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

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Short title: MiR-218-5p-CTNND2 pathway in asthma airway eosinophilia

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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 downregulated 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 analyzed 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 upregulated catenin compared to 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* upregulation 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 & 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.

Key words: asthma, δ -catenin, epithelial cell, eosinophilia, microRNA

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 contributes 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 miRNAs expression in cultured airway epithelial cells from severe asthma, and identified miR-19 family member that has 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 downregulated 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-signaling pathway [25, 26]. There are 12 catenin family members subdivided to α -catenin (*CTNNA1*, *CTNNA2*, *CTNNA3*), β -catenin (*CTNNB1*, *CTNNG*), and p120-catenin subfamilies (*CTNND1*, *CTNND2*, *ARVCF*, *P0071*, *PKP1*, *PKP2*, *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 α T-catenin) was identified as a promising locus for asthma exacerbation through multiple genome-wide association studies [29], and genetic loss of α T-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*) signaling has an established role in asthma airway remodeling [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 upregulated 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.

METHODS

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 (PD₂₀) of forced expiratory volume in the first second (FEV₁) < 2.505 mg and/or $\geq 12\%$ increase in FEV₁ following inhalation of 200 μ g salbutamol. Healthy control subjects had no respiratory symptoms, normal spirometric value and methacholine PD₂₀ $\geq$

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, 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.

Murine model of allergic airway inflammation

Eight- to 10-week-old female C57BL/6 mice (CDC, Hubei, China) were sensitized with intraperitoneal injection of OVA solution (100µg in 100µl saline; Sigma-Aldrich, USA) mixed with Al(OH)₃ as an adjuvant on days 0, 7, and 14. Mice were challenged with intranasal administration of OVA solution (1mg in 50µl saline) on days 21, 22, 23, 24, and 25. Mmu-miR-218-5p agomir (5nmol in 40µl saline; RiboBio, Guangzhou, China) or control agomir were 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, Montreal, Canada). 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.

Cell culture and treatment

Human bronchial epithelial (HBE) cells (ScienCell, San Diego, USA) were cultured at air–liquid interface as previously described [32] and stimulated with or without IL-13 (20ng/ml; Peprotech, Rocky Hill, USA). BEAS-2B cells (ATCC, USA) were stimulated with or without IL-13 and transfected with control or miR-218-5p mimic (50µM; RiboBio, Guangzhou, China), control or miR-218-5p inhibitor (100µM), scrambled control or *CTNND2* siRNA (80µM; Genecopoeia, Guangzhou, China), or empty or *CTNND2* cDNA expression vector (500ng/ml) using Lipofectamine 3000 (Invitrogen, Carlsbad, USA). The sequence of the sense strand of *CTNND2* siRNA was

5'-GCAGUGAGAUCGAUAGCAATTUUGCUAUCGAUCUCACUGCTT-3'. Forty-eight hours after IL-13 stimulation, cells were harvested for quantitative PCR and Western blotting. The cell culture media was collected for ELISA.

Quantitative PCR

Total RNA from bronchial epithelial brushing, mouse lungs and cultured cells was isolated using TRIzol (Invitrogen, Carlsbad, USA) and reverse transcribed using the PrimeScript RT reagent kit (Takara, Tokyo, Japan). The expression of each gene were determined using an ABI Prism 7500 PCR System (Applied Biosystems, Massachusetts, USA). The primers used were 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.

In situ hybridization

MiRNA-218-5p was detected in paraffin-embedded human bronchial biopsies or mouse lung tissue using biotin-labeled miR-218-5p miRNA probe (Qiagen, Shanghai, China). 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.

Histology and immunohistochemistry

Human bronchial biopsy and mouse lung sections were stained with hematoxylin and eosin (H&E). Observers who were blinded to the clinical status of the subjects counted numbers of eosinophils/mm² submucosa as previously described [34]. The methods for immunohistochemistry, assessment of airway inflammation, and PAS staining were described in Supporting Information.

Luciferase activity assay

Vector harboring wild-type, mutant *CTNND2* 3'-UTR or empty vector were 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, Madison, USA). Normalized relative light units represent firefly luciferase activity / renilla luciferase activity.

Western blotting

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

ELISA

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

Statistical Analysis

We analyzed data using Prism version 7 (GraphPad Software, San Diego, CA, USA). For normally distributed data, we calculated means $\pm$ 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 analyzed correlation using Spearman's rank order correlation. $P < 0.05$ was considered statistically significant.

RESULTS

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 were 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 to 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 to controls (Figure 1B).

Eosinophilic airway inflammation is a key feature of type 2-high asthma. We analyzed 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 was no correlations between epithelial miR-218-5p and sputum neutrophils in asthma patients (Figure S1). Our data indicate that epithelial miR-218-5p expression is downregulated 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).

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. MiRNAs regulates gene expression via acting on the 3'-UTR of the mRNA. When miR-218-5p mimic were co-transfected with luciferase reporter vector harboring wild type 3'-UTR of *CTNND2* in BEAS-2B cells, the luciferase activity was significantly reduced compared to the cells co-transfected with miR-218-5p mimic and the vector harboring mutant *CTNND2* 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 to 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).

CTNND2 is the most significantly upregulated 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 to 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 upregulated epithelial catenin in asthma patients (Figure 3E). The transcripts of *CTNNA3*, a catenin with established role in asthma airway inflammation [30], was 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 upregulated catenin after IL-13 stimulation in both cell cultures (Figure 3F; Figure S2B). *CTNNA3* transcripts was 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 upregulation in asthma.

Epithelial CTNND2 expression correlates with airway eosinophilia.

Given epithelial miR-218-5p expression correlated with airway eosinophilia in asthma, we analyzed 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.

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 of 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 of 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 downregulation, and suppressed *Ctnnd2* upregulation 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.

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 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* 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.

DISCUSSION

In the present study, we demonstrated that epithelial miR-218-5p expression was significantly downregulated 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 upregulated 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 chemokine CCL26 expression in epithelial cell culture.

In our earlier epithelial miRNA profiling data, miR-218-5p was the top 2 downregulated epithelial miRNAs in asthma [22]. Here, we confirmed that miR-218-5p expression was significantly decreased in bronchial epithelium in treatment-naïve asthma patients compared to controls. Consistently, miR-218-5p expression was decreased in both IL-13-stimulated human bronchial epithelial cells and OVA-challenged mice airways. It was reported that miR-218-5p was downregulated 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 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 gene. In agreement with this, 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 upregulated 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 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 α T-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 play 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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Disclosures

The authors declare no conflict of interest.

Author contributions

G.Z. designed the research, conceived of the manuscript and had primary responsibility for writing; Y.L., Y.F., W.W., C.C., D.C., and S.C. performed experiments; Y.L., Y.F., and G.Z. analyzed data; Y.L., Y.F., and G.Z. interpreted results of experiments; Y.L. and G.Z. prepared figures; Y.L. and G.Z. drafted manuscript; G.Z. edited and revised manuscript; all authors approved final version of manuscript.

References

1. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, Koth LL, Arron JR, Fahy JV, T-helper type 2-driven inflammation defines major subphenotypes of asthma. American journal of

respiratory and critical care medicine 2009;180: 388-95.

2. Ray A, Oriss TB, Wenzel SE, Emerging molecular phenotypes of asthma. American journal of physiology Lung cellular and molecular physiology 2015;308: L130-40.
3. Wenzel SE, Asthma phenotypes: the evolution from clinical to molecular approaches. Nature medicine 2012;18: 716-25.
4. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, Gilliet M, Ho S, Antonenko S, Lauerma A, Smith K, Gorman D, Zurawski S, Abrams J, Menon S, McClanahan T, de Waal-Malefyt Rd R, Bazan F, Kastelein RA, Liu YJ, Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat Immunol 2002;3: 673-80.
5. Lai Y, Altemeier WA, Vandree J, Piliponsky AM, Johnson B, Appel CL, Frevert CW, Hyde DM, Ziegler SF, Smith DE, Henderson WR, Jr., Gelb MH, Hallstrand TS, Increased density of intraepithelial mast cells in patients with exercise-induced bronchoconstriction regulated through epithelially derived thymic stromal lymphopoietin and IL-33. The Journal of allergy and clinical immunology 2014;133: 1448-55.
6. Suzukawa M, Morita H, Nambu A, Arae K, Shimura E, Shibui A, Yamaguchi S, Suzukawa K, Nakanishi W, Oboki K, Kajiwara N, Ohno T, Ishii A, Korner H, Cua DJ, Suto H, Yoshimoto T, Iwakura Y, Yamasoba T, Ohta K, Sudo K, Saito H, Okumura K, Broide DH, Matsumoto K, Nakae S, Epithelial cell-derived IL-25, but not Th17 cell-derived IL-17 or IL-17F, is crucial for murine asthma. J Immunol 2012;189: 3641-52.
7. Ballantyne SJ, Barlow JL, Jolin HE, Nath P, Williams AS, Chung KF, Sturton G, Wong SH, McKenzie AN, Blocking IL-25 prevents airway hyperresponsiveness in allergic asthma. The Journal

of allergy and clinical immunology 2007;120: 1324-31.

8. Gregory LG, Jones CP, Walker SA, Sawant D, Gowers KH, Campbell GA, McKenzie AN, Lloyd CM, IL-25 drives remodelling in allergic airways disease induced by house dust mite. *Thorax* 2013;68: 82-90.
9. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, Gorman DM, Bazan JF, Kastelein RA, IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005;23: 479-90.
10. Forssmann U, Ugucioni M, Loetscher P, Dahinden CA, Langen H, Thelen M, Baggiolini M, Eotaxin-2, a novel CC chemokine that is selective for the chemokine receptor CCR3, and acts like eotaxin on human eosinophil and basophil leukocytes. *The Journal of experimental medicine* 1997;185: 2171-6.
11. Coleman JM, Naik C, Holguin F, Ray A, Ray P, Trudeau JB, Wenzel SE, Epithelial eotaxin-2 and eotaxin-3 expression: relation to asthma severity, luminal eosinophilia and age at onset. *Thorax* 2012;67: 1061-6.
12. Provost V, Larose MC, Langlois A, Rola-Pleszczynski M, Flamand N, Laviolette M, CCL26/eotaxin-3 is more effective to induce the migration of eosinophils of asthmatics than CCL11/eotaxin-1 and CCL24/eotaxin-2. *Journal of leukocyte biology* 2013;94: 213-22.
13. Kitaura M, Nakajima T, Imai T, Harada S, Combadiere C, Tiffany HL, Murphy PM, Yoshie O, Molecular cloning of human eotaxin, an eosinophil-selective CC chemokine, and identification of a specific eosinophil eotaxin receptor, CC chemokine receptor 3. *The Journal of biological chemistry*

1996;271: 7725-30.

14. Bartel DP, MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116: 281-97.
15. Solberg OD, Ostrin EJ, Love MI, Peng JC, Bhakta NR, Hou L, Nguyen C, Solon M, Barczak AJ, Zlock LT, Blagev DP, Finkbeiner WE, Ansel KM, Arron JR, Erle DJ, Woodruff PG, Airway epithelial miRNA expression is altered in asthma. *American journal of respiratory and critical care medicine* 2012;186: 965-74.
16. Polikepahad S, Knight JM, Naghavi AO, Oplt T, Creighton CJ, Shaw C, Benham AL, Kim J, Soibam B, Harris RA, Coarfa C, Zariff A, Milosavljevic A, Batts LM, Kheradmand F, Gunaratne PH, Corry DB, Proinflammatory role for let-7 microRNAs in experimental asthma. *The Journal of biological chemistry* 2010;285: 30139-49.
17. Marcet B, Chevalier B, Luxardi G, Coraux C, Zaragosi LE, Cibois M, Robbe-Sermesant K, Jolly T, Cardinaud B, Moreilhon C, Giovannini-Chami L, Nawrocki-Raby B, Birembaut P, Waldmann R, Kodjabachian L, Barbry P, Control of vertebrate multiciliogenesis by miR-449 through direct repression of the Delta/Notch pathway. *Nat Cell Biol* 2011;13: 693-9.
18. Martinez-Nunez RT, Rupani H, Plate M, Niranjana M, Chambers RC, Howarth PH, Sanchez-Elsner T, Genome-Wide Posttranscriptional Dysregulation by MicroRNAs in Human Asthma as Revealed by Frac-seq. *J Immunol* 2018;201: 251-63.
19. Haj-Salem I, Fakhfakh R, Berube JC, Jacques E, Plante S, Simard MJ, Bosse Y, Chakir J, MicroRNA-19a enhances proliferation of bronchial epithelial cells by targeting TGFbetaR2 gene in severe asthma. *Allergy* 2015;70: 212-9.
20. Simpson LJ, Patel S, Bhakta NR, Choy DF, Brightbill HD, Ren X, Wang Y, Pua HH, Baumjohann D,

Montoya MM, Panduro M, Remedios KA, Huang X, Fahy JV, Arron JR, Woodruff PG, Ansel KM, A microRNA upregulated in asthma airway T cells promotes TH2 cytokine production. *Nat Immunol* 2014;15: 1162-70.

21. Zhang K, Liang Y, Feng Y, Wu W, Zhang H, He J, Hu Q, Zhao J, Xu Y, Liu Z, Zhen G, Decreased epithelial and sputum miR-221-3p associates with airway eosinophilic inflammation and CXCL17 expression in asthma. *American journal of physiology Lung cellular and molecular physiology* 2018;315: L253-L64.

22. Huo X, Zhang K, Yi L, Mo Y, Liang Y, Zhao J, Zhang Z, Xu Y, Zhen G, Decreased epithelial and plasma miR-181b-5p expression associates with airway eosinophilic inflammation in asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2016;46: 1281-90.

23. Conickx G, Mestdagh P, Avila Cobos F, Verhamme FM, Maes T, Vanaudenaerde BM, Seys LJ, Lahousse L, Kim RY, Hsu AC, Wark PA, Hansbro PM, Joos GF, Vandesompele J, Bracke KR, Brusselle GG, MicroRNA Profiling Reveals a Role for MicroRNA-218-5p in the Pathogenesis of Chronic Obstructive Pulmonary Disease. *American journal of respiratory and critical care medicine* 2017;195: 43-56.

24. Schembri F, Sridhar S, Perdomo C, Gustafson AM, Zhang X, Ergun A, Lu J, Liu G, Bowers J, Vaziri C, Ott K, Sensinger K, Collins JJ, Brody JS, Getts R, Lenburg ME, Spira A, MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium. *Proceedings of the National Academy of Sciences of the United States of America* 2009;106: 2319-24.

25. Yap AS, Crampton MS, Hardin J, Making and breaking contacts: the cellular biology of cadherin regulation. *Curr Opin Cell Biol* 2007;19: 508-14.
26. Hatzfeld M, Plakophilins: Multifunctional proteins or just regulators of desmosomal adhesion? *Biochim Biophys Acta* 2007;1773: 69-77.
27. McCrea PD, Gu D, The catenin family at a glance. *J Cell Sci* 2010;123: 637-42.
28. de Boer WI, Sharma HS, Baelemans SM, Hoogsteden HC, Lambrecht BN, Braunstahl GJ, Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation. *Can J Physiol Pharmacol* 2008;86: 105-12.
29. McGeachie MJ, Wu AC, Tse SM, Clemmer GL, Sordillo J, Himes BE, Lasky-Su J, Chase RP, Martinez FD, Weeke P, Shaffer CM, Xu H, Denny JC, Roden DM, Panettieri RA, Jr., Raby BA, Weiss ST, Tantisira KG, CTNNA3 and SEMA3D: Promising loci for asthma exacerbation identified through multiple genome-wide association studies. *The Journal of allergy and clinical immunology* 2015;136: 1503-10.
30. Folmsbee SS, Budinger GR, Bryce PJ, Gottardi CJ, The cardiomyocyte protein alphaT-catenin contributes to asthma through regulating pulmonary vein inflammation. *The Journal of allergy and clinical immunology* 2016;138: 123-29 e2.
31. Kumawat K, Koopmans T, Gosens R, beta-catenin as a regulator and therapeutic target for asthmatic airway remodeling. *Expert Opin Ther Targets* 2014;18: 1023-34.
32. Zhen G, Park SW, Nguyenvu LT, Rodriguez MW, Barbeau R, Paquet AC, Erle DJ, IL-13 and epidermal growth factor receptor have critical but distinct roles in epithelial cell mucin production. *Am J Respir Cell Mol Biol* 2007;36: 244-53.

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33. Livak KJ, Schmittgen TD, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25: 402-8.
 34. Cheng D, Xue Z, Yi L, Shi H, Zhang K, Huo X, Bonser LR, Zhao J, Xu Y, Erle DJ, Zhen G, Epithelial interleukin-25 is a key mediator in Th2-high, corticosteroid-responsive asthma. *American journal of respiratory and critical care medicine* 2014;190: 639-48.
 35. Peters MC, Mekonnen ZK, Yuan S, Bhakta NR, Woodruff PG, Fahy JV, Measures of gene expression in sputum cells can identify TH2-high and TH2-low subtypes of asthma. *The Journal of allergy and clinical immunology* 2014;133: 388-94.
 36. Kim SW, Park JI, Spring CM, Sater AK, Ji H, Otchere AA, Daniel JM, McCrea PD, Non-canonical Wnt signals are modulated by the Kaiso transcriptional repressor and p120-catenin. *Nat Cell Biol* 2004;6: 1212-20.
 37. Carnahan RH, Rokas A, Gaucher EA, Reynolds AB, The molecular evolution of the p120-catenin subfamily and its functional associations. *PLoS One* 2010;5: e15747.
 38. Rodova M, Kelly KF, VanSaun M, Daniel JM, Werle MJ, Regulation of the rapsyn promoter by kaiso and delta-catenin. *Mol Cell Biol* 2004;24: 7188-96.
 39. Koutras C, Lessard CB, Levesque G, A nuclear function for the presenilin 1 neuronal partner NPRAP/delta-catenin. *J Alzheimers Dis* 2011;27: 307-16.
 40. Israely I, Costa RM, Xie CW, Silva AJ, Kosik KS, Liu X, Deletion of the neuron-specific protein delta-catenin leads to severe cognitive and synaptic dysfunction. *Curr Biol* 2004;14: 1657-63.
 41. Turner TN, Sharma K, Oh EC, Liu YP, Collins RL, Sosa MX, Auer DR, Brand H, Sanders SJ, Moreno-De-Luca D, Pihur V, Plona T, Pike K, Soppet DR, Smith MW, Cheung SW, Martin CL,

State MW, Talkowski ME, Cook E, Hagan R, Katsanis N, Chakravarti A, Loss of delta-catenin function in severe autism. *Nature* 2015;520: 51-6.

42. Amin ND, Bai G, Klug JR, Bonanomi D, Pankratz MT, Gifford WD, Hinckley CA, Sternfeld MJ, Driscoll SP, Dominguez B, Lee KF, Jin X, Pfaff SL, Loss of motoneuron-specific microRNA-218 causes systemic neuromuscular failure. *Science* 2015;350: 1525-9.

43. Folmsbee SS, Morales-Nebreda L, Van Hengel J, Tyberghein K, Van Roy F, Budinger GR, Bryce PJ, Gottardi CJ, The cardiac protein alphaT-catenin contributes to chemical-induced asthma. *American journal of physiology Lung cellular and molecular physiology* 2015;308: L253-8.

Table 1. Subject characteristics

	Healthy controls	Asthma patients	<i>P</i> value
Number	15	50	
Age, yr	39.93 ± 11.01	40.00 ± 11.99	0.985
Sex, M:F (%F)	4:11 (73.3)	12:38 (76.0)	0.833
Body mass index	22.07 ± 2.82	22.52 ± 3.11	0.535
FEV ₁ , % predicted	95.66 ± 12.18	76.98 ± 19.71	0.0002
Methacholine PD ₂₀ , mg	2.505 ± 0	0.13 ± 0.24	<0.0001
Sputum eosinophil, %	0.72 ± 0.58	12.60 ± 12.71	<0.0001
Biopsy eosinophil, #/mm ²	1.07 ± 0.63	15.39 ± 15.58	<0.0001
FeNO, ppb	15.58 ± 13.68	82.08 ± 58.11	<0.0001

Values were presented as mean ± SD. FeNO, fraction of exhaled nitric oxide; FEV₁, forced expiratory volume in the first second; PD₂₀, provocative dosage required to cause a 20% decline in FEV₁. The minimal and maximal provocative dosages were 0.01 and 2.505 mg, respectively.

Figure Legends:

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 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 were 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 were 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 analyzed using ImageJ and the protein levels of *CTNND2* was indexed to GAPDH. Data are mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (one-way ANOVA followed by Tukey's multiple comparison test). The data are representative of three independent experiments.

Figure 3. Epithelial *CTNND2* is the most significantly upregulated 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 μ m. (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 μ m. (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*

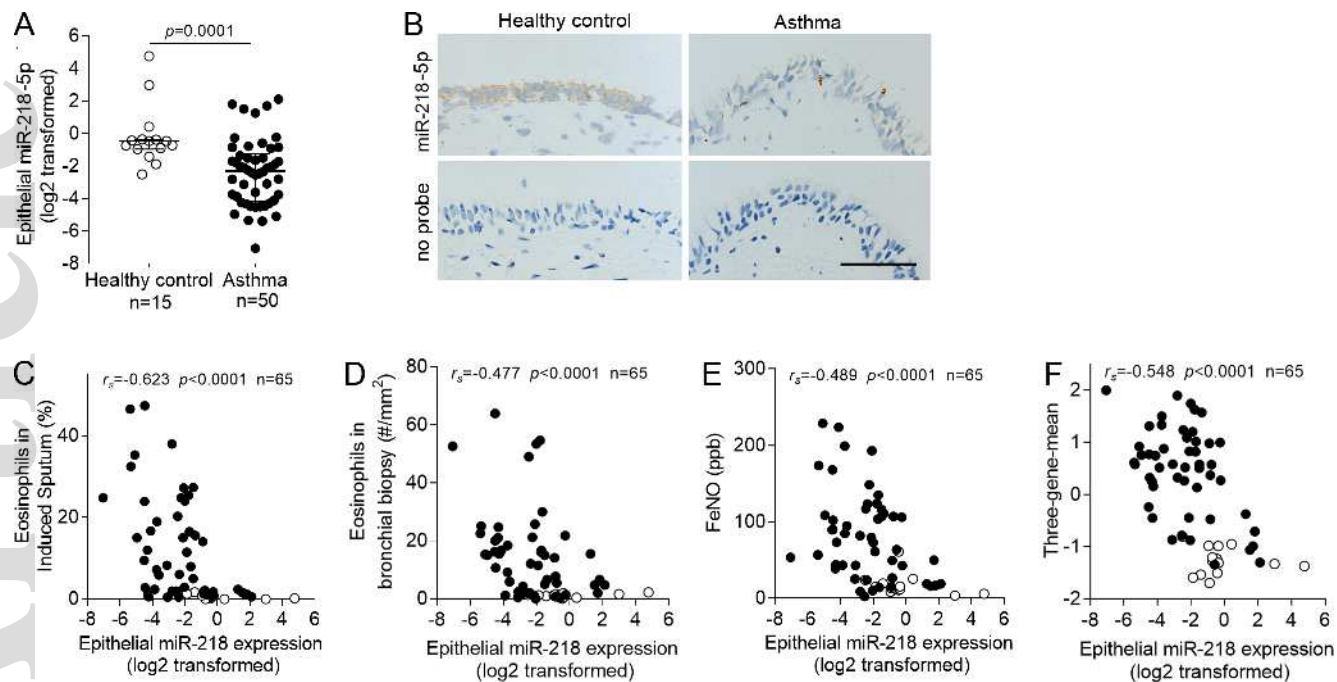
transcript levels and eosinophils in induced sputum (G) and bronchial biopsies (H), FeNO (I), three-gene-mean of *CLCA1*, *POSTN* and *SERPINB2* (J). Asthma patients were presented as dots and healthy controls as circles. Correlation assays were performed using Spearman's rank order correlation.

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 μ m. (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 $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (one-way ANOVA followed by Tukey's multiple comparison test). The data are representative of three independent experiments.

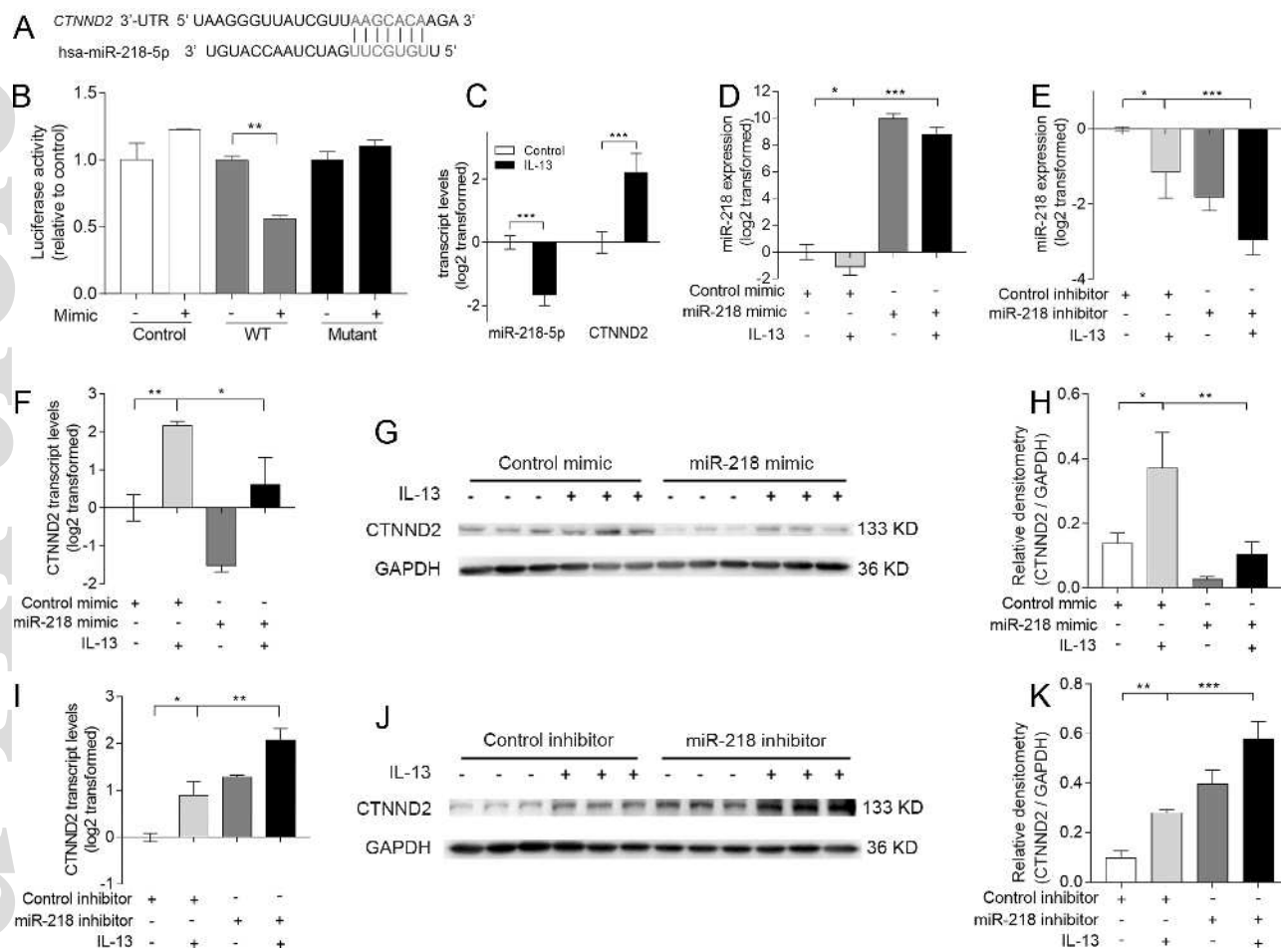
Figure 5. Mmu-miR-218-5p overexpression suppresses Ctnnd2 upregulation in the mouse model. (A) Representative images of in situ hybridization of miR-218-5p in mouse lung sections. Scale bar, 50 μ m. (B) MiR-218-5p transcript levels in mouse lungs were measured by quantitative PCR. (C) Representative images of Ctnnd2 immunofluorescence staining in mouse lung sections. Scale bar, 50 μ m. (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 $\pm$ SD. * $P < 0.05$; *** $P < 0.001$ (one-way ANOVA followed by Tukey's multiple comparison test). The data are representative of three independent experiments.

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

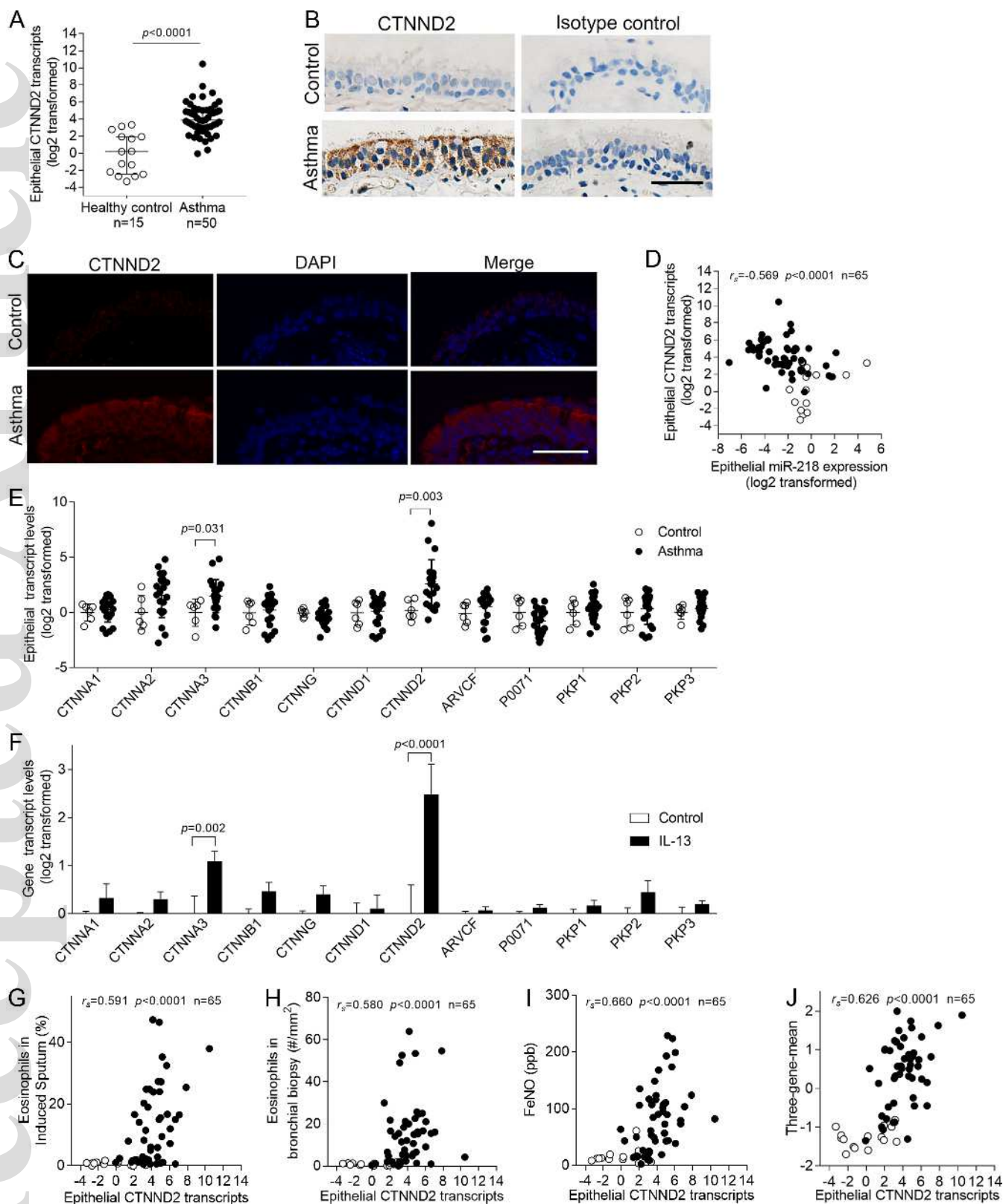
transfection with miR-218-5p inhibitor (*D*), empty or *CTNND2* cDNA expression vector (*F*), and control or *CTNND2* siRNA (*H*) with or without IL-13 stimulation were detected by ELISA. $n = 4$ wells per group. (E, G) The transcript levels of *CTNND2* after transfection with empty or *CTNND2* cDNA 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 $\pm$ SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (one-way ANOVA followed by Tukey's multiple comparison test). The data for cells and mice are representative of three independent experiments.



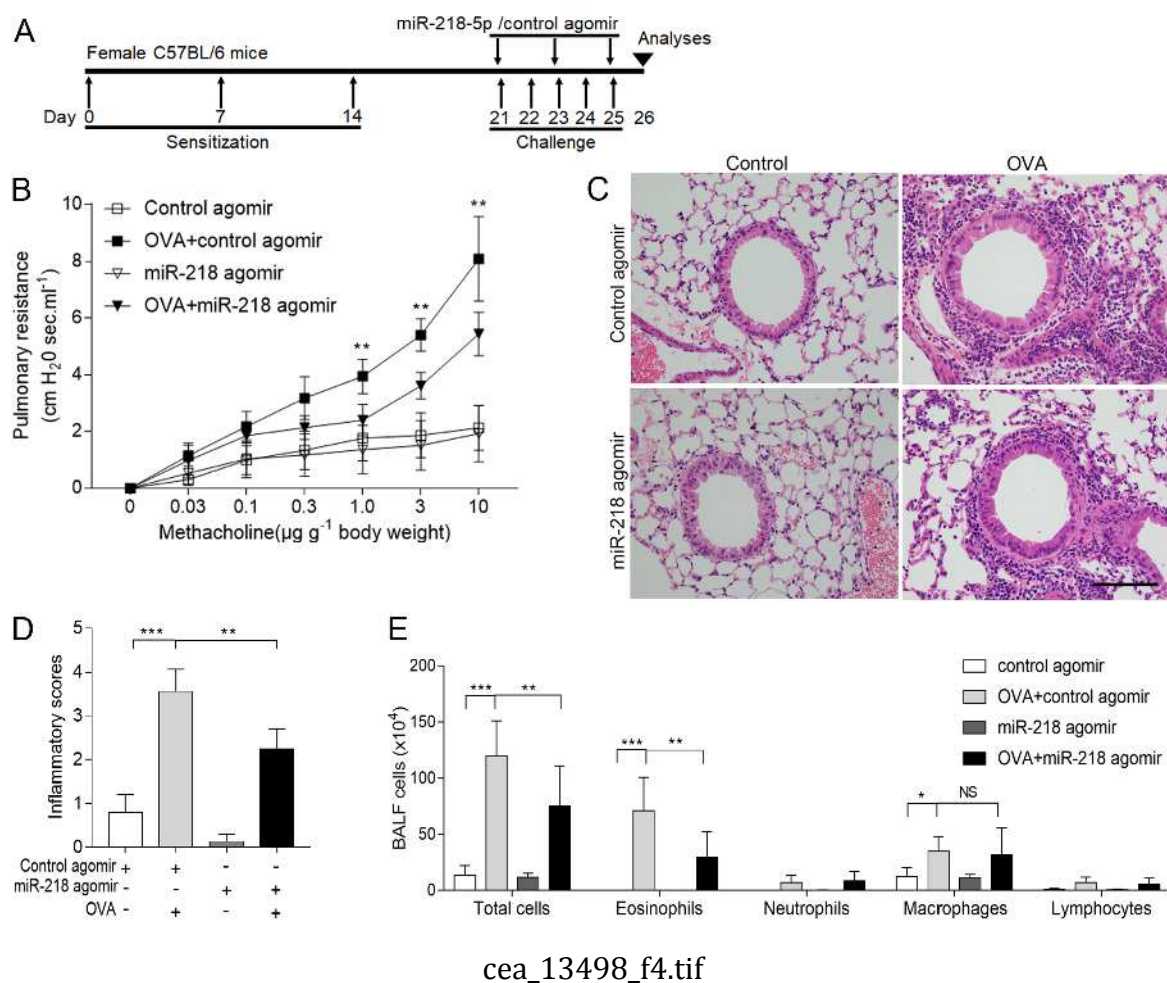
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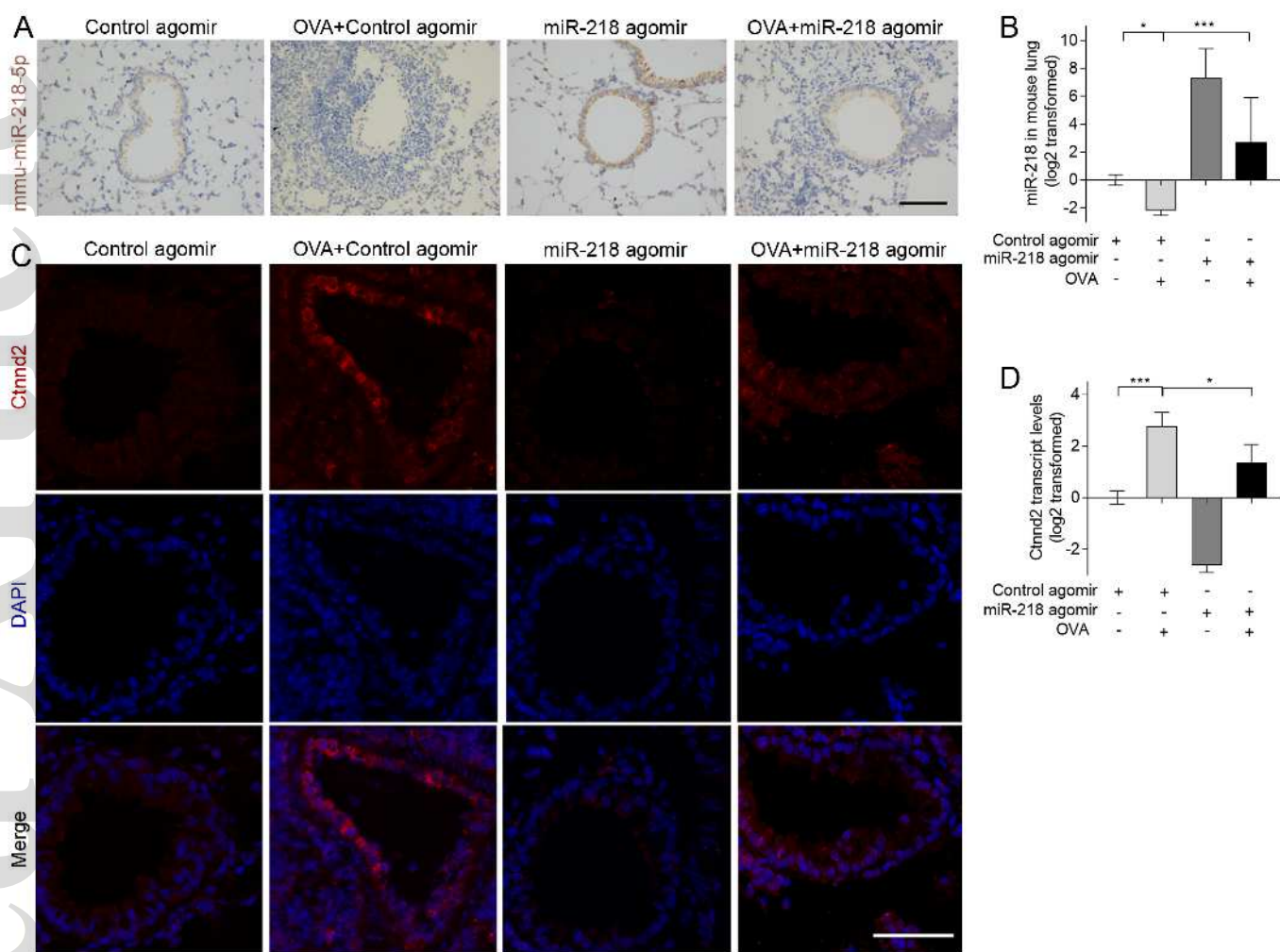
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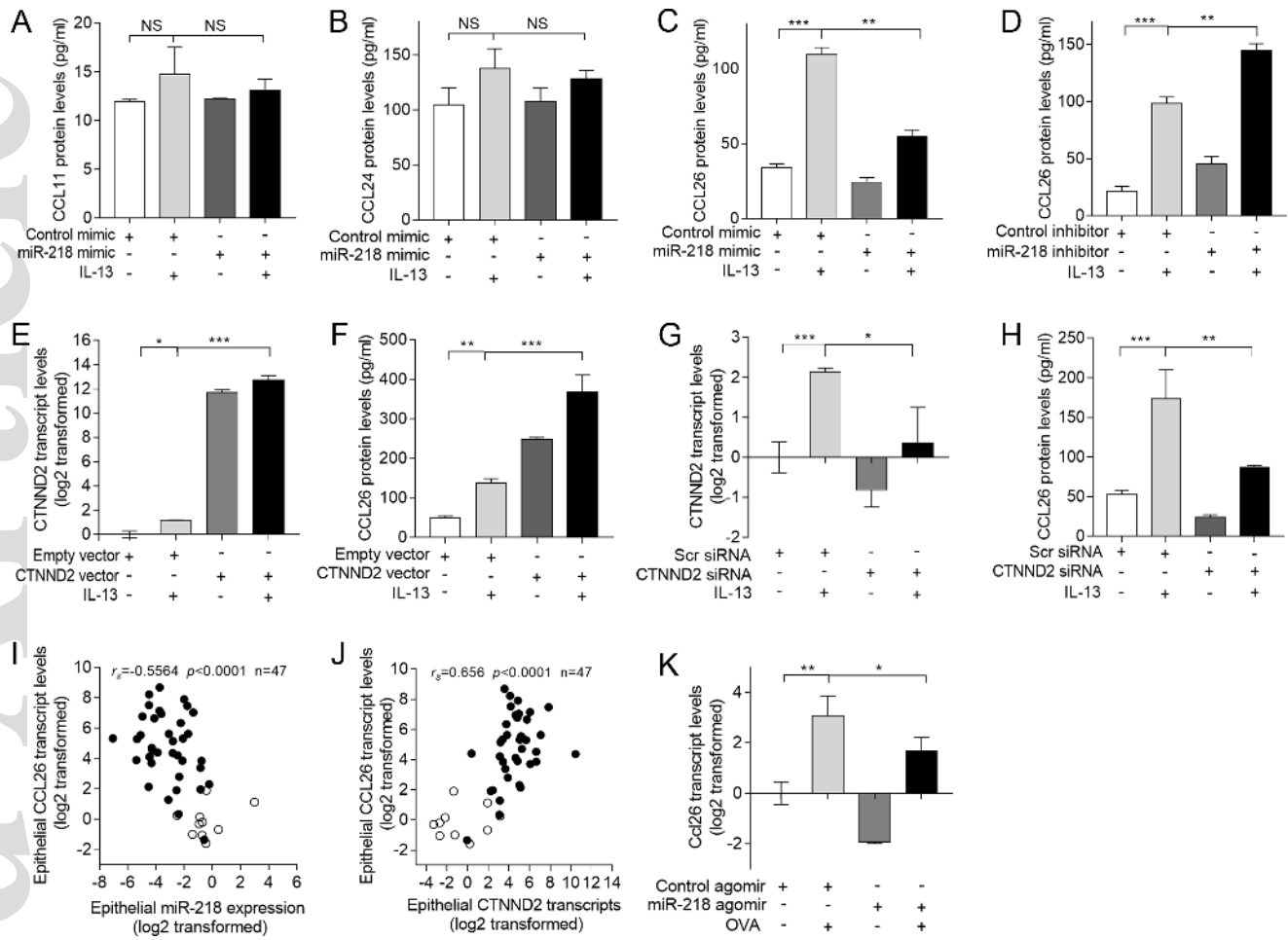
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SUPPORTING INFORMATION:

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

Yuxia Liang, Yuchen Feng, Wenliang Wu, Chenli Chang, Dian Chen, Shengchong
Chen, and Guohua Zhen

METHODS

Immunohistochemistry

Bronchial biopsy sections underwent antigen retrieval and were immunostained with mouse monoclonal δ -catenin antibody (Santa Cruz Biotechnology, Dallas, USA) at 1:100 dilution. Antibody was detected using the DAB kit (Boster, Wuhan, China) according to the manufacturer's instructions. For immunofluorescence staining, tissue sections were incubated with mouse monoclonal δ -catenin antibody (Santa Cruz Biotechnology, Dallas, USA) and then incubated with Cy3 goat anti-mouse secondary antibody (Boster, Wuhan, China). Nuclei were stained with DAPI. Photographs were taken by using a fluorescence microscope (NIKON Eclipse ci, Japan).

Assessment of airway inflammation

Cell counts for macrophages, eosinophils, lymphocytes, and neutrophils in bronchoalveolar lavage fluid (BALF) were performed. The severity of peribronchial inflammation in H&E-stained mouse lung sections was scored using the following features: 0, normal; 1, few cells; 2, a ring of inflammatory cells (1 cell layer deep); 3, a ring of inflammatory cells (2–4 cells deep); 4, a ring of inflammatory cells (> 4 cells deep).

Periodic acid-Schiff (PAS) staining

Lung sections were stained with PAS (Goodbio technology, Beijing, China) for detection of mucus. The number of PAS-staining-positive cells was counted in five random fields for each lung section at $\times 200$ magnification.

SUPPLEMENTARY TABLES

Table S1. Primers for quantitative PCR

Gene	Species		Sequence
<i>β-actin</i>	Human	Forward	GTCATTCCAAATATGAGATGCGT
		Reverse	GCTATCACCTCCCCTGTGTG
<i>ARVCF</i>	Human	Forward	CTATTGTCACATCCGAAGATGGC
		Reverse	CGTACTGTCCGAGTGGTCAC
<i>CCL26</i>	Human	Forward	TTGAGGCTGAGCCAAAGACC
		Reverse	GCCCTTCTCAGGTTTCTCCC
<i>CLCA1</i>	Human	Forward	ATGGCTATGAAGGCATTGTCTG
		Reverse	TGGCACATTGGGGTTCGATTG
<i>CTNNA1</i>	Human	Forward	TCCGATCCTCTATACTGCATCC
		Reverse	TGCTGTGAGGCATCGTCTG
<i>CTNNA2</i>	Human	Forward	GGACGCTAACAGTGGAAAGG
		Reverse	GAGTGGCTTGCTCTACAGAGG
<i>CTNNA3</i>	Human	Forward	AAGCTACTGGAGCCTCTCATAA
		Reverse	CTTTGCGAACTTCCTCAAGTGA
<i>CTNNB1</i>	Human	Forward	AGCTTCCAGACACGCTATCAT
		Reverse	CGGTACAACGAGCTGTTTCTAC
<i>CTNNG</i>	Human	Forward	ATCAATGAGCCCTATGGA
		Reverse	GGGCACATCGCTGGAGTACA
<i>CTNND1</i>	Human	Forward	ATGAAGATGGTTATCCAGGTGGC
		Reverse	CTGGGCCTATACCGCTCCT
<i>CTNND2</i>	Human	Forward	GAGCCCCGGCTTAAACACC
		Reverse	CCTGTTCTTTGACTGAGGCGA
<i>p0071</i>	Human	Forward	AGCTTCAGTTTCAGCGACTCA
		Reverse	TCTGCTCCAAGCCTACATCTT
<i>PKP1</i>	Human	Forward	TTTGCCGTCGGACCAAAAGAT
		Reverse	GAACCTCGATTGGAGTGGCTC
<i>PKP2</i>	Human	Forward	GTGGGCAACGGAAATCTTCAC
		Reverse	CCAGCCTTTAGCATGTCATAGG
<i>PKP3</i>	Human	Forward	ACGTGCATGGGTTCAACAG
		Reverse	CTGCTGAGGGCAGGTCAAT
<i>GAPDH</i>	Human	Forward	AAGGTGAAGGTCGGAGTCAAC
		Reverse	GGGGTCATTGATGGCAACAATA
<i>POSTN</i>	Human	Forward	GACCGTGTGCTTACACAAATTG
		Reverse	AAGTGACCGTCTCTTCCAAGG
<i>SERPINB2</i>	Human	Forward	TCCTGGGTCAAGACTCAAACC
		Reverse	CATCCTGGTATCCCCATCTACA
<i>β-actin</i>	Mouse	Forward	GTGACGTTGACATCCGTAAAGA
		Reverse	GCCGGACTCATCGTACTCC
<i>Ccl26</i>	Mouse	Forward	ATCCCATGGAGCTGGGTGTA

<i>Ctnnd2</i>	Mouse	Reverse	CCTGGCTGGACACAGAATTG
		Forward	GGACCCACAGGATTACTCCAC
		Reverse	CTGGCCGGATACTGGAAAGA
<i>Muc5ac</i>	Mouse	Forward	CAGGACTCTCTGAAATCGTACCA
		Reverse	GAAGGCTCGTACCACAGGG
<i>Gapdh</i>	Mouse	Forward	AGAGAGGCCCGCTACTCG
		Reverse	GGCACTGCACAAGAAGATGC

Table S2. Correlations between epithelial miR-218-5p transcripts and eosinophilic airway inflammation, and the three-gene-mean

Epithelial miR-218-5p	Asthma patients (n=50)	All subjects (n=65)
Sputum eosinophils	$r_s=-0.453, P=0.001$	$r_s=-0.623, P<0.0001$
Biopsy eosinophils	$r_s=-0.355, P=0.011$	$r_s=-0.477, P<0.0001$
FeNO	$r_s=-0.312, P=0.028$	$r_s=-0.489, P<0.0001$
Three-gene-mean	$r_s=-0.311, P=0.028$	$r_s=-0.548, P<0.0001$

Table S3. Predicted targets of miR-218-5p by online algorithms miRanda, miRWalk, PicTar and TargetScan

Gene symbol	Gene ID	Gene name
<i>ACTN1</i>	87	actinin alpha 1
<i>CDH2</i>	1000	cadherin 2
<i>CTNND2</i>	1501	catenin delta 2
<i>COL1A1</i>	1277	collagen type I alpha 1 chain
<i>EDEM1</i>	9695	ER degradation enhancing alpha-mannosidase like protein1
<i>EPHA8</i>	2046	EPH receptor A8
<i>FBN2</i>	2201	fibrillin 2
<i>MBNL1</i>	4154	muscleblind like splicing regulator 1
<i>NRXN1</i>	9378	neurexin 1
<i>NUMBL</i>	9253	NUMB like, endocytic adaptor protein
<i>PCDH8</i>	5100	protocadherin 8
<i>PCDHA1</i>	56147	protocadherin alpha 1
<i>PCDHA9</i>	9752	protocadherin alpha 9
<i>PCDHAC1</i>	56135	protocadherin alpha subfamily C, 1
<i>PCDHAC2</i>	56134	protocadherin alpha subfamily C, 2
<i>PLCG1</i>	5335	phospholipase C gamma 1
<i>PRKCE</i>	5581	protein kinase C epsilon
<i>PTPRM</i>	5797	protein tyrosine phosphatase, receptor type M
<i>RELN</i>	5649	reelin
<i>RET</i>	5979	ret proto-oncogene
<i>SEMA6A</i>	57556	semaphorin 6A
<i>SLC24A4</i>	123041	solute carrier family 24 member 4
<i>SNCB</i>	6620	synuclein beta
<i>SORBS1</i>	10580	sorbin and SH3 domain containing 1

Table S4. Correlations between epithelial *CTNND2* transcripts and miR-218-5p, and eosinophilic airway inflammation, and the three-gene-mean

Epithelial <i>CTNND2</i>	Asthma patients (n=50)	All subjects (n=65)
Epithelial miR-218-5p	$r_s=-0.448, P=0.001$	$r_s=-0.569, P<0.0001$
Sputum eosinophils	$r_s=0.420, P=0.002$	$r_s=0.591, P<0.0001$
Biopsy eosinophils	$r_s=0.322, P=0.023$	$r_s=0.580, P<0.0001$
FeNO	$r_s=0.498, P=0.0002$	$r_s=0.660, P<0.0001$
Three-gene-mean	$r_s=0.338, P=0.017$	$r_s=0.626, P<0.0001$

Table S5. Correlations between epithelial *CCL26* transcripts and miR-218-5p, and *CTNND2* transcripts

Epithelial <i>CCL26</i>	Asthma patients (n=37)	All subjects (n=47)
Epithelial miR-218-5p	$r_s=-0.330, P=0.046$	$r_s=-0.556, P<0.0001$
Epithelial <i>CTNND2</i>	$r_s=0.379, P=0.021$	$r_s=0.656, P<0.0001$

SUPPLEMENTARY FIGURES

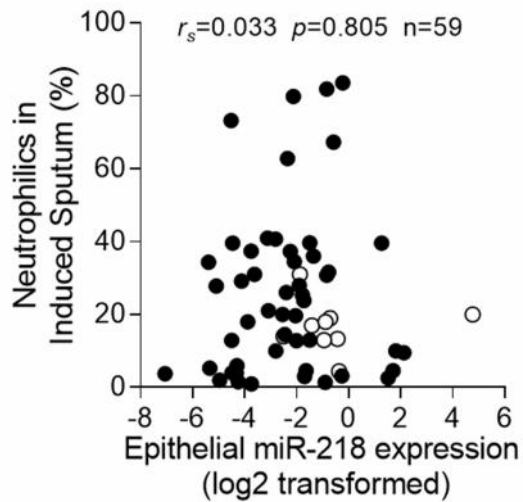


Figure S1. Epithelial miR-218-5p is not correlated with sputum neutrophil in asthma. Correlation assays between epithelial miR-218-5p transcript levels and the percentage of neutrophil in induced sputum. Asthma patients were presented as dots ($n = 50$), and healthy controls as circles ($n = 9$). Correlation assays were performed using Spearman's rank order correlation.

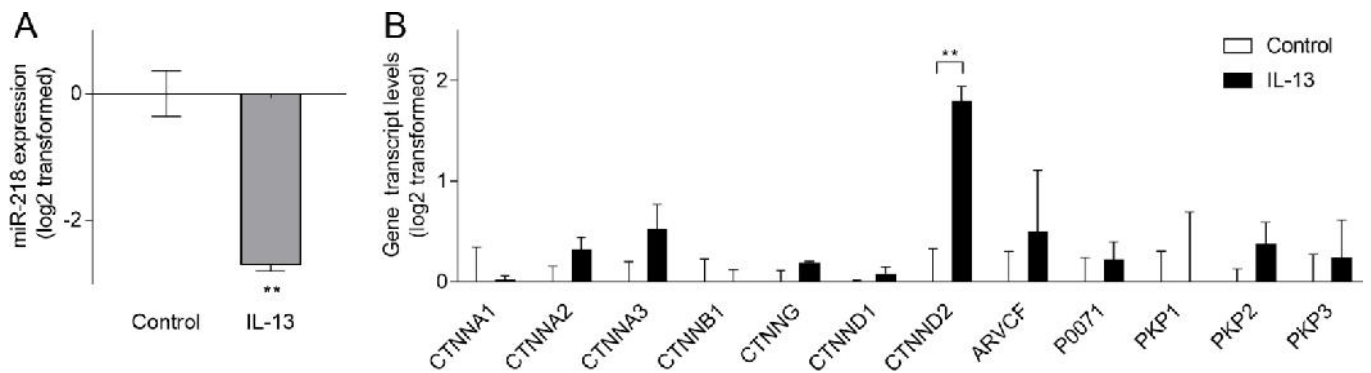


Figure S2. MiR-218-5p is downregulated and CTNND2 is the only upregulated catenin in IL-13-stimulated BEAS-2B cells. (A) MiR-218-5p transcripts in control and IL-13-stimulated BEAS-2B cells were measured by quantitative PCR. (B) The transcript levels of catenin family members in control and IL-13-stimulated BEAS-2B cells were measured by quantitative PCR. The transcript level was expressed as log2 transformed and relative to control. Data are mean $\pm$ SD. $**P < 0.01$, two-tailed student's *t* test.

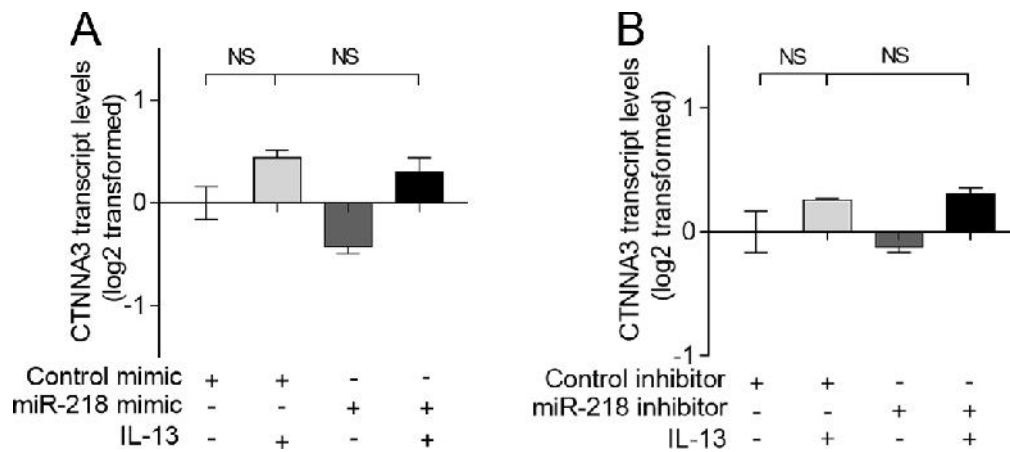


Figure S3. MiR-218-5p overexpression or inhibition do not alter *CTNNA3* expression. (A) *CTNNA3* transcripts in BEAS-2B cells after transfection with control or miR-218-5p mimic with or without IL-13 stimulation was measured by quantitative PCR. (B) *CTNNA3* transcripts in BEAS-2B cells after transfection with control or miR-218-5p inhibitor with or without IL-13 stimulation were measured by quantitative PCR. The transcript level was expressed as log2 transformed and relative to control. NS, not significant. One-way ANOVA followed by Tukey's multiple comparison test.

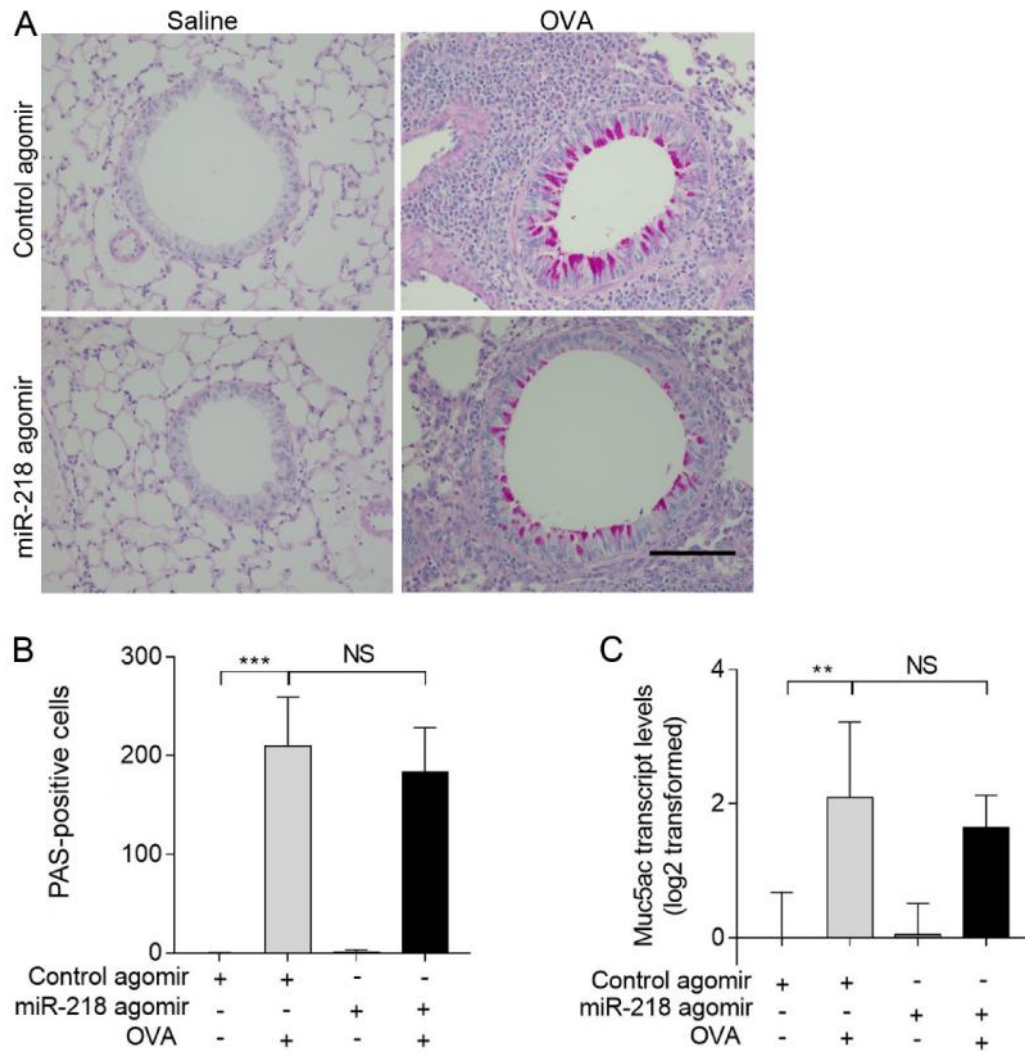


Figure S4. Mmu-miR-218-5p overexpression does not affect airway mucus overproduction in OVA sensitized and challenged mice. (A) Periodic acid-Schiff (PAS) staining for mucus in representative lung sections. Scale bar, 50 μ m. (B) The numbers of PAS-staining-positive cells were counted in five random fields for each lung section at $\times 200$ magnification, the average numbers of positive cells from five lung sections were shown. (C) *Muc5ac* transcripts levels in mouse lungs were determined by quantitative PCR. The transcript level was expressed as log2 transformed and relative to control. Data are mean $\pm$ SD. *** $P < 0.001$, ** $P < 0.01$. One-way ANOVA followed by Tukey's multiple comparison test.

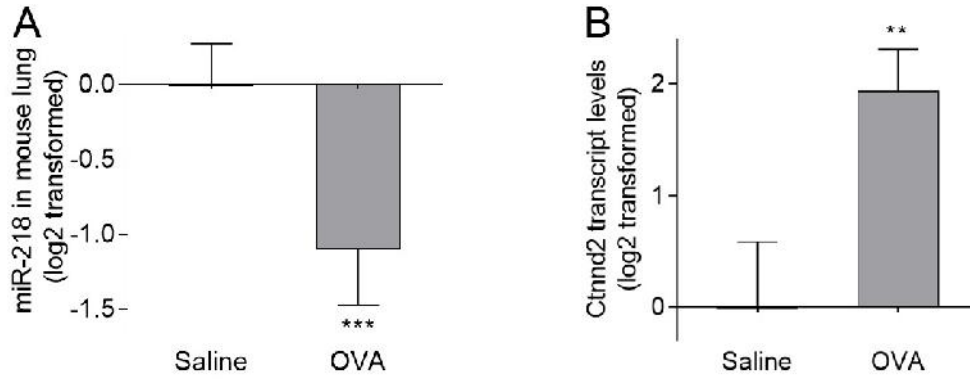


Figure S5. Mmu-miR-218-5p expression is decreased and Ctnnd2 expression is increased in OVA-challenged mice without intervention with control or miR-218-5p agomir. MiR-218-5p (A) and *Ctnnd2* (B) transcript levels in mouse lungs were measured by quantitative PCR. The transcript level was expressed as log2 transformed and relative to the mean of control group. $n = 6$ mice per group. Data are mean $\pm$ SD. ** $P < 0.01$; *** $P < 0.001$ (two-tailed student's t test).