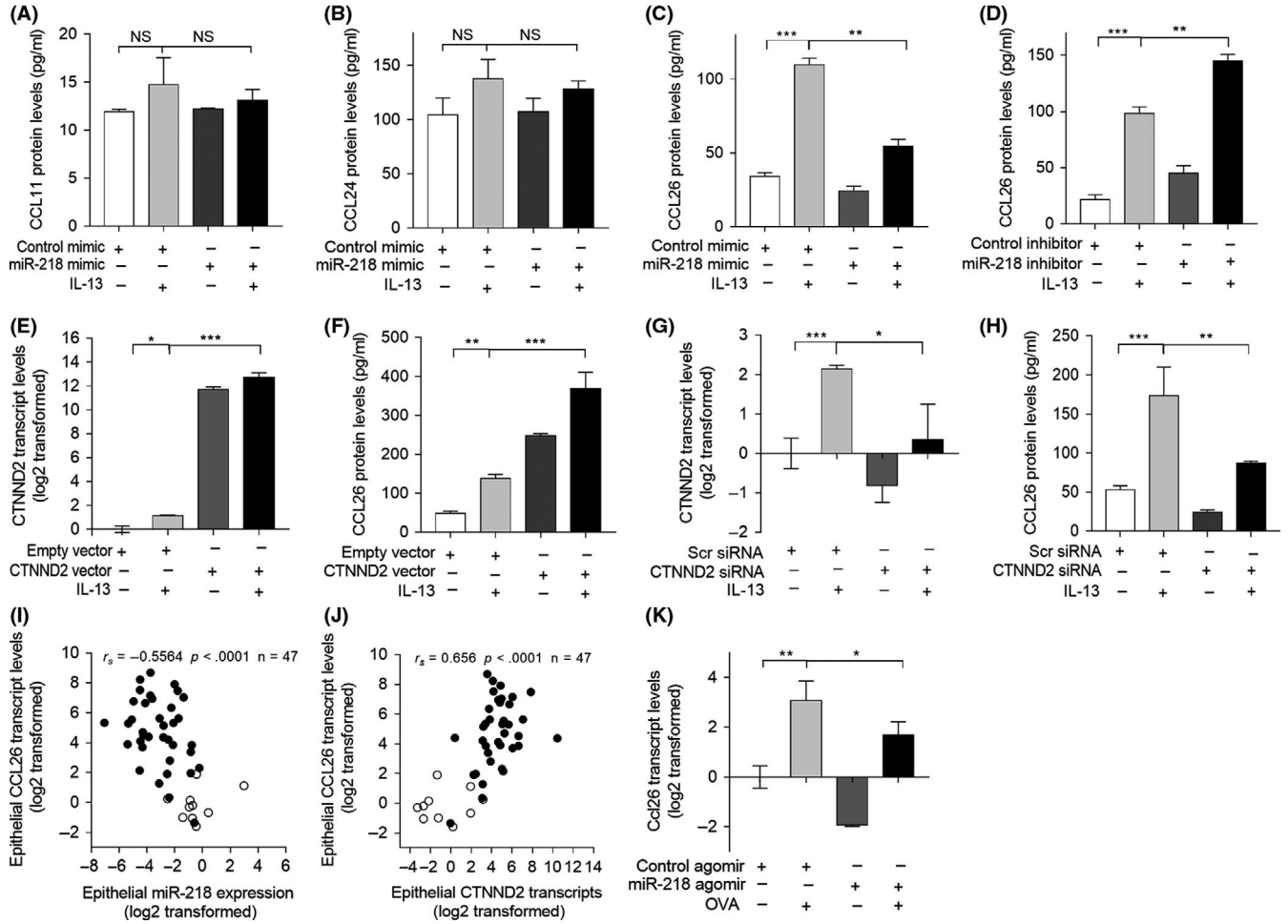


FIGURE 6 miR-218-5p-CTNND2 pathway regulates chemokine CCL26 expression. A-C, The protein levels of CCL11 (A), CCL24 (B) and CCL26 (C) in cell culture media after transfection with miR-218-5p mimic and stimulated with or without IL-13 stimulation were detected by ELISA. n = 4 wells per group. D, F, H, The protein levels of CCL26 in cell culture media after transfection with miR-218-5p inhibitor (D), empty or *CTNND2* cDNA expression vector (F), and control or *CTNND2* siRNA (H) with or without IL-13 stimulation were detected by ELISA. n = 4 wells per group. E, G, The transcript levels of *CTNND2* after transfection with empty or *CTNND2* cDNA expression vector (E), and control or *CTNND2* siRNA (G) with or without IL-13 stimulation were detected by quantitative PCR. n = 4 wells per group. I-J, Correlation assay between epithelial *CCL26* and *CTNND2* transcript levels (I), *CCL26* and miR-218-5p transcript levels (J) in controls and asthma patients. Asthma patients are presented as dots (n = 37), and healthy controls are presented as circles (n = 10). Correlation assays were performed using Spearman's rank-order correlation. K, *Ccl26* transcript levels in mouse lungs were detected by quantitative PCR. The transcript levels are expressed as log2 transformed and relative to the mean of control group. Data are mean ± SD. **P* < .05; ***P* < .01; ****P* < .001 (one-way ANOVA followed by Tukey's multiple comparison test). The data for cells and mice are representative of three independent experiments

expression of mmu-miR-218-5p was decreased and Ctnnd2 expression was increased in a murine model of allergic airway inflammation. Remarkably, airway overexpression of mmu-miR-218-5p suppressed allergen challenge-induced AHR and airway inflammation, specifically reduced the number of eosinophils in BALF. Our in vivo findings provide direct evidence for a protective role of miR-218-5p-CTNND2 pathway in eosinophilic airway inflammation in asthma. It was reported that *CTNNA3* (encoding αT-catenin) plays an important role in asthma airway inflammation because *CTNNA3* knockout mice manifested attenuated AHR and airway inflammation after allergen challenge.[30] Although *CTNNA3* expression was reported to be predominantly in cardiomyocytes surrounding pulmonary veins,[30,43] we demonstrated that *CTNNA3* transcripts were increased in asthma bronchial brushings and IL-13-stimulated HBE cells.

Finally, we demonstrated that miR-218-5p-CTNND2 pathway contributed to airway eosinophilia via regulating chemokine CCL26 expression. Provost *et al* reported that CCL26 was more effective to induce eosinophil migration of asthma patients than CCL11 and CCL24.[12] In our cohort of asthma, epithelial CCL26 expression correlated with miR-218-5p and CTNND2 expression. Perturbation of miR-218-5p or CTNND2 expression significantly altered IL-13-induced CCL26 expression in BEAS-2B cells, respectively. Moreover, mmu-miR-218-5p overexpression suppressed OVA challenge-induced Ccl26 expression as well as Ctnnd2 expression in mice lungs. However, the mechanism by which miR-218-5p-CTNND2 pathway contributes to airway eosinophilia and CCL26 expression requires further investigation. Since CTNND2 can regulate gene expression via nuclear translocation and interacting with transcriptional

factor,[38,39] CTNND2 may regulate the expression of chemokines and other inflammatory cytokines. The in vivo role of CTNND2 in eosinophilic airway inflammation requires further study using murine models of gain or loss of function.

There are several limitations of our study. First, a miRNA can post-transcriptionally regulate multiple genes' expression. Besides *CTNND2*, the alteration of other genes' expression may also contribute to the effect of miR-218-5p overexpression on airway inflammation in the murine model. Second, we did not examine the effect of miR-218-5p overexpression in a murine model where airway inflammation was already established.

Taken together, epithelial miR-218-5p plays a protective role in eosinophilic airway inflammation, at least in part, via targeting CTNND2, a novel catenin in asthma, and suppressing the chemokine CCL26 expression. miR-218-5p-CTNND2 pathway represents a promising therapeutic target of asthma eosinophilic airway inflammation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

GZ designed the research, conceived the manuscript and had primary responsibility for writing; YL, YF, WW, CC, DC and SC performed experiments; YL, YF and GZ analysed the data; YL, YF and GZ interpreted results of experiments; YL and GZ prepared figures; YL and GZ drafted the manuscript; GZ edited and revised the manuscript; and all authors approved final version of manuscript.

ORCID


Guohua Zhen https://orcid.org/0000-0001-5582-7900

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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