

RESEARCH ARTICLE | Biomarkers in Lung Diseases: From Pathogenesis to Prediction to New Therapies

Decreased epithelial and sputum miR-221-3p associates with airway eosinophilic inflammation and CXCL17 expression in asthma

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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. *Am J Physiol Lung Cell Mol Physiol* 315: L253–L264, 2018. First published April 12, 2018; doi:10.1152/ajplung.00567.2017.—Airway eosinophilic inflammation is a key feature of type 2 high asthma. The role of epithelial microRNA (miR) in airway eosinophilic inflammation remains unclear. We examined the expression of miR-221-3p in bronchial brushings, induced sputum, and plasma from 77 symptomatic, recently diagnosed, steroid-naïve subjects with asthma and 36 healthy controls by quantitative PCR and analyzed the correlation between miR-221-3p expression and airway eosinophilia. We found that epithelial, sputum, and plasma miR-221-3p expression was significantly decreased in subjects with asthma. Epithelial miR-221-3p correlated with eosinophil in induced sputum and bronchial biopsies, fraction of exhaled nitric oxide, blood eosinophil, epithelial gene signature of type 2 status, and methacholine provocative dosage required to cause a 20% decline in forced expiratory volume in the first second in subjects with asthma. Sputum miR-221-3p also correlated with airway eosinophilia and was partially restored after inhaled corticosteroid treatment. Inhibition of miR-221-3p expression suppressed chemokine (C-C motif) ligand (CCL) 24 (eotaxin-2), CCL26 (eotaxin-3), and periostin (POSTN) expression in BEAS-2B bronchial epithelial cells. We verified that chemokine (C-X-C motif) ligand (CXCL) 17, an anti-inflammatory chemokine, is a target of miR-221-3p, and epithelial CXCL17 expression significantly increased in asthma. CXCL17 inhibited CCL24, CCL26, and POSTN expression via the p38 MAPK pathway. Airway overexpression of miR-221-3p exacerbated airway eosinophilic inflammation, suppressed CXCL17 expression, and enhanced CCL24, CCL26, and POSTN expression in house dust mite-challenged mice. Taken together, epithelial and sputum miR-221-3p are novel biomarkers for airway eosinophilic inflammation in asthma. Decreased epithelial miR-221-3p may protect against airway eosinophilic inflammation by upregulating anti-inflammatory chemokine CXCL17.

asthma; CXCL17; eosinophilia; epithelial cells; microRNA

INTRODUCTION

Asthma is a heterogeneous disease with different clinical and molecular phenotypes (30). Types 2 low and high asthma are molecular phenotypes identified by the expression levels of type 2 cytokine and signature genes (28, 32). Airway eosinophilic inflammation is a key feature of type 2 high asthma. Airway epithelial cells are key players in the pathogenesis of airway eosinophilic inflammation in asthma by expressing inflammatory cytokines chemokine (C-C motif) ligand (CCL) 24 (eotaxin-2) and CCL26 (eotaxin-3), which play critical roles in recruiting eosinophils into the airway (9, 14, 27). Epithelial periostin (POSTN) is a signature gene of and plasma POSTN is a biomarker for type 2 high asthma (6, 31, 32). Periostin (encoded by POSTN) recruits eosinophils via binding integrin expressed on eosinophils and is required for airway inflammation in mice (2, 12).

MicroRNAs (miRNAs) are ~22-nucleotide RNAs that play pivotal roles in the regulation of gene expression by promoting mRNA degradation or inhibiting translation (1, 7). We and others have found that the miRNA expression profile in airway epithelial cells was altered in subjects with asthma (11, 29). Epithelial miRNAs play a role in the differentiation of airway epithelial cells (29) and promote airway eosinophilic inflammation by upregulating inflammatory cytokine expression in airway epithelial cells (11). miRNA (miR)-221-3p has been reported to regulate the proliferation of airway smooth muscle cells in severe asthma (25). miR-221-3p was upregulated upon activation of mast cells (20) and promotes the degranulation, migration, and adherence of mast cells (19). A recent study showed that plasma miR-221-3p was decreased in a murine model of allergic airway disease (21). However, the role of airway epithelial miR-221-3p in the pathogenesis of asthma remains unclear. Airway epithelial cells play a pivotal role in airway eosinophilic inflammation (14). We previously reported that epithelial miR-181b-5p is involved in airway eosinophilia (11). Therefore, we hypothesized that epithelial miR-

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221-3p plays a role in airway eosinophilic inflammation in asthma.

The epithelial cells can express anti-inflammatory molecules to keep the homeostasis of mucosal tissue (35). Chemokine (C-X-C motif) ligand (CXCL) 17 is a most recently reported anti-inflammatory chemokine (26) expressing at mucosal tissue of the stomach and trachea (4, 15). CXCL17 suppresses LPS-induced expression of inflammatory genes, including IL-6 and TNF α in macrophages (15). A recent study showed that CXCL17 suppressed skin inflammation by recruiting myeloid-derived suppressor cells and regulatory T cells (24). CXCL17 also has antimicrobial activity in the pathogenesis of interstitial lung disease (4). CXCL17 chemoattracts macrophages (15, 26), and CXCL17 knockout mice exhibit defects in macrophage recruitment to the lungs (4). In addition, CXCL17 plays a role in tumor progression (16, 18) or antitumor immune response (10).

In the present study, we found that epithelial, sputum, and plasma miR-221-3p expression was decreased in subjects with asthma. Epithelial and sputum miR-221-3p expression was correlated with airway eosinophilic inflammation. In vitro, inhibition of miR-221-3p expression suppressed basal and IL-13-induced CCL24, CCL26, and POSTN expression in airway epithelial cells. We verified that CXCL17 is a target of miR-221-3p, and epithelial CXCL17 expression was increased in subjects with asthma. In vivo, airway overexpression of miR-221-3p decreased CXCL17 expression and exacerbated airway eosinophilic inflammation in house dust mite (HDM)-challenged mice.

MATERIALS AND METHODS

Subjects. We recruited 36 healthy control subjects and 77 symptomatic, recently diagnosed subjects with asthma. All subjects were Chinese and were recruited from Tongji Hospital. Subjects with asthma were diagnosed by a physician, had symptoms of episodic cough, had wheezing and/or dyspnea, and had an 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. Physicians referred the subjects with asthma to the study personnel. After baseline evaluation, subjects initiated inhaled corticosteroids (ICS) prescribed by the referring physician. Healthy control subjects had no

respiratory symptoms, normal spirometric value, and methacholine PD₂₀ ≥ 2.505 mg. None of the subjects had ever smoked or received inhaled or oral corticosteroid or leukotriene antagonist. Written informed consent was obtained from all subjects. The ethics committee of Tongji Hospital, Huazhong University of Science and Technology approved the study.

Baseline evaluation. For each subject, demographic information was recorded, blood and induced sputum samples were collected, and spirometry and fraction of exhaled nitric oxide (F_{ENO}) and serum IgE were measured at study entry. Bronchoscopy with brushing and endobronchial biopsies were performed within 1 wk of study entry. Biopsy techniques and methods for histology, pulmonary function testing, and F_{ENO} measurement were described previously (5).

ICS treatment. All subjects with asthma were placed on a standard ICS regimen (200 μ g budesonide twice a day) by their personal physicians. Subjects began using ICS immediately after the bronchoscopy procedure and continued to use ICS throughout the 4-wk study period. All subjects underwent pulmonary function testing and F_{ENO} measurement and had blood and induced sputum samples collected at 4 wk.

Histology and immunohistochemistry. We stained 5- μ m-thick sections with hematoxylin and eosin or monoclonal mouse-antihuman CXCL17 antibody (MAB-4207, R&D Systems, Minneapolis, MN). Two independent investigators who were ignorant of the clinical status counted the numbers of eosinophils/mm² submucosa epithelium.

Cell culture and treatment. BEAS-2B bronchial epithelial cells were stimulated with IL-13 (20 ng/ml, Peprotech, Rocky Hill, NJ) and transfected with control or miR-221-3p mimic, control or miR-221-3p inhibitor (RiboBio, Guangzhou, China), scrambled or CXCL17 siRNA, and empty or CXCL17 cDNA expression vector (Genecopoeia, Guangzhou, China). For dexamethasone and SB-203580 treatment, dexamethasone (10 μ M, Sigma, Darmstadt, Germany) and SB-203580 (50 μ M, MCE) were added before IL-13 exposure. Forty-eight hours after IL-13 stimulation, cells were harvested for quantitative PCR and Western blot analysis, and cell culture media were collected for ELISA.

Mouse model of allergic airway inflammation. Animal experiments were approved by the ethics committee of Tongji Hospital, Huazhong University of Science and Technology. Female C57BL/6 mice received intraperitoneal injections with 100 μ l of the solution of lyophilized HDM extract (0.1 mg/ml, Greer, Lenoir, NC) and Al(OH)₃ as an adjuvant on days 0, 7, and 14, and received 40 μ l HDM solution (3 mg/ml) or saline intranasally on days 21, 22, and 23. miR-221-3p agomir (5 nmol in 40 μ l saline) or control agomir was

Table 1. Subject characteristics

	Healthy Control Subjects	Subjects with Asthma	P Value
<i>n</i>	36	77	
Age, yr	34.13 $\pm$ 9.49	37.51 $\pm$ 11.14	0.30
Sex, M:F, %F	12:24, 67	23:54, 70	0.71
FEV ₁ , %predicted	99.28 $\pm$ 9.05	83.47 $\pm$ 15.34	<0.0001
Methacholine PD ₂₀ , mg	2.505 $\pm$ 0.00	0.20 $\pm$ 0.38	<0.0001
Sputum eosinophil, % (<i>n</i>)	0.54 $\pm$ 0.53 (31)	21.35 $\pm$ 25.41 (69)	<0.0001
Biopsy eosinophil, #/mm ² (<i>n</i>)	2.00 $\pm$ 3.03 (21)	15.12 $\pm$ 10.88 (61)	<0.0001
F _{ENO} (<i>n</i>)	17.18 $\pm$ 12.17 (31)	102.3 $\pm$ 65.36 (69)	<0.0001
Blood eosinophil	75.08 $\pm$ 58.35	367.90 $\pm$ 225.0	<0.0001
Serum IgE	35.71 $\pm$ 35.36	229.4 $\pm$ 263.6	<0.0001
Methodology			
Quantitative PCR for epithelial brushings, <i>n</i>	25	60	
Quantitative PCR for plasma samples, <i>n</i>	36	77	
Quantitative PCR for induced sputum, <i>n</i>	31	69	

Values are means $\pm$ SD. F_{ENO}, 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. In the case of missing data, the number of subjects for whom data exist is noted.

administered intranasally on days 20 and 22. Mice were euthanized 24 h after the last HDM challenge. Cell counts for macrophages, eosinophils, lymphocytes, and neutrophils in bronchoalveolar lavage fluid were performed as previously described (13). The severity of peribronchial inflammation was scored by a blinded observer 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 to 4 cells deep; 4, a ring of inflammatory cells of more than 1 cells deep (22).

Quantitative PCR. For quantification of hsa-miR-221-3p in epithelial brushings, induced sputum, and plasma samples; hsa-miR-221-3p expression in BEAS-2B cells; mmu-miR-221-3p expression in mouse lungs; CCL24, CCL26, chloride channel accessory (CLCA) 1, CXCL17, POSTN, and plasminogen activator inhibitor-2 (SERPINB2) mRNA expression in epithelial brushings and BEAS-2B cells; and CCL24, CCL26, CXCL17, POSTN mRNA expression in mouse lungs, total RNA was isolated and reverse transcribed, and quantitative PCR was performed using an ABI Prism 7500 HT Fast Real-time PCR System (Applied Biosystems, Waltham, MA). The

cycle threshold (Ct) of each gene transcript was normalized to the Ct of cel-miR-239b, U6, or U48 for miRNA, and to β -actin or GAPDH for mRNA, respectively. Relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method (17). The transcript level of each gene is expressed as log2 transformed and relative to the median of healthy control subjects or the mean of control group.

In situ hybridization. We performed in situ hybridization of hsa- and mmu-miR-221-3p on paraffin-embedded sections using biotin-labeled hsa- and mmu-miR-221-3p miRCURY LNA miRNA Detection probe (Qiagen, Shanghai, China). The sequence of the probes for both hsa- and mmu-miR-221-3p was 5'-GAAACCCAG-CAGACAATGTAGCT-3'. The protocol was according to the manufacturer's instructions.

Luciferase activity assay. Vector harboring wild-type, mutant CXCL17 3'-untranslated region (UTR) or no 3'-UTR (control) were cotransfected with miR-221-3p mimic or nontargeting control miRNA into BEAS-2B cells. Luciferase activity was detected by Dual Luciferase Reporter Assay system (Promega, Madison, WI). Normalized

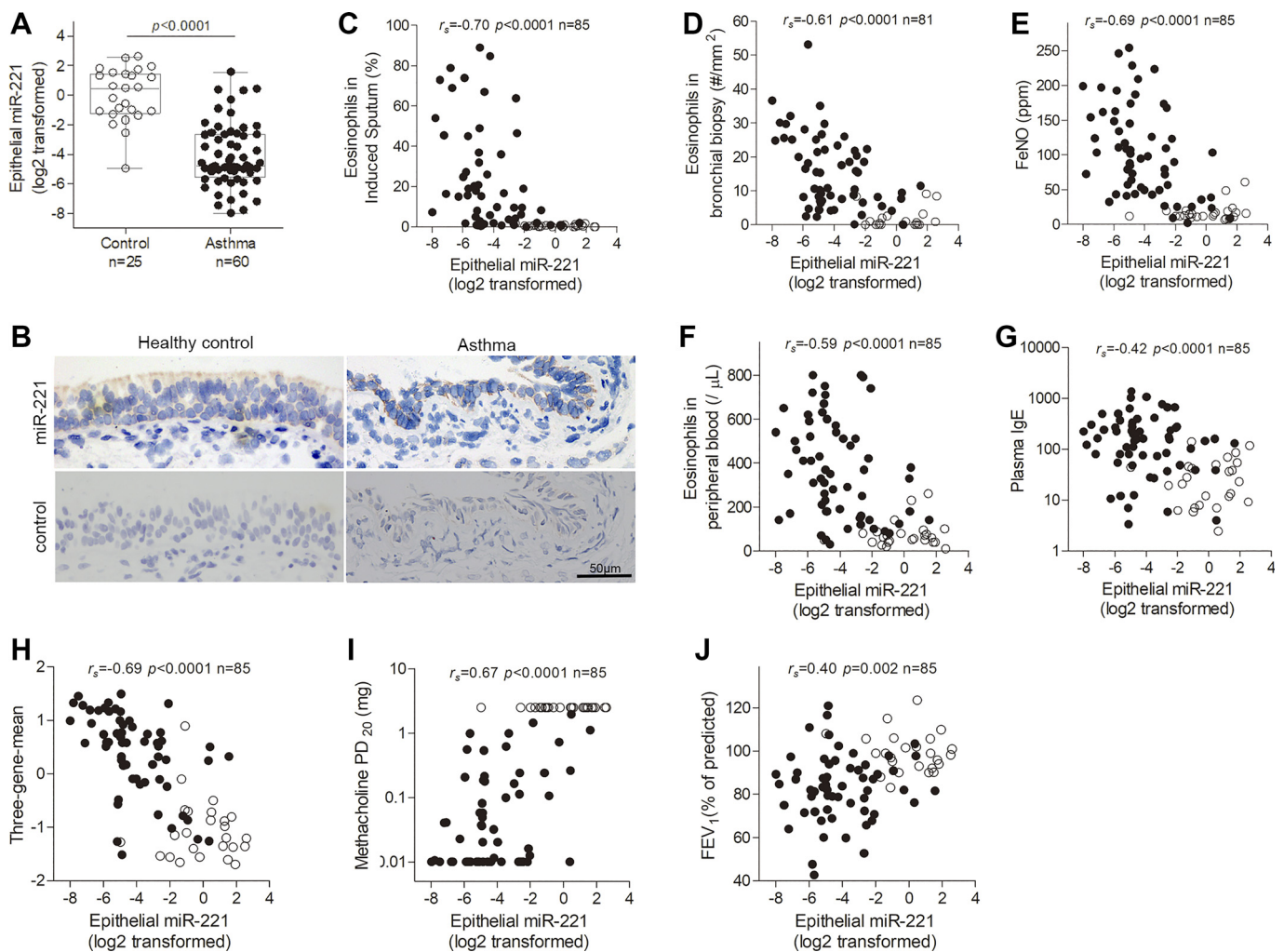


Fig. 1. Epithelial miR-221-3p is decreased and associates with airway eosinophilic inflammation and type 2 status in asthma. miR-221-3p transcript levels in bronchial brushings from subjects with asthma ($n = 60$) and healthy controls ($n = 25$) were determined by quantitative PCR (A). Transcript level is expressed as log2 transformed and relative to the median value for healthy controls (two-tailed Mann-Whitney test). Representative images of in situ hybridization of miR-221-3p in epithelium of bronchial biopsies from subjects with asthma and healthy controls. Original magnification $\times 400$ (B). Correlation assays between epithelial miR-221-3p transcript levels and eosinophil in induced sputum (C) and bronchial biopsies (D), FeNO (E), blood eosinophil (F), plasma IgE (G), epithelial gene signature of type 2 status (three-gene-mean of *CLCA1*, *POSTN*, and *SERPINB2*) (H), methacholine PD_{20} (I), and FEV_1 (J). Subjects with asthma are presented as closed circles ($n = 60$), and healthy controls are presented as open circles ($n = 25$). For eosinophil in bronchial biopsies, $n = 21$ for healthy controls and $n = 60$ for patients with asthma. Correlation assays were performed using Spearman's rank order correlation. *CLCA*, chloride channel accessory; FeNO , fraction of exhaled nitric oxide; miR, microRNA; PD_{20} , provocative dosage required to cause a 20% decline in FEV_1 ; *POSTN*, periostin; *SERPINB2*, plasminogen activator inhibitor-2.

relative light units represent firefly luciferase activity/renilla luciferase activity.

Western blot analysis. The protein expression of CXCL17 (1 μ g/ml, MAB-4207, R&D Systems), and total and phospho-p38 MAPK (1:1,000, Cell Signaling Technology, Boston, MA) in BEAS-2B cells was detected by Western blot analysis as described previously (33).

ELISA. CXCL17, CCL24, CCL26, and POSTN in BEAS-2B cell culture media were analyzed by ELISA (RayBiotech, Atlanta, GA; Eton Bioscience, San Diego, CA). Plasma IgE levels of mouse were determined by ELISA (Dakewe Biotech, Shenzhen, China). ELISA was performed according to the manufacturer's instructions. All samples and standards were measured in duplicate.

Statistical analysis. We analyzed data using Prism version 5 (GraphPad Software, San Diego, CA). For normally distributed data, we calculated means $\pm$ SD, and we used parametric tests (1-way analysis of variance with Tukey's correction or unpaired *t*-test) to compare across groups. For nonnormally distributed data, we calculated medians (with interquartile ranges) and used nonparametric tests (Kruskal-Wallis test with Dunn intergroup comparison or Mann-Whitney test). We analyzed correlation using Spearman's rank order correlation.

RESULTS

Subject characteristics. Subject characteristics are summarized in Table 1. We recruited 77 symptomatic, recently diagnosed subjects with asthma who had never been treated with a steroid or leukotriene antagonist and 36 healthy control subjects. Subjects with asthma had lower FEV₁ and methacholine PD₂₀ and higher F_{ENO}, sputum, biopsy, and blood eosinophils, and serum IgE levels than healthy control subjects.

Epithelial miR-221-3p expression is decreased and associates with airway eosinophilic inflammation and type 2 status in asthma. We measured the expression of miR-221-3p in endobronchial brushing samples by quantitative PCR. Endobronchial brushing samples predominantly consisted of epithelial cells ($97.1 \pm 4.2\%$). There was no significant difference in the percentage of epithelial cells between control subjects ($96.9 \pm 3.9\%$, $n = 25$ subjects) and subjects with asthma ($97.2 \pm 4.4\%$, $n = 60$, $P = 0.18$). We found that epithelial miR-221-3p expression was significantly decreased in subjects with asthma compared with healthy controls (Fig. 1A). Using in situ hybridization, we found that miR-221-3p was mainly located in the cytoplasm of airway epithelial cells, and the miR-221-3p-positive staining was much less in subjects with asthma compared with controls (Fig. 1B).

Airway eosinophilic inflammation is a key feature of type 2 high asthma (32). We found strong inverse correlations between epithelial miR-221-3p expression and three measures of airway eosinophilic inflammation, including the percentage of eosinophils in induced sputum ($r_s = -0.70$, $P < 0.0001$, $n = 85$ for all subjects; $r_s = -0.48$, $P = 0.0001$, $n = 60$ for asthma), the number of eosinophils in bronchial biopsies ($r_s = -0.61$, $P < 0.0001$, $n = 81$ for all subjects; $r_s = -0.39$, $P = 0.002$, $n = 60$ for asthma), and F_{ENO} ($r_s = -0.69$, $P < 0.0001$, $n = 85$ for all subjects; $r_s = -0.47$, $P = 0.0002$, $n = 60$ for asthma) (Fig. 1, C–E). This indicates that epithelial miR-221-3p expression associates with airway eosinophilic inflammation in asthma.

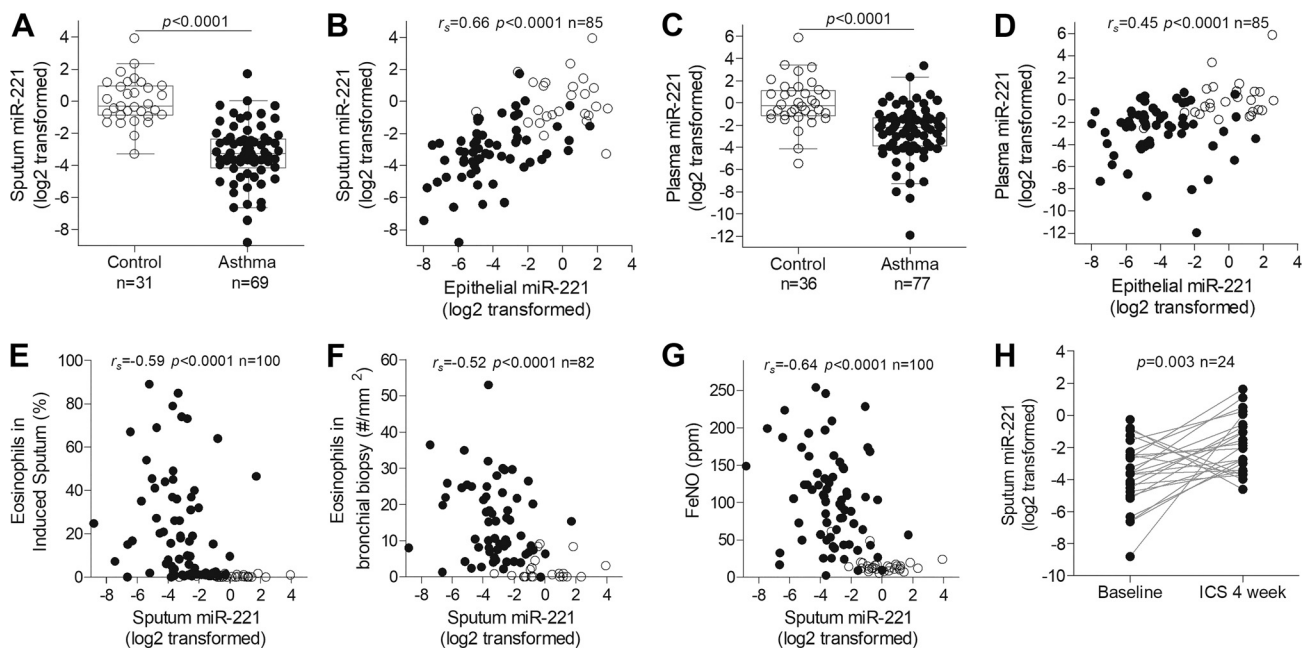


Fig. 2. Sputum and plasma miR-221-3p is decreased in asthma, and sputum miR-221-3p associates with airway eosinophilia and is restored after ICS treatment. miR-221-3p transcript levels in induced sputum from subjects with asthma ($n = 69$) and healthy controls ($n = 31$) were determined by quantitative PCR (A). Transcript level is expressed as log2 transformed and relative to the median value for healthy controls (two-tailed Mann-Whitney test). Correlation assay between epithelial and sputum miR-221-3p transcript levels in subjects with asthma ($n = 60$) and healthy controls ($n = 25$) (B). miR-221-3p transcript levels in plasma samples from subjects with asthma ($n = 77$) and healthy controls ($n = 36$) were determined by quantitative PCR (two-tailed Mann-Whitney test) (C). Correlation assay between epithelial and plasma miR-221-3p transcript levels in subjects with asthma ($n = 60$) and healthy controls ($n = 25$) (D). Correlation assays between sputum miR-221-3p transcript levels and eosinophil in induced sputum (E) and bronchial biopsies (F) and F_{ENO} (G). Subjects with asthma are presented as closed circles ($n = 69$), and healthy controls are presented as open circles ($n = 31$). For eosinophil in bronchial biopsies, $n = 21$ for healthy controls and $n = 61$ for asthma. Correlation assays were performed using Spearman's rank order correlation. Change of sputum miR-221-3p transcript levels in subjects with asthma after ICS treatment for 4 wk ($n = 24$, two-tailed Student's *t*-test) (H). F_{ENO}, fraction of exhaled nitric oxide; ICS, inhaled corticosteroids; miR, microRNA.

Eosinophils in peripheral blood and serum IgE are measures of allergic status and reflect the type 2 status of asthma (8). We found that epithelial miR-221-3p expression inversely correlated with blood eosinophil counts ($r_s = -0.59$, $P < 0.0001$, $n = 85$ for all subjects; $r_s = -0.31$, $P = 0.015$, $n = 60$ for asthma) and serum IgE levels ($r_s = -0.42$, $P < 0.0001$, $n = 85$ for all subjects; $r_s = -0.13$, $P = 0.33$, $n = 60$ for asthma) (Fig. 1, *F* and *G*). Furthermore, by measuring the expression of three IL-13-induced epithelial genes (*CLCA1*, *POSTN*, and *SERPINB2*) in endobronchial brushings using quantitative PCR, and by combination of these measurements to calculate a three-gene-mean for each subject (3), we found that epithelial miR-221-3p expression correlated with the expression of this epithelial gene signature for type 2 status ($r_s = -0.69$, $P < 0.0001$, $n = 85$ for all subjects; $r_s = -0.56$, $P < 0.0001$, $n = 60$ for asthma) (Fig. 1*H*). Our data indicate that epithelial miR-221-3p expression associates with the type 2 status in asthma.

In addition, we found that epithelial miR-221-3p correlated with methacholine PD₂₀ ($r_s = 0.67$, $P < 0.0001$, $n = 85$ for all subjects; $r_s = 0.30$, $P = 0.02$, $n = 60$ for asthma). Epithelial miR-221-3p correlated with FEV₁ in all subjects ($r_s = 0.40$, $P = 0.002$, $n = 85$) but not in subjects with asthma ($r_s = 0.10$, $P = 0.45$, $n = 60$) (Fig. 1, *I* and *J*).

Sputum and plasma miR-221-3p is decreased in asthma, and sputum miR-221-3p associates with airway eosinophilia and is restored after ICS treatment. miRNAs are attractive candidates for biomarker development because they are stable and can be measured in extracellular fluids (21). We found that sputum and plasma miR-221-3p levels were significantly lower in subjects with asthma compared with healthy controls (Fig. 2, *A* and *C*). Sputum and plasma miR-221-3p levels correlated with epithelial miR-221-3p expression (Fig. 2, *B* and *D*). Moreover, sputum miR-221-3p levels correlated with the percentage of eosinophils in induced sputum ($r_s = -0.59$, $P < 0.0001$, $n = 100$ for all subjects; $r_s = -0.31$, $P = 0.01$, $n = 69$ for asthma),

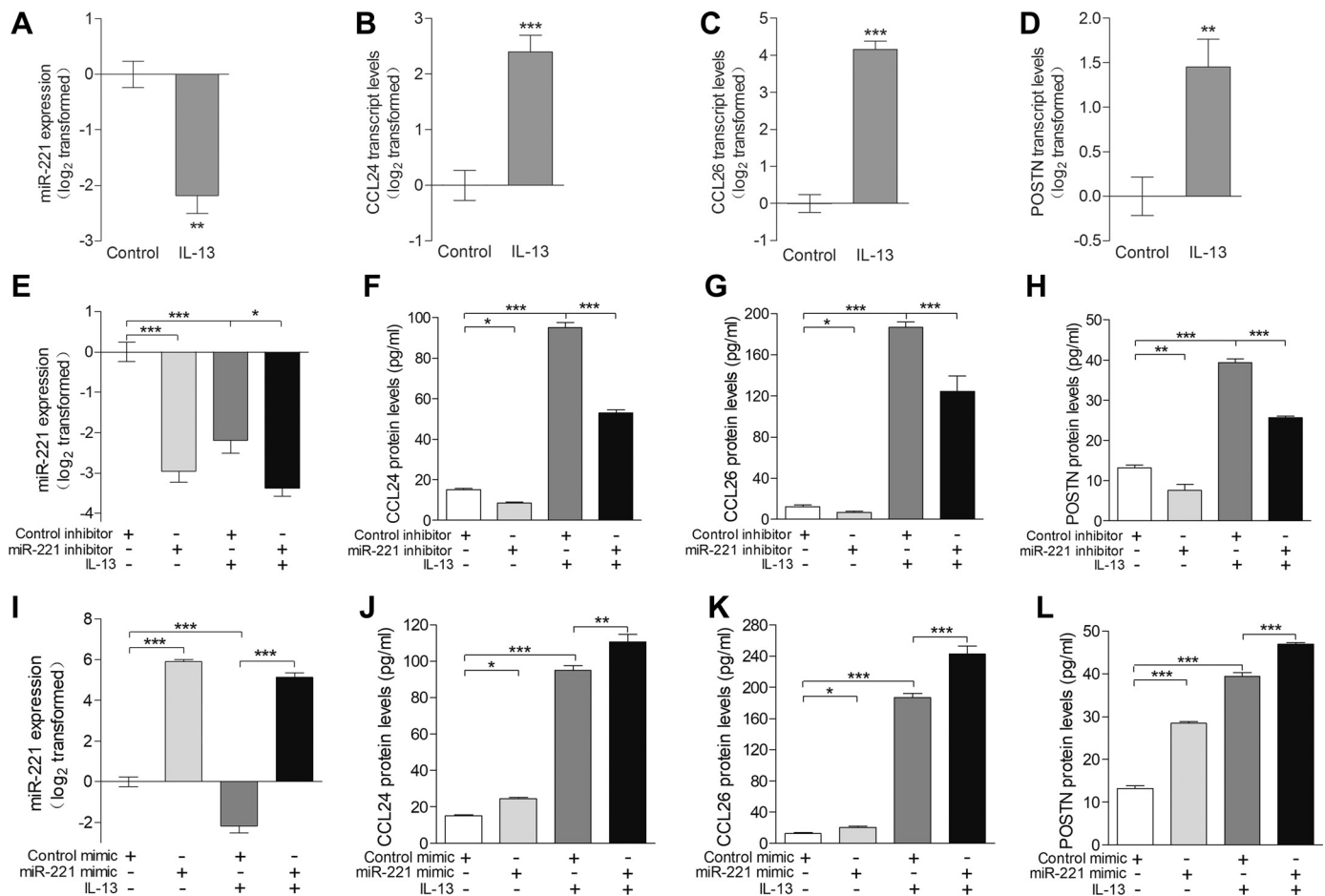


Fig. 3. Repression of miR-221-3p suppresses, but overexpression of miR-221-3p enhances, baseline and IL-13-induced CCL24, CCL26, and POSTN expression in BEAS-2B cells. Transcript levels of baseline and IL-13-induced miR-221-3p, CCL24, CCL26, and POSTN in BEAS-2B cells were determined by quantitative PCR (A–D). Transcript level is expressed as log₂ transformed and relative to the mean of control group (two-tailed Student's *t*-test). Expression of miR-221-3p after control or miR-221-3p inhibitor transfection with or without IL-13 stimulation was determined by quantitative PCR (E). Protein levels of CCL24 (F), CCL26 (G), and POSTN (H) in cell culture media after control or miR-221-3p inhibitor transfection with or without IL-13 stimulation were determined by ELISA. Expression of miR-221-3p after control or miR-221-3p mimic transfection with or without IL-13 stimulation was determined by quantitative PCR (I). Protein levels of CCL24 (J), CCL26 (K), and POSTN (L) in cell culture media after control or miR-221-3p mimic transfection with or without IL-13 stimulation were determined by ELISA. $n = 4$ wells per group. Data are means $\pm$ SD; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (one-way ANOVA followed by Tukey's multiple comparison test). Data are representative of three independent experiments. CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; miR, microRNA; POSTN, periostin.

the number of eosinophils in bronchial biopsies ($r_s = -0.52$, $P < 0.0001$, $n = 82$ for all subjects; $r_s = -0.25$, $P = 0.049$, $n = 61$ for asthma), and F_{ENO} ($r_s = -0.64$, $P < 0.0001$, $n = 100$ for all subjects; $r_s = -0.29$, $P = 0.02$, $n = 69$ for asthma) (Fig. 2, E–G). Our data indicate that sputum miR-221-3p may be a useful noninvasive biomarker for airway eosinophilic inflammation.

Interestingly, we found that sputum miR-221-3p levels were increased after ICS treatment for 4 wk compared with baseline in subjects with asthma (Fig. 2H). Because ICS ameliorates airway eosinophilic inflammation (23), the partial restoration of sputum miR-221-3p after ICS treatment may reflect the recovery of airway inflammation in asthma.

Inhibition of miR-221-3p suppresses epithelial CCL24, CCL26, and POSTN expression. To investigate the mechanism by which epithelial miR-221-3p associates with airway eosinophilic inflammation, we used IL-13, a key type 2 cytokine, to stimulate BEAS-2B bronchial epithelial cells. IL-13 decreased miR-221-3p expression but increased the expression of CCL24, CCL26, and POSTN in BEAS-2B cells (Fig. 3, A–D). Inhibition of miR-221-3p expression by transfecting with miR-221-3p inhibitor suppressed baseline and IL-13-induced CCL24, CCL26, and POSTN protein levels in culture media (Fig. 3, E–H). This indicates that decreased epithelial miR-221-3p expression may contribute to resolve airway inflammation for return to airway homeostasis in asthma by suppressing CCL24, CCL26, and POSTN expression. In support of this, reversal of IL-13-induced decrease of miR-221-3p by transfecting miR-221-3p mimic further enhanced IL-13-induced CCL24, CCL26, and POSTN expression (Fig. 3, I–L).

Because ICS treatment partially restored decreased sputum miR-221-3p in subjects with asthma, we treated BEAS-2B cells with dexamethasone and found that dexamethasone restored IL-13-induced miR-221-3p downregulation and suppressed IL-13-induced upregulation of CCL24, CCL26, and POSTN (Fig. 4, A–D).

CXCL17 is a target of miR-221-3p, and epithelial CXCL17 expression is increased in asthma. We predicted the candidate target genes of miR-221-3p by using online algorithms DIANA-microT, miRanda, miRWalk, PicTar, and TargetScan. CXCL17, a recently reported anti-inflammatory chemokine (15), is one of the candidates. The 3'-UTR of CXCL17 contains a region that matches the seed sequence of hsa-miR-221-3p (Fig. 5A). Transfection of miR-221-3p

mimic decreased the luciferase activity when cotransfected with the vector harboring wild-type CXCL17 3'-UTR but had no effect on the luciferase activity when cotransfected with the vector containing mutant 3'-UTR or empty vector (Fig. 5B). This indicates that miR-221-3p acts directly on the 3'-UTR of the CXCL17 gene. IL-13 stimulated CXCL17 expression in BEAS-2B cells. Baseline and IL-13-induced CXCL17 mRNA and protein expression was decreased after transfection with miR-221-3p mimic but enhanced after transfection with miR-221-3p inhibitor (Fig. 5, C–E). Our data suggest that CXCL17 is a target of miR-221-3p.

The expression of CXCL17 in the airway of subjects with asthma has never, to our knowledge, been reported. We found that epithelial CXCL17 transcript levels were significantly increased in subjects with asthma compared with controls (Fig. 5F). Immunohistochemistry revealed that there were few CXCL17-positive epithelial cells in healthy control subjects, whereas CXCL17-positive staining markedly increased in subjects with asthma (Fig. 5H). Moreover, epithelial CXCL17 transcript levels were significantly inversely correlated with epithelial miR-221-3p expression ($r_s = -0.55$, $P < 0.0001$, $n = 85$ for all subjects; $r_s = -0.45$, $P = 0.0003$, $n = 60$ for asthma; Fig. 5G).

CXCL17 suppresses IL-13-induced CCL24, CCL26, and POSTN expression via p38 MAPK pathway in airway epithelial cells. CXCL17 is reported to suppress inflammatory gene expression (15). We found that overexpression of CXCL17 by transfecting with a CXCL17 cDNA expression vector decreased baseline and IL-13-induced CCL24, CCL26, and POSTN protein levels in culture media (Fig. 6, A–C). In contrast, knockdown of CXCL17 expression by transfecting with siRNA against CXCL17 enhanced baseline and IL-13-induced CCL24, CCL26, and POSTN expression (Fig. 6, D–F). Our data indicate that CXCL17 suppresses the production of epithelial proinflammatory cytokines, suggesting that epithelial CXCL17 acts as an anti-inflammatory factor to keep the airway homeostasis in asthma. In addition, IL-13-induced CXCL17 expression was decreased after dexamethasone treatment in BEAS-2B cells (data not shown). This is consistent with the recovery of IL-13-reduced miR-221-3p expression after dexamethasone treatment.

We further investigated the signaling pathway through which CXCL17 suppresses the expression of inflammatory cytokines. SB-203580, an inhibitor of p38 MAPK, blocked

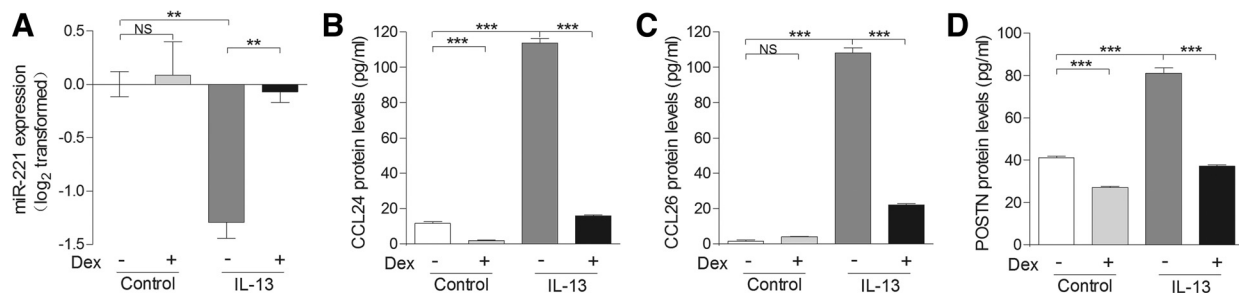


Fig. 4. Steroid restores miR-221-3p expression and suppresses CCL24, CCL26, and POSTN expression in BEAS-2B cells. A: transcript levels of miR-221-3p after dexamethasone (Dex) treatment with or without IL-13 stimulation were determined by quantitative PCR. Transcript level is expressed as log2 transformed and relative to the mean of control group. B–D: protein levels of CCL24, CCL26, and POSTN in culture media after dexamethasone treatment with or without IL-13 stimulation were determined by ELISA. $n = 4$ wells per group. Data are means $\pm$ SD. NS, not significant; $**P < 0.01$; $***P < 0.001$ (one-way ANOVA followed by Tukey's multiple comparison test). Data are representative of three independent experiments. CCL, chemokine (C-C motif) ligand; miR, microRNA; POSTN, periostin.

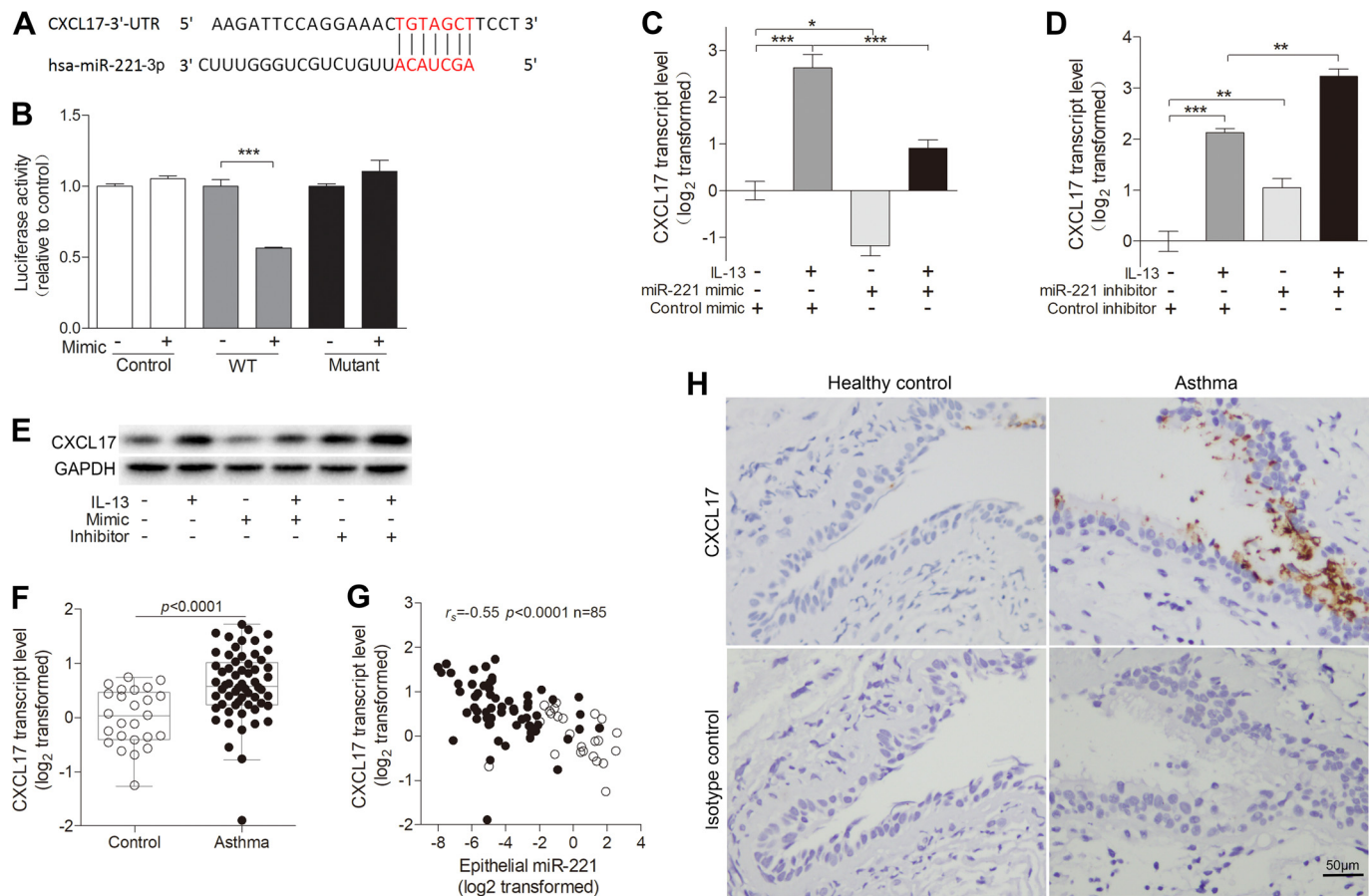


Fig. 5. CXCL17, a target of miR-221-3p, is increased and correlated with epithelial miR-221-3p expression in asthma. 3'-UTR of CXCL17 contains a region that matches the seed sequence of hsa-miR-221-3p (A). 3'-UTR luciferase report assay with vector harboring wild-type (WT), mutant CXCL17 3'-UTR, or no 3'-UTR (control) cotransfected with miR-221-3p mimic or nontargeting miRNA (B). Luciferase activity was measured by dual-luciferase reporter assay system. Firefly luciferase activity was normalized to renilla luciferase activity. $n = 4$ per group. CXCL17 transcript levels were determined by quantitative PCR in BEAS-2B cells after transfection with miR-221-3p mimic (C) and inhibitor (D) and stimulated with or without IL-13. Transcript level is expressed as log₂ transformed and relative to the mean of control group. $n = 4$ per group. Data are means $\pm$ SD; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (one-way ANOVA followed by Tukey's multiple comparison test). Data are representative of three independent experiments. CXCL17 protein expression in BEAS-2B cells after transfection with miR-221-3p mimic and inhibitor and stimulated with or without IL-13, which is determined by Western blotting and representative of 3 independent experiments (E). CXCL17 transcript levels in bronchial brushings from subjects with asthma ($n = 60$) and healthy controls ($n = 25$) were determined by quantitative PCR (F). Transcript level is expressed as log₂ transformed and relative to control (two-tailed Student's t -test). Correlation assay between epithelial CXCL17 and miR-221-3p transcript levels in subjects with asthma ($n = 60$) and healthy controls ($n = 25$) (G). Correlation assays were performed using Spearman's rank order correlation. Representative images of CXCL17 immunohistochemistry in bronchial biopsies from healthy control subjects and subjects with asthma (H). Original magnification $\times 400$. CXCL, chemokine (C-X-C motif) ligand; miR, microRNA; UTR, untranslated region.

baseline and IL-13-induced CCL24, CCL26, and POSTN expression (Fig. 6, G–I) but did not affect CXCL17 expression (data not shown). Meanwhile, IL-13 treatment induced the phosphorylation of p38 MAPK (Fig. 6J). This indicates that the activation of p38 MAPK is required for IL-13-induced CCL24, CCL26, and POSTN expression in epithelial cells. Furthermore, overexpression of CXCL17 suppressed, whereas knock-down of CXCL17 expression enhanced, baseline and IL-13-induced p38 MAPK phosphorylation (Fig. 6J). Our data suggest that CXCL17 suppresses CCL24, CCL26, and POSTN expression via inhibition of p38 MAPK activation.

Airway overexpression of miR-221-3p exacerbates airway eosinophilic inflammation in murine model of allergic airway inflammation. We further investigated the role of miR-221-3p in airway eosinophilic inflammation in a murine model of allergic airway inflammation induced by HDM. Using in situ hybridization and quantitative PCR, we found that miR-221-3p expression was decreased in HDM-challenged mice compared

with control. Airway administration of miR-221-3p agomir markedly increased airway epithelial miR-221-3p expression (Fig. 7, A and B). HDM challenge significantly increased lung inflammatory scores and the number of total cells and eosinophils in bronchoalveolar lavage fluid compared with control mice. Airway administration of miR-221-3p agomir further increased lung eosinophilic inflammation in HDM-challenged mice (Fig. 7, C–E). Our data indicate that airway overexpression of miR-221-3p exacerbates allergen-induced airway eosinophilic inflammation in mice.

Furthermore, we found that CXCL17, CCL24, CCL26, and POSTN expression was increased after HDM sensitization and challenge. Airway overexpression of miR-221-3p inhibited baseline and HDM-induced CXCL17 expression but enhanced baseline and HDM-induced CCL24, CCL26, and POSTN expression in mouse lungs (Fig. 8, A–D). This indicates that miR-221-3p overexpression exacerbates airway eosinophilic inflammation by suppressing CXCL17 and enhancing inflam-

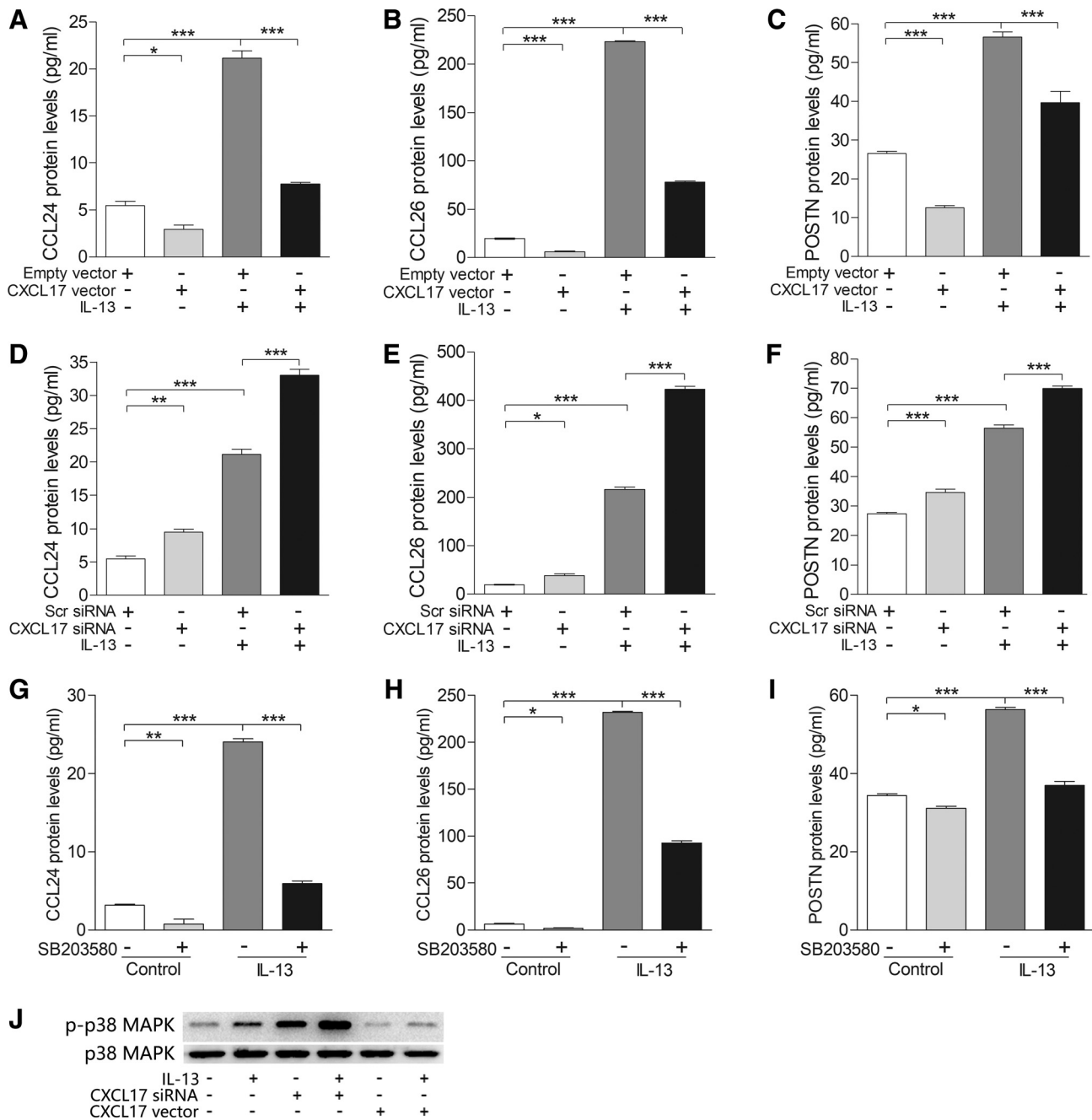


Fig. 6. CXCL17 suppresses IL-13-induced CCL24, CCL26, and POSTN expression via p38 MAPK pathway in airway epithelial cells. Protein levels of CCL24 (A), CCL26 (B), and POSTN (C) in culture media after transfection with empty or CXCL17 expression vector and stimulated with or without IL-13 were determined by ELISA. Protein levels of CCL24 (D), CCL26 (E), and POSTN (F) in culture media after transfection with scrambled or CXCL17 siRNA and stimulated with or without IL-13 were determined by ELISA. The protein levels of CCL24 (G), CCL26 (H), and POSTN (I) in culture media after treatment with SB-203580, an inhibitor of p38 MAPK pathway, and stimulated with or without IL-13 were determined by ELISA. $n = 4$ wells per group. Data are means $\pm$ SD; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (one-way ANOVA followed by Tukey's multiple comparison test). Phosphorylation of p38 MAPK protein in BEAS-2B cells after transfection with CXCL17 siRNA or CXCL17 expression vector and stimulated with or without IL-13 was determined by Western blotting (J). Data are representative of at least three independent experiments. CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; miR, microRNA; POSTN, periostin.

matory cytokine expression. Moreover, airway overexpression of miR-221-3p increased baseline and HDM-induced IL-4, IL-5, and IL-13 mRNA expression in mouse lungs (Fig. 8, E–G) and enhanced serum IgE levels in HDM-challenged mice (Fig. 8H). This is consistent with our findings that epithelial miR-221 expression correlates the type 2 status in asthma.

DISCUSSION

In the present study, we demonstrated that epithelial, sputum, and plasma miR-221-3p expression was decreased in subjects with asthma who were newly diagnosed and untreated with ICS or leukotriene receptor antagonist. Epithelial miR-

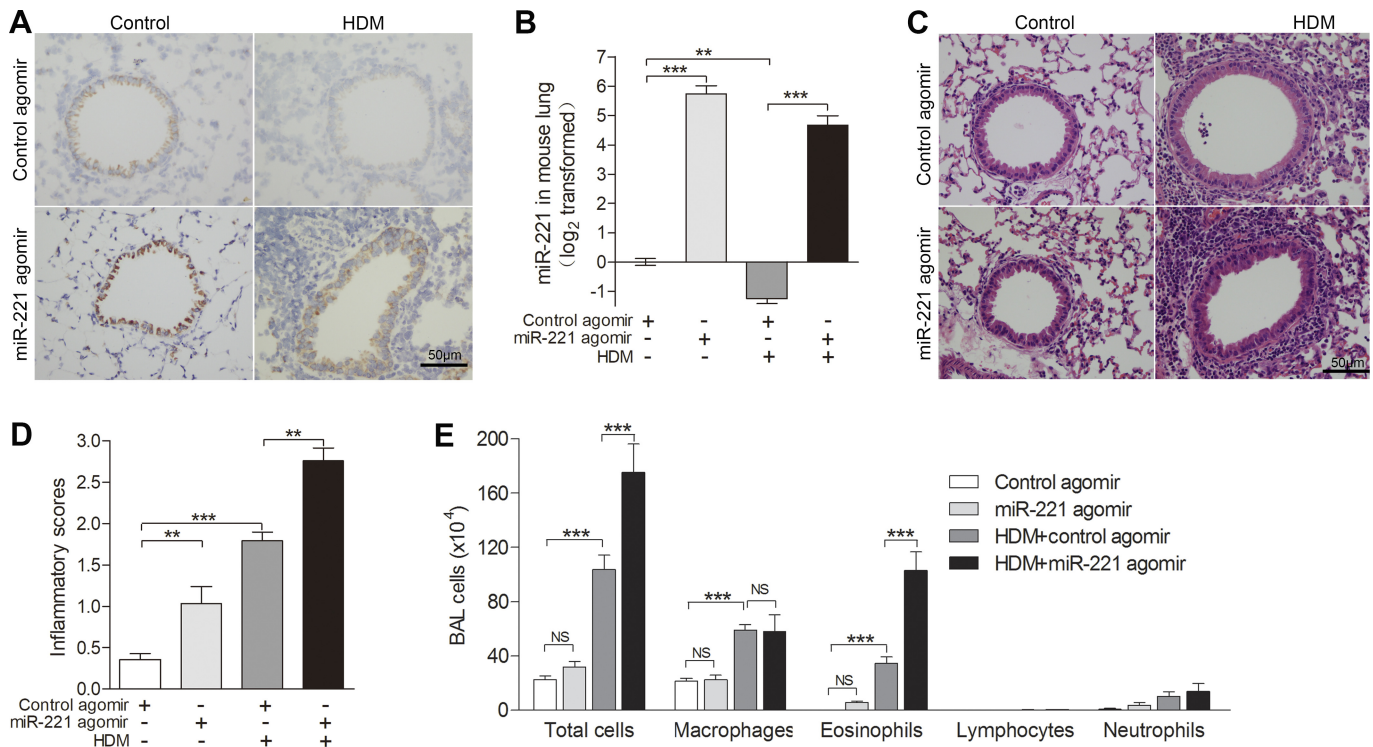


Fig. 7. Airway overexpression of miR-221-3p exacerbates airway eosinophilic inflammation in mice sensitized and challenged with HDM. **A:** representative images of in situ hybridization of miR-221-3p in mouse lung sections. Original magnification $\times 200$. **B:** transcript levels of miR-221-3p in mouse lungs were determined by quantitative PCR. Transcript level is expressed as log₂ transformed and relative to the mean of control group. **C:** representative images of H&E staining of mouse lung sections. Original magnification $\times 200$. **D:** lung inflammatory scores were calculated as described in MATERIALS AND METHODS. **E:** counts for macrophages, eosinophils, lymphocytes, and neutrophils in BAL fluid. $n = 6$ –10 mice per group. Data are means $\pm$ SD; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (one-way ANOVA followed by Tukey's multiple comparison test). BAL, bronchoalveolar lavage; HDM, house dust mite; H&E, hematoxylin-eosin; miR, microRNA.

221-3p significantly correlated with eosinophils in induced sputum and bronchial biopsies, F_{ENO} , blood eosinophil, epithelial gene signature of type 2 status, and methacholine PD₂₀. Moreover, sputum miR-221-3p correlated with airway eosinophilic inflammation and was partially restored after ICS treatment.

Asthma can be classified into type 2 low and -high asthma. Airway eosinophilic inflammation is a key feature of type 2-high asthma (32). Herein, we reported that epithelial miR-221-3p expression was significantly correlated with airway eosinophilic inflammation and biomarkers for type 2 status, including blood eosinophil count and epithelial gene signature (*CLCA1*, *SERPINE2*, *POSTN*) of type 2 status. This suggests that epithelial miR-221-3p plays a role in airway eosinophilic inflammation and associates with type 2 status in asthma. Perry et al. (25) reported that miR-221-3p was upregulated and contributed to the proliferation of cultured airway smooth muscle cells from subjects with severe asthma, suggesting that the expression of miR-221-3p is differentially regulated among different cell types of the asthmatic airway. In vitro studies showed that miR-221-3p plays a role in mast cell activation, degranulation, migration, and adherence (19, 20), and miR-221-3p contributed to IL-4 expression in mast cells (34).

miRNAs are attractive candidates for biomarker development because miRNAs are stable and can be measured in extracellular fluids (21). We demonstrated that sputum and plasma miR-221-3p expression was decreased in subjects with asthma. Moreover, sputum and epithelial miR-221-3p expres-

sion correlated with each other, and sputum miR-221-3p correlated with airway eosinophilic inflammation. Of note, decreased sputum miR-221-3p expression was partially recovered after ICS treatment for 4 wk in subjects with asthma, which may reflect the improvement of airway eosinophilic inflammation after ICS treatment. Therefore, sputum miR-221-3p can be a useful noninvasive biomarker for assessing airway eosinophilic inflammation. Consistent with our findings, Milger et al. (21) reported that a set of plasma miRNAs including miR-221-3p were decreased in allergen-challenged mice. Because eosinophil accumulated in sputum in asthma, eosinophil could be another source of sputum miR-221-3p in addition to epithelial cells, and miR-221-3p expression may also be decreased in eosinophils.

We verified that CXCL17, a recently identified anti-inflammatory chemokine (15), is a target of miR-221-3p. For the first time, we demonstrated that CXCL17 expression in airway epithelial cells was markedly increased in subjects with asthma compared with control subjects. Repression of miR-221-3p expression or overexpression of CXCL17 suppressed baseline and IL-13-induced CCL24, CCL26, and POSTN expression in airway epithelial cells. Because these cytokines play critical roles in the recruitment of eosinophils to the airway, CXCL17 may contribute to the resolution of airway eosinophilic inflammation for the airway to return to homeostasis. Consistently, Lee et al. (15) reported that CXCL17 inhibited inflammatory gene expression in macrophages. Our data suggest that down-regulated epithelial miR-221 inhibits inflammatory cytokine

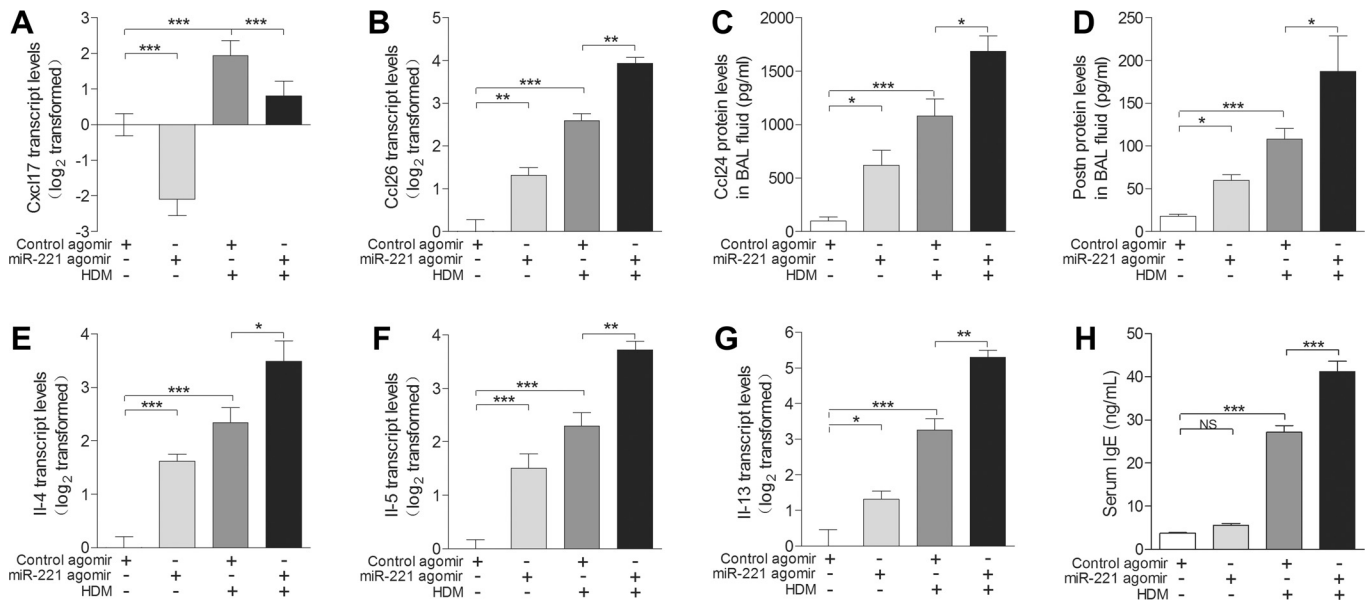


Fig. 8. Airway overexpression of miR-221-3p suppresses CXCL17 and enhances inflammatory and type 2 cytokines expression and serum IgE levels. *A* and *B*: transcript levels of CXCL17 and CCL26 in mouse lungs were determined by quantitative PCR. Transcript level is expressed as log₂ transformed and relative to the mean of control group. *C* and *D*: protein levels of CCL24 and POSTN in BALF were determined by ELISA. *E–G*: transcript levels of IL-4, IL-5, and IL-13 in mouse lungs were determined by quantitative PCR. *H*: serum IgE levels in peripheral blood were determined by ELISA. *n* = 6–10 mice per group. Data are means $\pm$ SD; **P* < 0.05; ***P* < 0.01; ****P* < 0.001 (one-way ANOVA followed by Tukey's multiple comparison test). BALF, bronchoalveolar lavage fluid; CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; HDM, house dust mite; miR, microRNA; POSTN, periostin.

expression by upregulating anti-inflammatory chemokine CXCL17 expression in airway epithelial cells (Fig. 9). Interestingly, we found that corticosteroids suppressed CCL24, CCL26, and POSTN expression, and restored downregulated miR-221-3p and upregulated CXCL17 in bronchial epithelial cells. This is in line with our finding that sputum miR-221-3p was partially restored in subjects with asthma after ICS treatment and suggests that epithelial miR-221-3p and CXCL17 may respond to upregulated proinflammatory cytokine expression in a feedback manner.

Furthermore, we demonstrated that airway overexpression of miR-221-3p inhibited CXCL17 expression, enhanced CCL24, CCL26, and POSTN expression, and exacerbated airway eosinophilic inflammation in HDM-challenged mice. This supports that decreased epithelial miR-221-3p expression

protects against airway eosinophilic inflammation by upregulating CXCL17. Besides inhibition of inflammatory cytokines expression, CXCL17 has been reported to play a role in recruitment of macrophages (4, 26). However, we found the number of eosinophils, but not other cell types, in bronchoalveolar lavage fluid was increased in control or HDM-challenged mice after airway overexpression of miR-221-3p. Because miR-221-3p was predominantly overexpressed in mouse airway epithelial cells (Fig. 7A), the exacerbated airway eosinophilia was mainly attributed to miR-221-3p overexpression in epithelial cells. However, we cannot exclude the possibility that miR-221-3p overexpression may also affect the function of mast cells in airway epithelium.

Our study has several limitations. First, the subjects in our study had mild-to-moderate asthma; therefore, the expression of epithelial miR-221-3p in severe asthma needs further investigation. Second, we cannot exclude the possibility that miR-221-3p regulates airway inflammation by targeting other genes besides CXCL17 because an miRNA targets multiple genes' expression.

Taken together, we conclude that decreased epithelial and sputum miR-221-3p are novel biomarkers for airway eosinophilic inflammation in asthma. Decreased miR-221-3p expression may protect against airway eosinophilic inflammation by upregulating anti-inflammatory chemokine CXCL17.

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GRANTS

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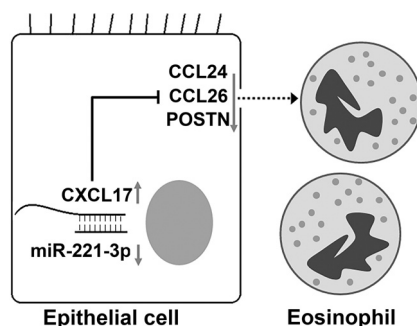


Fig. 9. Model of the interactions between miR-221-3p, CXCL17, CCL24, CCL26, and POSTN in airway epithelial cells in asthma. Epithelial miR-221-3p expression is decreased in asthma, leading to the upregulation of anti-inflammatory chemokine CXCL17. CXCL17 suppresses the expression of CCL24, CCL26, and POSTN, which play a role in eosinophilic airway inflammation in asthma. CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; miR, microRNA; POSTN, periostin.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

G.Z. conceived and designed research; K.Z., Y.L., Y.F., and W.W. performed experiments; K.Z., Y.L., Y.F., W.W., and G.Z. analyzed data; K.Z., Y.L., H.Z., J.H., Q.H., J.Z., Y.X., Z.L., and G.Z. interpreted results of experiments; K.Z. and G.Z. prepared figures; K.Z. and G.Z. drafted manuscript; G.Z. edited and revised manuscript; K.Z., Y.L., H.Z., Y.F., H.Z., J.H., Q.H., J.Z., Y.X., Z.L., and G.Z. approved final version of manuscript.

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