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RESEARCH ARTICLE

MiR-223 is increased in lungs of patients with COPD and modulates cigarette smoke-induced pulmonary inflammation

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Abstract

Since microRNA (miR)-223-3p modulates inflammatory responses and chronic obstructive pulmonary disease (COPD) is associated with amplified pulmonary inflammation, we hypothesized that miR-223-3p plays a role in COPD pathogenesis. Expression of miR-223-3p was measured in lung tissue of two independent cohorts with patients with GOLD stage II–IV COPD, never smokers, and smokers without COPD. The functional role of miR-223-3p was studied in deficient mice and on overexpression in airway epithelial cells from COPD and controls. We observed higher miR-223-3p levels in patients with COPD stage II–IV compared with (non)-smoking controls, and levels were associated with higher neutrophil numbers in bronchial biopsies of patients with COPD. MiR-223-3p expression was also increased in lungs and bronchoalveolar lavage of cigarette smoke (CS)-exposed mice. CS-induced neutrophil and monocyte lung infiltration was stronger in miR-223-deficient mice on acute (5 days) exposure, but attenuated on subchronic (4 wk) exposure. Additionally, miR-223 deficiency attenuated acute and subchronic CS-induced lung infiltration of dendritic cells and T lymphocytes. Finally, *in vitro* overexpression of miR-223-3p in non-COPD airway epithelial cells suppressed C-X-C motif chemokine ligand 8 (CXCL8) and granulocyte monocyte-colony stimulation factor (GM-CSF) secretion and gene expression of the proinflammatory transcription factor *TRAF6*. Importantly, this suppressive effect of miR-223-3p was compromised in COPD-derived cultures. In conclusion, we demonstrate that miR-223-3p is increased in lungs of patients with COPD and CS-exposed mice and is associated with neutrophilic inflammation. *In vivo* data indicate that miR-223 acts as negative regulator of acute CS-induced neutrophilic and monocytic inflammation. *In vitro* data suggest that miR-223-3p does so by suppressing proinflammatory airway epithelial responses, which is less effective in COPD epithelium.

COPD; inflammation; miRNA

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a progressive and disabling respiratory disease characterized by chronic airway inflammation, chronic bronchitis, and/or destruction of alveolar tissue (emphysema), leading to irreversible airflow limitation (1). The development of COPD is caused by chronic exposure to noxious particles and gases, of which cigarette smoke (CS) is the major risk factor. Chronic inhalation of CS damages and activates the airway epithelium, leading to secretion of cytokines, chemokines and growth factors, including C-X-C motif chemokine ligand 8 (CXCL8) and granulocyte monocyte-colony stimulation factor (GM-CSF), which attract neutrophils, monocytes, and dendritic cells (DCs) (2, 3). Activated neutrophils release neutrophil proteases, for example, neutrophil elastase and

metalloproteases, which results in destruction of alveolar tissue by degradation of extracellular matrix components (4).

In addition to exposure to environmental factors, (epi)genetic susceptibility plays a role in the pathogenesis of COPD (5). MicroRNAs (miRNAs) are highly conserved, small noncoding RNA molecules that regulate gene expression by binding to target mRNAs, leading to either mRNA degradation or suppression of protein translation (6). MiRNAs are involved in a wide variety of biological processes, including regulation of inflammatory responses (7), and have been implicated in the pathogenesis of several diseases (8).

Multiple miRNAs have been shown to be differentially expressed in lungs of patients with COPD (9–11). Ezzie et al. (11) observed that miR-223-3p is increased with the highest fold-change in lung tissue samples of smokers with COPD compared with smokers without COPD. A small *in vivo* study

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reported increased expression of miR-223 in lung tissue of mice exposed to CS for 3 wk (12). In contrast, decreased expression levels of miR-223 were found in bronchial airway epithelial cells obtained from bronchial brushings of current smokers compared with never smokers (13). Previously, we showed that levels of miR-223-3p are increased in sputum of patients with severe neutrophilic asthma, compared with nonsmoking healthy controls, and are associated with impaired lung function and sputum neutrophilia (14). Furthermore, several studies have demonstrated that miR-223 is involved in the regulation of inflammatory processes as well as in myelopoiesis by regulating granulocyte differentiation and activation (15). MiR-223 is mainly expressed in hematopoietic cells and was shown to colocalize with neutrophil elastase positive cells in the lamina propria in bronchial biopsies (14, 16). Importantly, miR-223 can transfer to other cell types, including epithelial cells, via the secretion of neutrophil- and macrophage-derived microvesicles (17, 18).

We hypothesized that miR-223-3p regulates CS-induced pulmonary inflammation and contributes to the pathogenesis of COPD. Therefore, we first measured the expression of miR-223-3p in lung tissue in two independent cohorts ($n = 92$ and $n = 89$) of never smokers and smokers with and without COPD, as well as in lung tissue and bronchoalveolar lavage of mice exposed to CS. Next, to assess the functional role of miR-223 in pulmonary inflammation, we exposed wild-type and miR-223-deficient mice *in vivo* to CS for 5 days and 4 wk and quantified different inflammatory cell types, cytokines and chemokines in bronchoalveolar lavage fluid (BAL), and lung tissue. Finally, to gain further insight into the functional role of miR-223 and the potential relevance of transfer of miR-223 to the airway epithelium, we studied whether *in vitro* overexpression of miR-223 had an effect on proinflammatory responses in airway epithelial cells derived from patients with and without COPD.

MATERIALS AND METHODS

Human Subjects and Study Design of the Cohorts

MiR-223-3p expression was measured by qPCR in lung tissue samples from patients with COPD and non-COPD control subjects from two independent study cohorts. The screening cohort ($n = 92$) consisted of 14 never-smokers, 38 smokers without airflow limitation, 20 patients with GOLD stage II COPD, and 20 patients with GOLD stage IV COPD, obtained at the University Medical Center Groningen (UMCG). Samples were collected according the research code of the UMCG and the national ethical and professional guidelines on the use of human body material (19). The validation cohort ($n = 89$) consisted of 16 never-smokers, 26 smokers without airflow limitation, 33 patients with GOLD stage II COPD, and 14 patients with GOLD stage III–IV COPD, obtained at Ghent University Hospital and University Hospital Gasthuisberg, Leuven. Sample collection was approved by the medical ethics committee of the Ghent University Hospital (2016/0132) and the University Hospital Gasthuisberg, Leuven (S51577). All subjects in the validation cohort provided written informed consent. Lung tissue samples from patients with GOLD stage II COPD and non-COPD control subjects with normal lung

function were derived after tumor resection surgery, taken as far as possible from the tumor and controlled for abnormalities by an experienced pathologist. Lung tissue from patients with GOLD stage III–IV COPD was obtained after lung transplantation. All subject characteristics are shown in Table 1.

Correlation analysis between miR-223-3p expression and inflammatory cells was performed in bronchial biopsies from 63 patients with COPD participating in the Groningen Leiden Universities Corticosteroids in Obstructive Lung Disease (GLUCOLD) study. The inclusion criteria, study design, and inflammatory cell counts have previously been described (20).

Primary Human Airway Epithelial Cell Culture and Treatment

Airway epithelial cells (AECs) were isolated by enzymatic treatment from tracheobronchial tissue of six transplanted patients with severe COPD and 11 control donors as described previously (21). The patients with COPD had an average age of 59, a smoking history of 30 pack-years and a forced expiratory volume in 1 s (FEV₁; % predicted) of 20% (a full list of characteristics is provided in Table 2). No information was available on the control donors. The study protocol was according the research code of the UMCG (19). AECs were cultured as described previously in bronchial epithelial cell growth medium (BEGM; Lonza, Walkersville, MD) and used in *passage 3* (22).

For experiments, AECs were seeded in 24-wells plate at 5×10^4 cells/well in 500- μ L BEGM. After the cells reached $\sim 70\%$ confluence, they were transfected with 1 nM pre-miRNA-precursor miR-223-3p (Ambion) or 1 nM mimic control (Qiagen) using RNAimax (Invitrogen) in Gibco Opti-MEM (Thermo Fisher Scientific) for 4 h. Subsequently, the transfection reagent was removed and cells were incubated for another 16 h until $\sim 90\%$ confluence in hormone/growth factor-deprived medium. Thereafter, cells were stimulated with 20% CS extract (CSE). The optimal concentration of CSE was selected based on previous experiments, showing effects on proinflammatory responses without affecting cell viability (23). After 6 h, cells were collected for RNA isolation and after 24 h, cell-free supernatants were collected for ELISA.

Preparation of Cigarette Smoke Extract

CSE was prepared as described previously (24). In short, Kentucky 2R4F research cigarettes (The Tobacco Research Institute, University of Kentucky, Lexington, KY) were used without filter. The smoke of two cigarettes was bubbled through 25-mL bronchial epithelial cell basal medium. This was considered as 100% CSE. The extract was prepared freshly and used within 15 min.

Mice

B6.Cg-Ptprc^a Mir223^{tm1Fcam}/J [miR-223 knockout (KO), for miR-223-3p and miR-223-5p] and B6.SJL-Ptprc^a Pepc^b/BoyJ [wild-type (WT) control] were obtained from Jackson Laboratories (Bar Harbor, ME). Male mice were used for all the experiments. The local ethics committee for animal experimentation of the faculty of Medicine and Health Sciences (Ghent University, Ghent, Belgium, ECD19-89) approved the experimental protocol.

Table 1. Characteristics of screening and validation cohort

	Screening Cohort				Validation Cohort			
	Never smokers	Smokers	COPD GOLD II	COPD GOLD IV	Never Smokers	Smokers	COPD GOLD II	COPD GOLD III-IV
<i>n</i>	14	38	19	21	16	26	33	14
Age, yr	63 [53–72]	62 [56–68]	69 [58–71]	55 [52–58]§	64 [56–70]	63 [55–70]	65 [58–68]	56 [54–60]§
Sex (male/female)*	6/8	17/21	14/5	7/14§	6/10	19/7+	30/3+	8/6§
Smoking status (current/ex-smokers)*		18/20+	5/14	0/21‡§		16/10+	22/11‡	0/14‡§
Pack-years	0	33 [20–50]†	42 [29–51]†	30 [25–31]†	0 [0–0]	28 [15–44]†	45 [40–60]†‡	30 [25–30]†§
FEV ₁ , % predicted	102 [91–116]	89 [76–103]	60 [57–69]†‡	17 [14–25]†‡	97 [92–107]	95 [93–111]	69 [61–75]†‡	26 [21–32]†‡§
FEV ₁ /FVC	78 [73–83]	75 [72–79]	53 [45–63]†‡	28 [25–42]†‡	78 [75–82]	75 [71–79]	56 [54–60]†‡	32 [28–34]†‡§
DL _{CO} , % predicted	NA	NA	NA	NA	98 [84–105]	80 [65–99]	70 [52–86]†	35 [33–40]†‡§
K _{CO} , % predicted	NA	NA	NA	NA	111 [93–123]	91 [73–106]	90 [63–107]†	59 [51–63]†‡§
ICS (yes/no/NA)*	0/14/0	0/38/0	5/9/5†‡	20/0/1†‡§	1/15/0	0/26/0	15/18/0†‡	13/1/0†‡§

Values are median with interquartile range. Mann–Whitney *U* test was performed, unless otherwise described. *Fisher’s exact test was performed: †*P* < 0.05 vs. never-smokers, ‡*P* < 0.05 vs. smokers, §*P* < 0.05 vs. COPD GOLD II. COPD, chronic obstructive pulmonary disease; DL_{CO}, diffusing capacity for carbon monoxide; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, inhaled corticosteroids; K_{CO}, ratio of DL_{CO} to alveolar volume; NA, not available.

Murine Cigarette Smoke Exposure Model

miR-223 KO and WT control mice (*n* = 8–10, 8–11 wk old) were exposed whole body to the tobacco smoke of five cigarettes (References Cigarette 3R4F without filter, University of Kentucky, Lexington, KY), four times a day with 30 min smoke-free intervals (25). The mice were exposed 5 days (acute exposure) or 5 days per week for 4 wk (subchronic exposure), which is known to induce inflammation but no airway remodeling or emphysema (25). The control groups were exposed to ambient air. The 4 wk CS-exposure model was performed twice. The results were similar, and therefore only one experiment was shown.

Collection of Blood, Bronchoalveolar Lavage, and Lung Tissue

Blood was drawn by retro-orbital bleeding, collected in EDTA-coated tubes, and subjected to red blood cell lysis. BAL was collected and cells were counted as described previously (25) and in the Supplemental Data (all Supplemental Material is available at <https://doi.org/10.6084/m9.figshare.14754132.v1>). The small lobe of the right lung was used for RNA extraction. The major lobe of the right lung was used for preparation of single-cell suspensions. The lobe was minced, digested, and subjected to red blood cell lysis, and cells were counted using a Z2 particle counter (Beckman-Coulter, Fullerton, CA) (26).

Table 2. Patients characteristics of COPD-derived AECs

	Patients with COPD (<i>n</i> = 6)
Age, yr	59 [54–61]
Sex (male/female)	2/4
Smoking status (current/ex-smokers)	1/5
Pack years	30 [11–39]
FEV ₁ , % predicted	20 [15–22]
FEV ₁ /FVC	23 [21–29]
ICS (yes/no/NA)	3/0/3

Values are median with interquartile range. AECs, airway epithelial cells; COPD, chronic obstructive pulmonary disease; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, inhaled corticosteroids; NA, not available.

Flow Cytometric Analysis of Blood, BAL, and Lung Cells

Cells from blood, BAL, and lung tissue digests were stained with a combination of anti-mouse fluorochrome-conjugated mAbs to quantify inflammatory cells using flow cytometry as described in the Supplemental Material.

RNA Extractions

Total RNA from the human lung tissue samples of the screening cohort and primary human AECs was isolated by the TRIzol reagent method (Molecular Research Center, Cincinnati, OH) according to manufacturer’s guidelines. RNA from human lung tissue samples of the validation cohort and murine lung tissue samples as well as BAL cells and cell-free BAL supernatant was isolated using the miRNeasy mini kit (Qiagen, Hilden, Germany).

Quantification of miRNA and mRNA Expression

Human miR-223-3p expression in lung tissue samples and primary human AECs and murine miR-223-3p expression in lung tissue, BAL cells, and BAL cell-free supernatant was analyzed with qPCR. Messenger RNA (mRNA) expression of cytokines and chemokines [CXCL8, colony stimulation factor 2 (CSF2), tumor necrosis factor receptor associated factor 6 (TRAF), and murine *Cxcl1*, *Ccl2*, *Csf2*, *Ccl20*] was also analyzed with qPCR and related to the expression of housekeeping genes *B2M* and *PPIA*. Taqman PCR was performed as described in the Supplemental Material.

Protein Measurements

Murine CXCL1, CCL2, GM-CSF, and neutrophil elastase were measured in cell-free BAL supernatant, and human CXCL8 and GM-CSF were measured in cell-free supernatant from AECs cultures using ELISA kits (R&D Systems, Abingdon, UK) according to manufacturer’s protocol.

Statistical Analysis

The Kruskal–Wallis test, followed by Mann–Whitney *U* tests, was used to determine significant differences between the different human subject groups and between the different experimental groups of the murine cigarette smoke model. Linear regression analysis was used to determine the

impact of age, sex, smoking status, and inhaled corticosteroids (ICS) treatment on miR-223-3p expression. Wilcoxon signed-rank test was used for paired comparisons between different conditions within the *in vitro* experiments. The Spearman's rank correlation coefficient was used to determine the association between miR-223-3p expression and lung function parameters [FEV₁, FEV₁/forced vital capacity (FVC), diffusing capacity for carbon monoxide (DL_{CO}), and ratio of DL_{CO} to alveolar volume (K_{CO})] and neutrophils in tissue. Statistical analyses were performed with SPSS (version 26.0; IBM, Armonk, NY) and GraphPad Prism 7.04 Software (GraphPad Software Inc, La Jolla, CA). *P* values <0.05 were considered as significant.

RESULTS

Characteristics of the Human Study Cohorts

Table 1 shows the subject characteristics of the screening (*n* = 92) and validation (*n* = 89) cohorts. In both cohorts, patients with COPD had a significantly lower forced expiratory volume in 1 s (FEV₁) % predicted and FEV₁/forced vital capacity (FVC) ratio and used significantly more often ICS compared with never-smokers and smokers without airflow limitation. Furthermore, patients with GOLD stage III–IV COPD were significantly younger and included less males compared with patients with GOLD stage II COPD.

Expression of miR-223-3p Is Increased in Lung Tissue of Patients with COPD

We investigated miR-223-3p expression in lung tissue of patients with COPD and non-COPD controls and correlated the pulmonary miR-223-3p expression with lung function parameters of airway obstruction and diffusing capacity. In the screening cohort, we detected significantly higher miR-223-3p expression in lung tissue of patients with GOLD stage II COPD as well as GOLD stage IV compared with never-smokers and smokers without airflow limitation (Fig. 1A). In the validation cohort, we confirmed the significantly higher expression of miR-223-3p in lung tissue of patients with GOLD III–IV COPD but not in patients with GOLD II COPD (Fig. 1B). Additional linear regression analysis demonstrated that the increased expression of miR-223-3p in lungs of patients with COPD persisted after adjusting for age, sex, and smoking status. In the screening cohort, ICS treatment had a negative effect on miR-223-3p expression (Supplemental Table S1).

In both cohorts, we observed that higher levels of miR-223-3p were associated with more severe airflow limitation (FEV₁% predicted and FEV₁/FVC, Fig. 1, C–F). In addition, higher miR-223-3p expression in the validation cohort correlated with lower diffusing capacity for carbon monoxide (DL_{CO}; Fig. 1G).

MiR-223-3p Expression in Bronchial Biopsies of Patients with COPD Correlates Significantly with Neutrophil Counts

We have previously shown that the increased miR-223-3p expression in sputum supernatant of severe asthmatics was associated with sputum neutrophilia (14). Using data from the Groningen Leiden Universities Corticosteroids in Obstruc-

tive Lung Disease (GLUCOLD) study (20), we investigated whether miR-223-3p expression is associated with neutrophilic inflammation in COPD. Interestingly, also in bronchial biopsies from patients with COPD from the GLUCOLD study, we observed a positive correlation between miR-223-3p expression and neutrophil counts (Fig. 1I).

MiR-223-3p Expression Is Increased in Lung Tissue and Bronchoalveolar Lavage of Mice Exposed to CS

To determine the effect of CS exposure on pulmonary miR-223-3p expression, we analyzed its expression in lung tissue and BAL cells of mice exposed to air or CS for 5 days and 4 wk. In lung tissue, miR-223-3p expression was not altered on 5 days of CS exposure but was significantly increased on 4 wk of CS exposure compared with air-exposed controls (Fig. 2A). In BAL cells, miR-223-3p expression was already significantly increased on 5 days of CS exposure (Fig. 2B), and its levels correlated positively with the neutrophil counts (Fig. 2C). Finally, to determine whether miR-223-3p is released into the BAL, we assessed its levels in cell-free BAL supernatant and observed that CS exposure increased the expression of miR-223-3p in 4-wk CS-exposed mice, compared with air-exposed controls (Fig. 2D).

Nuclear Hypersegmentation in Neutrophils Derived from miR-223-Deficient Mice

It was previously described that peripheral blood neutrophils from miR-223-deficient mice display hypersegmented nuclei, indicating a hypermature morphology (27). We made similar observations in peripheral blood-derived neutrophils from miR-223 knockout (KO) mice, both at baseline and after CS-exposure (data not shown). Interestingly, in our study, we also observed that BAL neutrophils from miR-223 KO mice have this unusual morphology with hypersegmented nuclei, which was not observed in neutrophils derived from wild type (WT) mice (Fig. 2E).

Effect of miR-223 Deficiency on CS-Induced Pulmonary Inflammation

To investigate the role of increased miR-223-3p in the regulation of CS-induced pulmonary inflammation, we compared the effects of CS exposure in miR-223 KO mice and WT mice. Efficient knockout of miR-223 was confirmed by the absence of detectable levels of miR-223-3p in lung tissue (data not shown).

In BAL, exposure to CS for 5 days increased the numbers of neutrophils, monocytes, DCs, and CD4⁺ and CD8⁺ T lymphocytes compared with air-exposed control mice (Fig. 3, A–H). Exposure to CS for 4 wk increased the numbers of total BAL cells, macrophages, neutrophils, eosinophils, monocytes, DCs, and CD4⁺ and CD8⁺ T lymphocytes compared with air-exposed control mice (Fig. 3, A–H). Interestingly, on 5-day exposure, the CS-induced increase in number of neutrophils and monocytes was significantly higher in miR-223 KO mice compared with WT mice (Fig. 3, C and E). In contrast, on 4-wk exposure, the CS-induced increases in numbers of macrophages and monocytes were significantly attenuated in miR-223 KO mice, whereas no difference in CS-induced neutrophilia was detected (Fig. 3, B–E). In addition, compared with CS-exposed WT mice, miR-223 KO mice

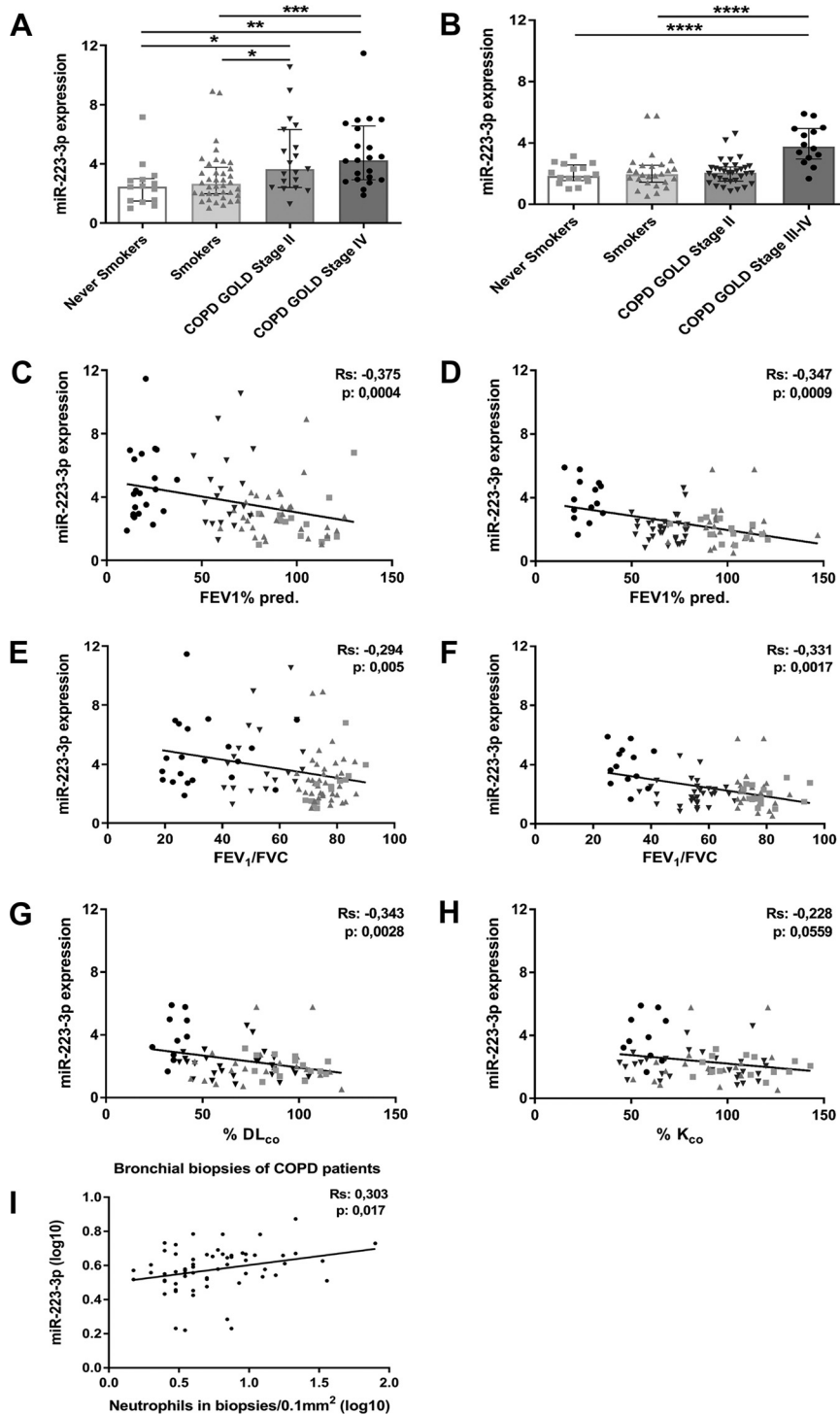
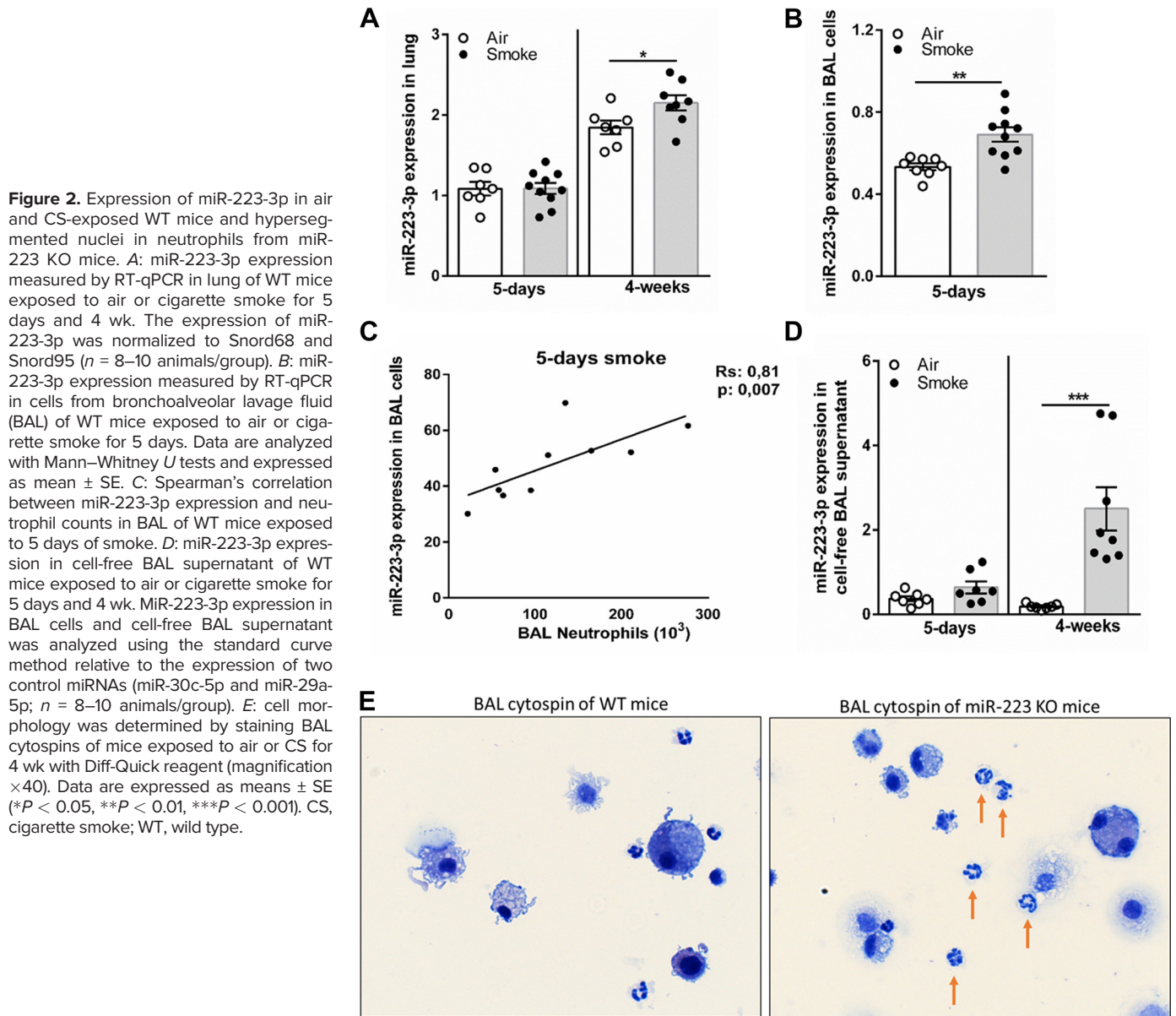


Figure 1. Expression of miR-223-3p in lung tissue of patients with COPD and non-COPD control subjects and correlation with lung function parameters and neutrophil counts. Expression of miR-223-3p in lung tissue samples of the screening cohort (A) and validation cohort (B), as determined by qRT-PCR. Data were analyzed using the delta CT method (ratio to control genes RNU44/RNU48) and Mann–Whitney *U* tests and shown as median with interquartile range. Spearman’s correlation analysis of miR-223-3p expression with the postbronchodilator values of forced expiratory volume in 1 s (FEV₁) % predicted in the screening cohort (C) and validation cohort (D), and with the FEV₁/forced vital capacity (FVC) ratio in screening cohort (E) and validation cohort (F). Spearman’s correlation of miR-223-3p expression in lung tissue of subjects from the validation cohort with carbon monoxide diffusing capacity (DL_{co}; H) and the ratio of DL_{co} to alveolar volume (K_{co}; G). I: Spearman’s correlation of miR-223-3p expression and neutrophil counts in bronchial biopsies from the GLUCOLD study; *n* = 63 subjects (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 between the indicated values). COPD, chronic obstructive pulmonary disease; GLUCOLD, Groningen Leiden Universities Corticosteroids in Obstructive Lung Disease.

showed an attenuated increase in numbers of DCs on 5-day and 4-wk CS exposure, as well as an attenuated increase in numbers of CD4⁺ and CD8⁺ T lymphocytes on 4-wk CS exposure (Fig. 3, F–H). Investigating the percentages of inflammatory cell types in BAL revealed that at baseline, BAL consisted of ~99% of macrophages for both WT and miR-223 KO mice. After 5-day and 4-wk CS exposure, significantly higher percentages of neutrophils and lower percentages of

macrophages were observed in miR-223 KO mice compared with WT mice, indicating that CS exposure induces predominantly an increase in neutrophilic inflammation in miR-223 KO mice compared with WT mice (Fig. 3I).

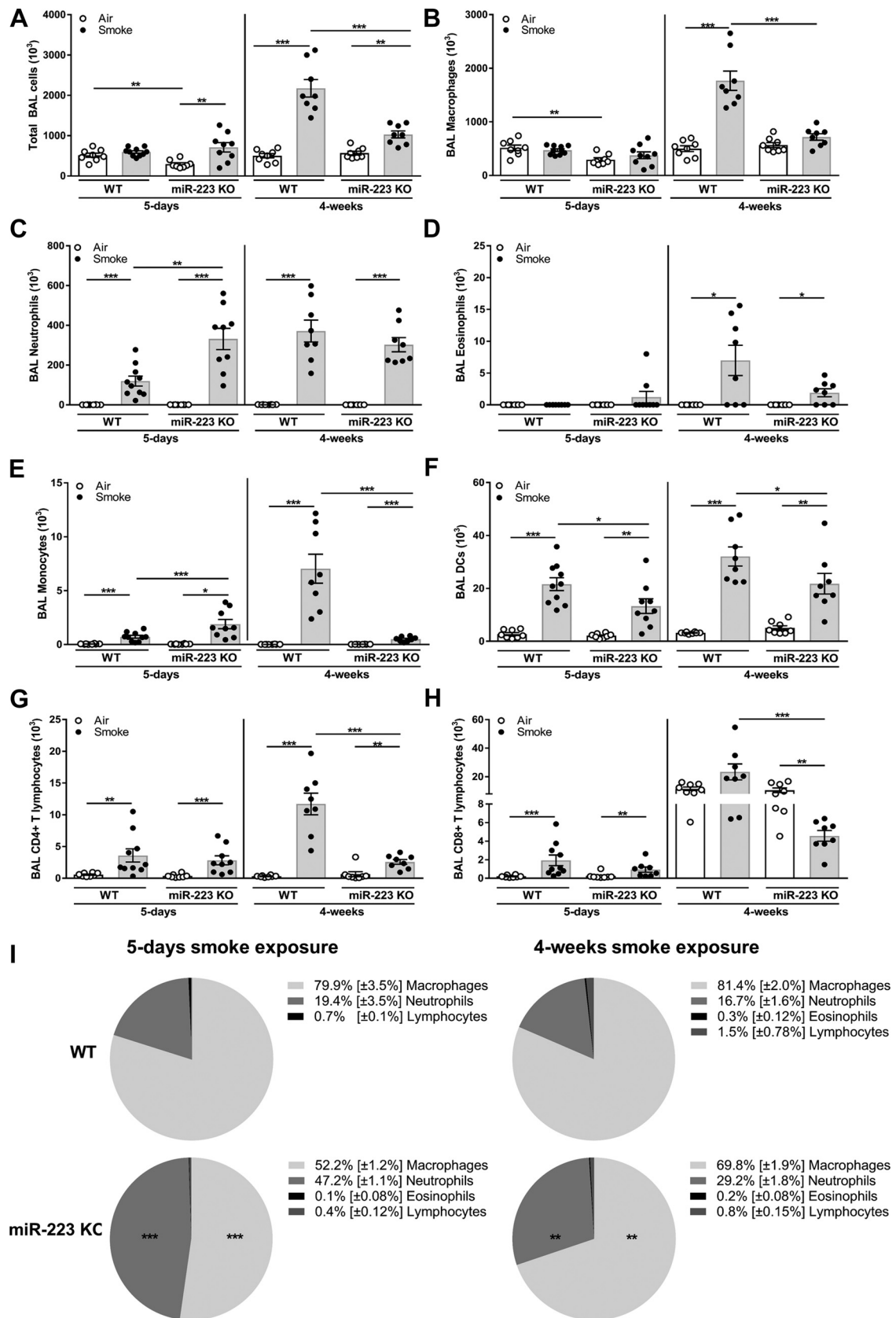
In lung tissue, we only assessed effects of 4-wk CS exposure and observed an increased percentage of monocytes, eosinophils, DCs, and CD4⁺ and CD8⁺ T lymphocytes compared with air-exposed mice. Similar to BAL, the CS-



induced increase in the percentage of CD4⁺ T lymphocytes was significantly attenuated in miR-223 KO mice compared with WT controls, whereas the percentage of neutrophils did not significantly change (Supplemental Fig. S1). Furthermore, on hematoxylin and eosin staining of lung tissue, we did not observe structural differences between WT and KO mice, at baseline or after CS exposure (Supplemental Fig. S2).

Next, we determined whether the effect of miR-223 deficiency on CS-induced inflammation is also reflected in the levels of chemokines and cytokines attracting neutrophils, monocytes, and DCs. Therefore, we measured mRNA expression in lung tissue and protein levels in BAL supernatant of CXCL1 (the homologue of human CXCL8), CCL2, survival factor GM-CSF, and CCL20 in miR-223 KO and WT mice. Both 5-day and 4-wk exposure to CS significantly increased the lung mRNA expression and/or BAL protein levels of CXCL1, CCL2,

GM-CSF, and CCL20 in WT mice, compared with air-exposed controls (Fig. 4, A–G). Interestingly, in line with the numbers of BAL neutrophils and monocytes, miR-223 KO showed an amplification of the CS-induced increase in CXCL1 and CCL2 on 5-day exposure, but an attenuation of the CS-induced increase of these chemokines on 4-wk exposure (Fig. 4, A, B, E, and F). For GM-CSF, expression was similar in both strains on 5-day CS exposure, but reduced levels were observed in miR-223 KO mice on 4-wk CS exposure compared with WT mice (Fig. 4, C and G). For *Ccl20* gene expression, no differences were found between miR-223 KO mice and WT mice. In addition, we measured protein levels of neutrophil elastase (NE) in BAL supernatant as a marker of neutrophilic inflammation and again observed an amplified response on 5 days of CS exposure in miR-223 KO mice compared with WT controls (Fig. 4H). Moreover, the levels of NE in BAL supernatant were significantly associated with higher BAL



neutrophil numbers (5 days: $R_s = 0.78$ and $P \leq 0.0001$ and 4 wk: $R_s = 0.64$ and $P = 0.009$; Supplemental Fig. S3). Given the role of miR-223 in myelopoiesis and hematopoiesis, we assessed whether the contradictory results (in innate inflammation in BAL) between acute (5 day) and subchronic (4 wk) CS exposure may be due to exhaustion of the hematopoietic cell pool in miR-223 KO mice. Therefore, we investigated the white blood cell profile in young (age 9–10 wk) and elderly (age > 1 yr) miR-223 KO and WT mice (Fig. 5). We observed that in contrast to younger miR-223 KO mice, specifically in elderly mice, miR-223 deficiency resulted in lower percentage of monocytes and eosinophils, with a trend toward higher percentage of CD3⁺ T lymphocytes in peripheral blood compared with WT mice (Fig. 5, A–F). In contrast, miR-223 deficiency in young mice resulted in higher percentage of blood eosinophils. Taken together, these in vivo data demonstrate that miR-223 is a negative regulator of innate inflammation (neutrophilic and monocytic) on acute CS exposure. This effect was no longer observed (and even reversed) on subchronic CS exposure, possibly due to exhausted myelopoiesis and hematopoiesis in elderly miR-223 KO mice.

miR-223-3p Acts as an Anti-Inflammatory miRNA by Reducing CXCL8 and GM-CSF Levels in Airway Epithelial Cells

We hypothesized that the negative regulation of the acute CS-induced pulmonary inflammation by miR-223 is at least partly due to its effects on airway epithelium, after transfer of the microRNA via neutrophil- and macrophage-derived microvesicles (17, 18). Therefore, we investigated whether overexpression of miR-223-3p is able to suppress CS extract-induced proinflammatory responses in AECs derived from non-COPD controls and patients with COPD. We focused on CXCL8 (a neutrophil attracting chemokine) and the validated targets of miR-223 GM-CSF (prolonging survival of neutrophils) and TRAF6 (that promotes NF- κ B-mediated transcriptional activation of many proinflammatory genes, including CXCL8 and GM-CSF) (28–30), which are important COPD features, and higher levels of CXCL8 and GM-CSF have been observed in sputum of patients with COPD (2, 31).

Although AECs hardly expressed miR-223-3p at baseline conditions, transfection with the miR-223-3p mimic resulted in robustly increased miR-223-3p expression levels in both non-COPD and COPD-derived AECs, compared with mimic control (Fig. 6A). At baseline and after CSE stimulation, miR-223-3p overexpression significantly suppressed CXCL8 and GM-CSF secretion compared with mimic control in non-COPD-derived AECs (Fig. 6, B and C). Interestingly, in COPD-derived AECs, overexpression of miR-223-3p significantly attenuated secretion of GM-CSF at baseline and after CSE stimulation, but not of CXCL8 (Fig. 6, B and C). The protein concentrations of CXCL8 and GM-CSF are displayed in Supplemental Fig. S4. Next, we measured the mRNA

expression of CXCL8 and CSF2 (coding for GM-CSF) in non-COPD and COPD-derived AECs (Fig. 6, D and E). In line with protein secretion, overexpression of miR-223-3p significantly suppressed CSF2 expression at baseline and on CSE stimulation in both non-COPD and COPD-derived AECs, whereas expression of CXCL8 was only suppressed in non-COPD-derived AECs (Fig. 6, D and E). In addition, overexpression of miR-223-3p significantly downregulated mRNA expression of TRAF6 in non-COPD AECs but upregulated TRAF6 expression in COPD-derived AECs at baseline, with a similar trend on CSE stimulation (Fig. 6F).

Taken together, these in vitro data demonstrate that miR-223-3p negatively regulates proinflammatory responses in airway epithelium of control subjects without COPD. Interestingly, the ability of miR-223-3p to suppress CXCL8 release is less effective in airway epithelium from patients with COPD (see Fig. 6G for proposed mechanism).

DISCUSSION

In the present study, we investigated the role of miR-223-3p in the regulation of CS-induced inflammation in COPD. We detected higher levels of miR-223-3p in lung tissue of patients with COPD, as well as in lungs of CS-exposed mice. In COPD biopsies, we showed a correlation with higher infiltration of neutrophils. Mice data indicated that miR-223 is a negative regulator of innate lung inflammation on acute CS exposure, whereas the attenuation of inflammatory lung infiltration in miR-223-deficient mice on subchronic exposure may be due to exhaustion of the hematopoietic pool. Furthermore, we demonstrated (in vitro) that in AEC cultures from subjects with non-COPD, miR-223-3p attenuates proinflammatory responses by reducing GM-CSF, CXCL8, and TRAF6 levels, whereas specifically, the proposed feedback mechanism to reduce TRAF6 expression and CXCL8 production was attenuated in airway epithelium of patients with COPD (Fig. 6G). Together, these findings suggest that on acute CS exposure, neutrophilic infiltration leads to higher miR-223 levels, which act on airway epithelium in a negative feedback loop to reduce attraction of neutrophils. In airway epithelium of patients with COPD, this regulatory mechanism is defective, which may contribute to increased neutrophilic inflammation in COPD lungs.

We detected higher levels of miR-223-3p in lung tissue of patients with COPD in two independent cohorts and in lung tissue, BAL cells and cell-free BAL supernatant of CS-exposed mice, which is in line with previous studies in lung tissue (11) and BAL (32) of human subjects and in lung tissue of mice (12). Furthermore, we showed that higher miR-223-3p levels are associated with impaired lung function and with higher neutrophil counts in human bronchial biopsies and murine BAL cells. Similar correlations between miR-223-3p expression and neutrophilia were observed in sputum and serum of asthma patients and healthy controls (14, 33,

Figure 3. Effect of miR-223 deficiency on cigarette smoke (CS)-induced inflammation in bronchoalveolar lavage (BAL) in mice. Wild type (WT) and miR-223 knockout (KO) mice ($n = 8–10$ animals/group, all male mice) were exposed to air or cigarette smoke (CS) for 5 days and 4 wk. Number of total BAL cells (A), macrophages (B), neutrophils (C), and eosinophils (D) were determined on cytospin. Monocytes (E), dendritic cells (F), CD4⁺ T lymphocytes (G), and CD8⁺ T lymphocytes (H) were determined with flowcytometry. *I*: pie chart representing the percentage of inflammatory cells in BAL on cytospins. The 4-wk CS-exposure model was performed twice. The results were similar, and therefore only 1 experiment was shown. Data are analyzed with Kruskal–Wallis and Mann–Whitney *U* tests and expressed as means \pm SE ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

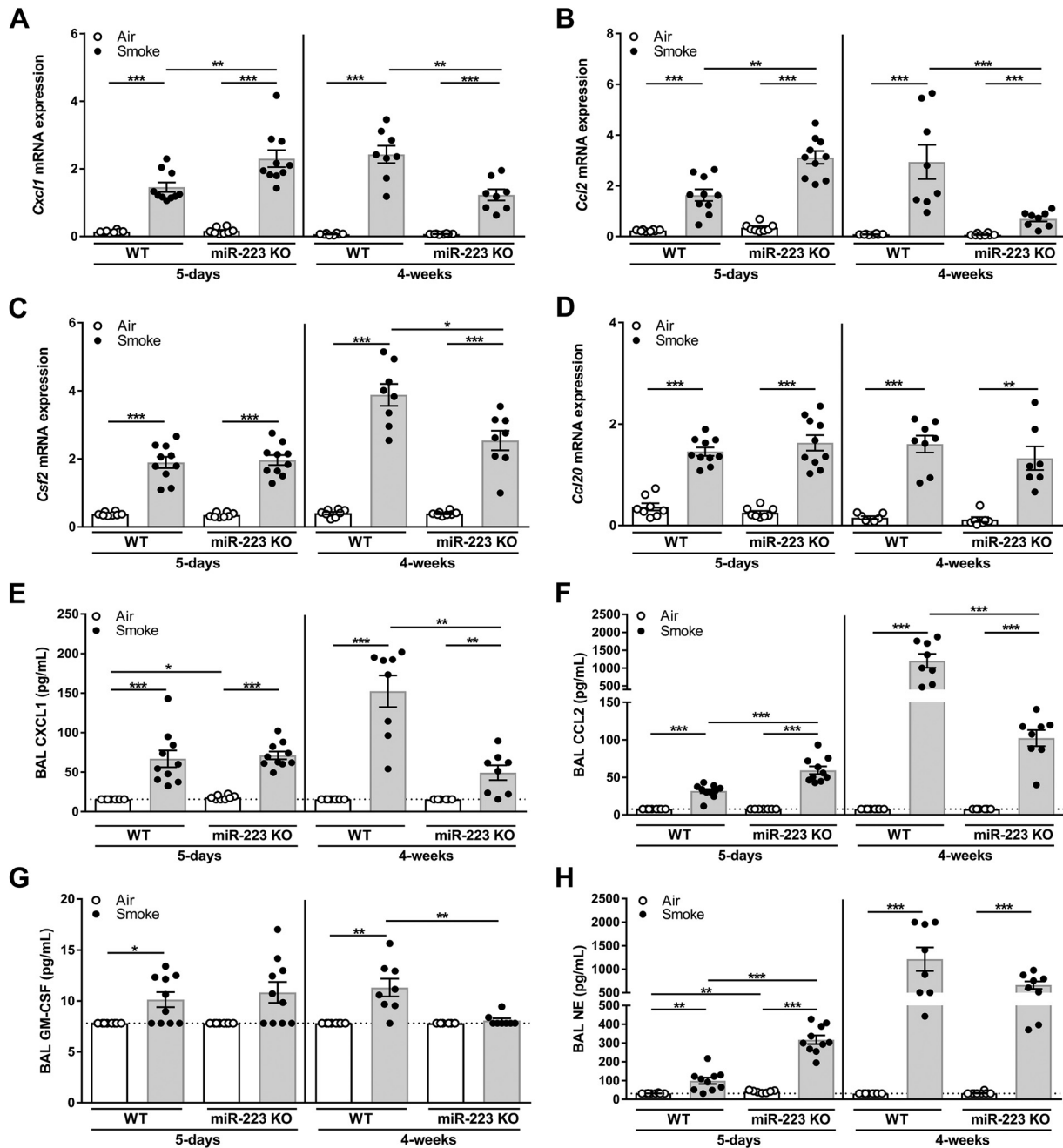


Figure 4. Effect of miR-223 deficiency on CS-induced changes in cytokine/chemokine levels in murine lung tissue and BAL supernatant. mRNA expression *Cxcl1* (A), *Ccl2* (B), *Csf2* (C), and *Ccl20* (D) measured by RT-qPCR in lung tissue of WT and miR-223 KO mice on 5-day and 4-wk exposure to air or CS ($n = 7-10$ animals/group, all male mice). Protein levels of CXCL1 (E), CCL2 (F), and neutrophil elastase (NE; H) measured by ELISA in BAL supernatant of WT and miR-223 KO mice on 5-day and 4-wk exposure to air or CS ($n = 8-10$ animals/group). Dotted line is detection limit. Data are analyzed with Kruskal–Wallis and Mann–Whitney U tests and expressed as means \pm SE (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). BAL, bronchoalveolar lavage; CS, cigarette smoke; KO, knockout; WT, wild type.

34). It will be of interest to investigate the expression levels of miR-223-3p in patients with an overlapping asthma-COPD phenotype in future studies.

Since neutrophils are known to express high levels of miR-223-3p (27, 34, 35), we suggest that the higher levels of miR-223-3p in lungs of patients with COPD and CS-exposed mice are a result of the increased infiltration of neutrophils, which may lead to more severe lung function impairment. Therefore, we conclude that miR-223 is not a

specific biomarker for COPD but rather for neutrophilic inflammation in obstructive lung diseases.

To investigate the role of miR-223 in CS-induced pulmonary inflammation, we exposed WT and miR-223-deficient mice to air or CS. On acute CS exposure, miR-223 deficiency amplified the innate inflammatory response, as evidenced by higher levels of monocytes and neutrophil chemoattractants, resulting in amplified monocyte and neutrophil infiltration and a predominantly neutrophilic inflammation.

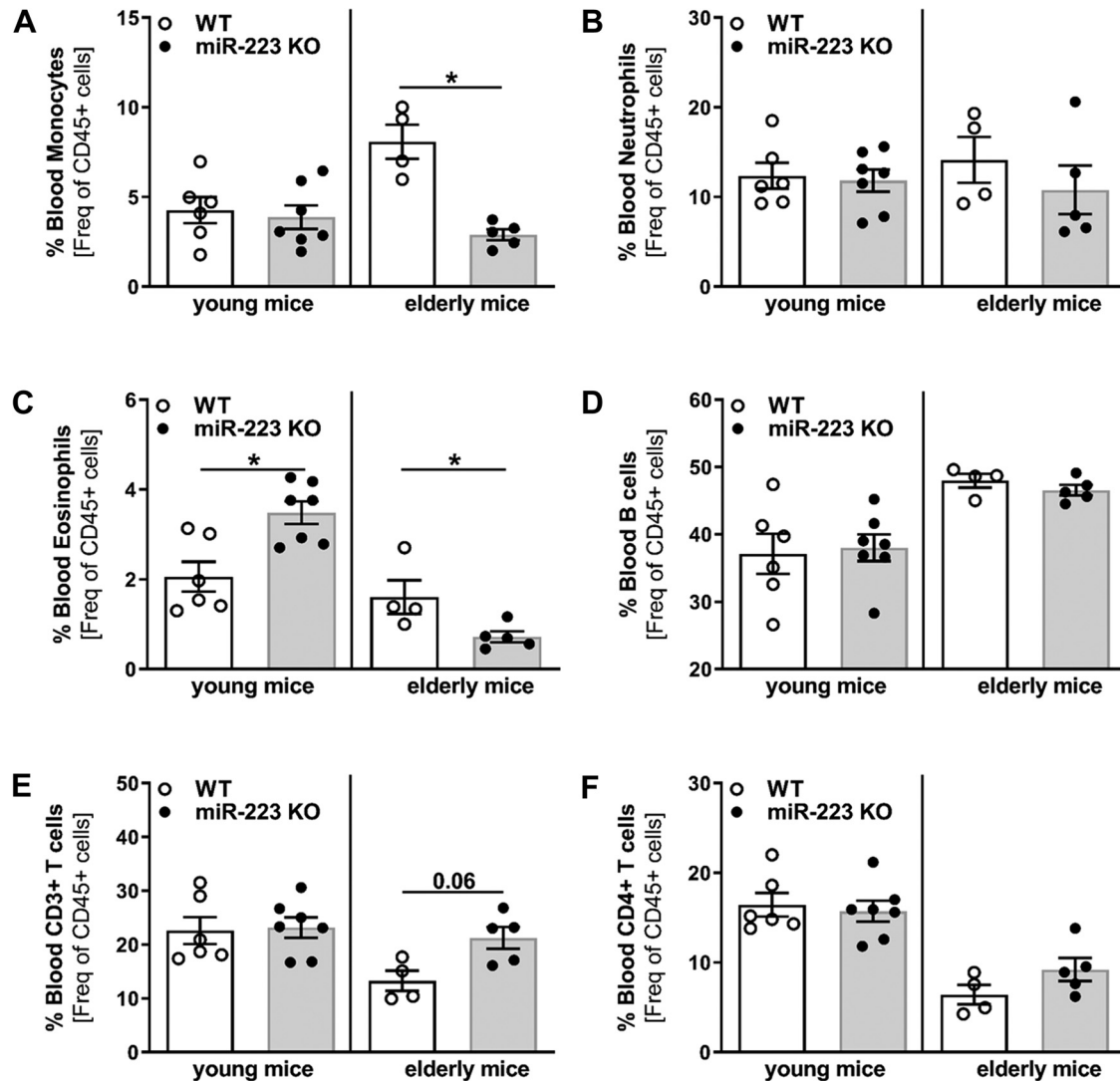


Figure 5. White blood cell profile of young and elderly miR-223 knockout (KO) mice compared with wild-type (WT) mice. Peripheral blood was isolated from young (age 9–10 wk) and elderly (age > 1 yr) WT and miR-223 KO mice. Percentage of monocytes (A), neutrophils (B), eosinophils (C), B cells (D), CD3⁺ T lymphocytes (E), and CD4⁺ T lymphocytes ($n = 4\text{--}7$ animals/group, all male mice; F). Data are analyzed with Mann–Whitney U test and expressed as means \pm SE (* $P < 0.05$).

This suppressive effect of miR-223 on innate inflammation was also observed in murine acute liver and lung injury models, where miR-223-deficient mice have highly elevated infiltration of neutrophils, monocytes, and macrophages in liver and lung, whereas overexpression of miR-223 reduced infiltration of neutrophils and macrophages (36–38). Moreover, miR-223 has been described as myeloid specific miRNA, regulating proliferation of granulocyte/macrophages progenitors and inhibiting differentiation of those progenitors into peripheral blood neutrophils. Interestingly, at baseline, miR-223-deficient mice displayed higher numbers of granulocyte/macrophages progenitors (27), whereas after acute stimulation with mitochondrial damage-associated molecular patterns, miR-223 knockout heterozygous mice displayed lower percentages of granulocyte/macrophage progenitors in bone marrow, together with higher percentages of neutrophils and monocytes in peripheral blood and BAL (37). These data suggest that miR-223-3p controls the differentiation

and activation of neutrophils and monocytes. In addition, we propose that during acute CS-induced inflammation, miR-223 acts in a negative feedback mechanism, inhibiting the production of neutrophil attractant CXCL8 and subsequent infiltration of neutrophils.

Interestingly, we observed reversed effects on subchronic CS exposure, where miR-223-deficient mice had an attenuated increase in innate inflammatory cells together with lower levels of cytokines and chemokines that not only act as chemoattractants but are also expressed by those inflammatory cells. To our knowledge, only Dorhoi et al. (39) investigated the role of miR-223 in a murine chronic lung inflammation model. They observed 80%–100% mortality in miR-223-deficient mice after chronic *Mycobacterium tuberculosis* exposure and neutrophilic infiltration, whereas depletion of neutrophils prolonged the survival of miR-223-deficient mice during induced-tuberculosis (39). Since miR-223 is involved in granulocyte proliferation and differentiation and since we observed

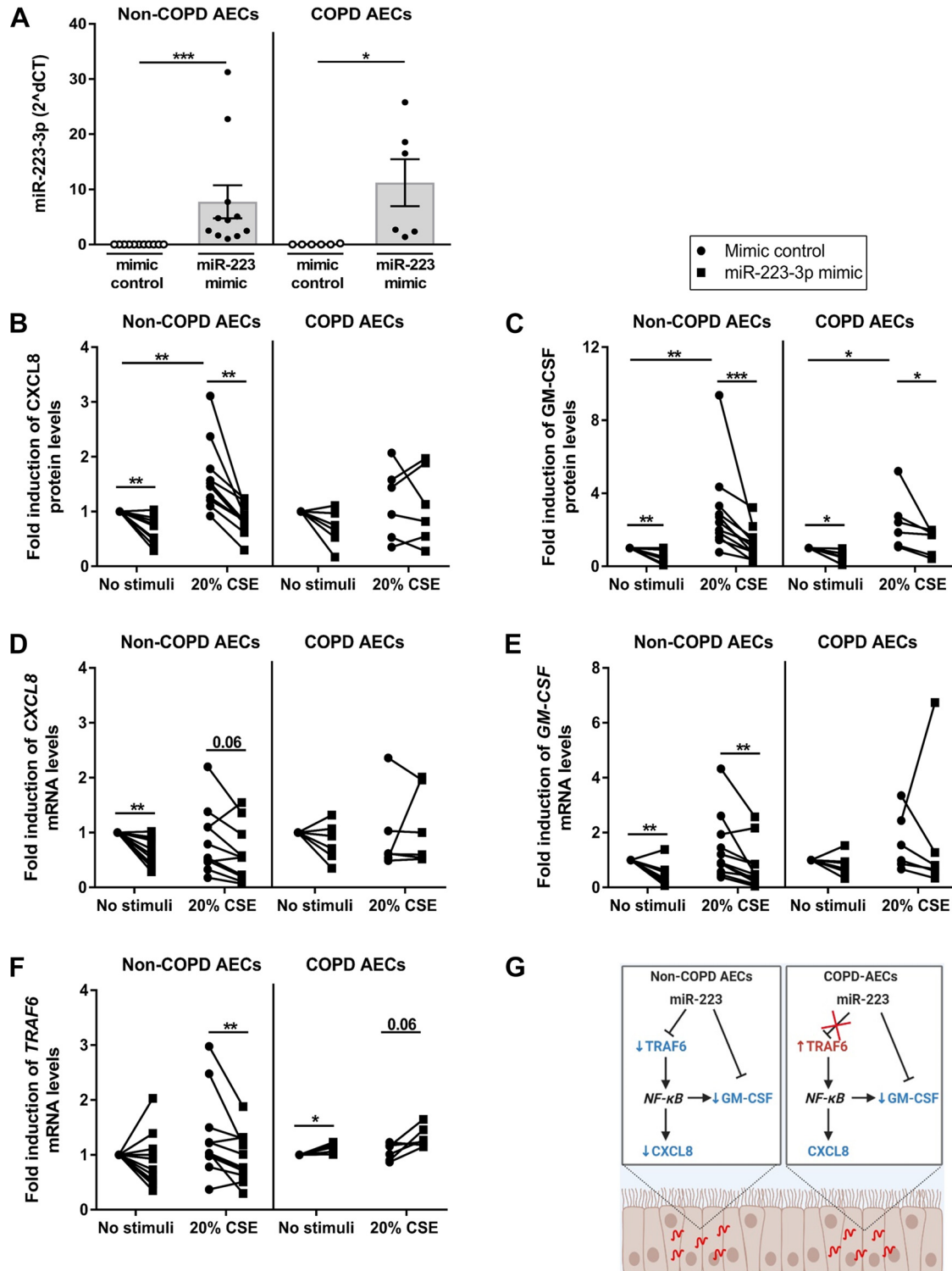


Figure 6. Effect of miR-223-3p overexpression on cytokine/chemokine levels in primary human airway epithelial cells, in vitro stimulated with cigarette smoke extract. Airway epithelial cells (AECs) derived from non-COPD controls ($n = 10-11$) or patients with COPD ($n = 6$) were seeded in duplicates, grown to $\sim 70\%$, transfected with 1 nM miR-223-3p or mimic control for 4 h, growth factor deprived overnight and stimulated with 20% cigarette smoke extract (CSE) for 6 and 24 h. RNA was isolated and cell-free supernatants were collected. **A:** miR-223-3p expression was assessed and related to RNU48. The miRNA levels were expressed as means \pm SE ($n = 6-11$). Protein levels in culture supernatant of CXCL8 (**B**) and GM-CSF (**C**), 24 h after CSE stimulation as measured by ELISA and mRNA expression of *Cxcl8* (**D**), *Csf2* (**E**), and *Traf6* (**F**), 6 h after CSE stimulation as measured by RT-qPCR. **G:** graphical representation of the proposed role of miR-223-3p in AECs-derived from patients with non-COPD and patients with COPD. Data were analyzed with Wilcoxon test and (**B-F**) assessed as fold induction relative to mimic control no stimuli of each donor (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). COPD, chronic obstructive pulmonary disease. [Created with BioRender.com.]

that 1-yr-old miR-223-deficient mice had lower numbers of peripheral monocytes and eosinophils, we propose that over time, the enhanced neutrophil differentiation in these constitutive miR-223-deficient mice may lead to an exhausted hematopoietic cell pool. This may result in less monocytic and granulocytic infiltration on subchronic CS exposure and explain the opposing outcomes of acute versus subchronic CS exposure.

Mice deficient for miR-223 also showed an attenuated adaptive immune response on both acute and subchronic CS exposure, with lower numbers of DCs and CD4⁺ and CD8⁺ T lymphocytes. However, we did not observe differences in the CS-induced increase in expression of CCL20, a chemoattractant for immature DCs and lymphocytes (40), in lung tissue of miR-223-deficient mice compared with WT controls. Although little is known regarding the underlining mechanism of miR-223 in adaptive immune responses, Satoorian et al. (41) described that miR-223 is involved in DC activation and maturation as well as in naive T-cell differentiation.

We propose that miR-223 may contribute to the regulation of proinflammatory responses by acting on airway epithelial cells to suppress the production of neutrophil attractant CXCL8 and GM-CSF. Airway epithelium barely expresses miR-223-3p; however, previous studies described that macrophages and neutrophils can secrete microvesicles that contain high levels of miR-223, which can subsequently be taken up by target cells, including airway epithelial cells (17, 18). Our data support the notion that inflammatory cells secrete miR-223-3p, as we observed substantial levels of miR-223-3p in cell-free BAL supernatant, which increased on CS exposure. As neutrophils highly express miR-223-3p, we suggest that CS-induced infiltration of neutrophils leads to higher secretion of miR-223-3p in microvesicles, which on uptake by airway epithelial cells induces a negative feedback mechanism. Indeed, our *in vitro* findings confirm that overexpression of miR-223-3p in AECs leads to suppression of CXCL8 and GM-CSF, which may further explain the increase in neutrophil inflammation in the miR-223 KO cells on CS. This may be mediated by the reduction of TRAF6, a validated target gene of miR-223-3p that is known to be involved in transcriptional activation of many proinflammatory genes including CXCL8 and GM-CSF (30). In AECs from non-COPD donors, this overexpression of miR-223-3p reduced the mRNA and protein levels of CXCL8 and the miR-223-3p targets GM-CSF and TRAF6. This suppressive effect of miR-223-3p on expression and secretion of CXCL8 and GM-CSF, essential for, respectively, attracting and prolonging survival of neutrophils, is in line with our *in vivo* observations on acute CS exposure and with previous studies that suggest that miR-223-3p suppresses inflammatory responses (18, 42, 43). Interestingly, this suppressive effect of miR-223-3p was compromised in AECs from COPD donors, especially for TRAF6 expression, leading to an increase instead of decrease expression of TRAF6. Therefore, we propose that the direct regulation of GM-CSF by miR-223, suppressing the translation of GM-CSF at protein level, may not be impaired in COPD, only the TRAF6-dependent expression, which prevents the reduction of CXCL8 and GM-CSF mRNA levels (proposed mechanism in Fig. 6G). There are various possible explanations why miR-223-3p acts differently in normal versus diseased cells, including differences in miRNA binding sites and sensitivity of target genes (44, 45). It will be interesting in future experiments to

investigate the binding of miR-223 to TRAF6 in AECs from control and patients with COPD using a luciferase reporter assay and to investigate whether single nucleotide polymorphisms are involved in the reduced binding of miR-223 to TRAF6. Our findings suggest that airway epithelium in COPD is less sensitive to this negative feedback regulation by miR-223-3p, which may lead to higher neutrophilic infiltration as observed in patients with COPD on cigarette smoking.

There are also limitations in this study that should be mentioned. The lung tissue of never-smokers, smokers, and patients with GOLD stage II COPD from the validation cohort were obtained in Ghent University Hospital, whereas patients with GOLD stage III–IV COPD were obtained in Catholic University Leuven. Furthermore, there are no clinical characteristics available of the non-COPD donors for epithelial cultures, except that those participants had a normal lung function. Another limitation of this study is that 5-day and 4-wk exposure to CS are too short to induce structural changes to the lung. Therefore, it was not possible to investigate the role of miR-223 in remodeling or emphysema, which are important COPD features in addition to the inflammation that we focused on here.

In conclusion, we demonstrate that miR-223-3p is increased in lung tissue of patients with COPD. Data from *in vivo* and *in vitro* experiments indicate that this increase in miR-223-3p acts in a negative feedback mechanism suppressing airway epithelial production of proinflammatory cytokines to counteract CS-induced innate inflammatory responses in subjects without COPD. Importantly, we demonstrate that this miR-223-3p feedback mechanism is impaired in airway epithelium of patients with COPD.

SUPPLEMENTAL DATA

Supplemental Methods, Supplemental Table S1, and Supplemental Figs. S1–S4: <https://doi.org/10.6084/m9.figshare.14754132.v1>.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

M.P.R., T.M., I.H.H., and K.R.B. conceived and designed research; M.P.R. performed experiments; M.P.R. analyzed data; M.P.R., T.M., C.-A.B., M.v.d.B., B.M.V., G.F.J., G.G.B., I.H.H., and K.R.B. interpreted results of experiments; M.P.R. prepared figures; M.P.R. and K.R.B. drafted manuscript; M.P.R., T.M., C.-A.B., M.v.d.B., B.M.V., G.F.J., G.G.B., I.H.H., and K.R.B. edited and revised manuscript; M.P.R., T.M., C.-A.B., M.v.d.B., B.M.V., G.F.J., G.G.B., I.H.H., and K.R.B. approved final version of manuscript.

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