

Correlation between CCL26 production by human bronchial epithelial cells and airway eosinophils: Involvement in patients with severe eosinophilic asthma

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Background: High pulmonary eosinophil counts are associated with asthma symptoms and severity. Bronchial epithelial cells (BECs) produce CC chemokines, notably CCL26 (eotaxin-3), which recruits and activates eosinophils from asthmatic patients. This suggests that CCL26 production by BECs might be involved in persistent eosinophilia in patients with severe asthma despite treatment with high corticosteroid doses. **Objective:** We sought to determine whether CCL26 levels correlate with eosinophilia and asthma severity. **Methods:** Human CC chemokine expression was assessed by means of quantitative PCR or a quantitative PCR array in vehicle- or IL-13–treated BECs. CCL26 was quantitated by means of ELISA. Immunohistochemistry analyses of CCL26 and major basic protein were done on bronchial biopsy specimens. **Results:** IL-13 selectively induced CCL26 expression by BECs. This increase was time-dependent and more prominent in BECs from patients with severe eosinophilic asthma. CCL26 levels measured in supernatants of IL-13–stimulated BECs also increased with asthma severity as follows: patients with severe eosinophilic asthma > patients with mild asthma ≈ healthy subjects. Immunohistochemistry analyses of bronchial biopsy specimens confirmed increased levels of CCL26 in the epithelium of patients with mild and those with severe eosinophilic asthma. Tissue eosinophil counts did not correlate with CCL26 staining. However, sputum CCL26 levels significantly correlated with sputum eosinophil counts ($P < .0001$), suggesting that CCL26 participates in the movement of eosinophils from the tissues to the airway lumen. **Conclusions:** These results show a relation between CCL26 production by IL-13–stimulated BECs, sputum eosinophil

counts, and asthma severity. They also suggest a role for CCL26 in the sustained inflammation observed in patients with severe eosinophilic asthma and reveal CCL26 as a potential target for treating patients with eosinophilic asthma that are refractory to classic therapies. (*J Allergy Clin Immunol* 2015;■■■:■■■-■■■.)

Key words: Asthma, bronchial epithelial cells, chemokine, eotaxin, CCL26, IL-13, bronchial mucosa, inflammation, severe asthma

The recruitment of eosinophils to the airways is a recognized feature of asthma and increases with disease intensity and severity.¹⁻⁵ In most asthmatic patients, corticosteroids decrease eosinophil counts and control asthma symptoms. However, some patients with severe asthma have persistent increased sputum eosinophil counts and remain symptomatic despite being treated with high-dose corticosteroids.^{6,7} This stresses the need to decipher the mechanisms of eosinophil recruitment to the airways to develop novel pharmaceutical tools that would effectively block this recruitment.

Bronchial and inflammatory cells can release eosinophil chemotactic factors, notably CC chemokines.⁸⁻¹⁰ Among them, CCL11 (eotaxin-1), CCL24 (eotaxin-2), and CCL26 (eotaxin-3) chiefly recruit and activate eosinophils.¹¹⁻²² Although they induce a similar migration rate of freshly isolated eosinophils from healthy donors, their effects on eosinophil counts from patients with mild asthma differ, with CCL26 being more effective than CCL11 and CCL24.²³ Increased CCL26 levels are found in patients with eosinophilic diseases, such as atopic dermatitis, chronic rhinosinusitis, eosinophilic esophagitis, and Churg-Strauss vasculitis, suggesting a primordial role for CCL26 in the pathogenesis of eosinophilic diseases.²⁴⁻³⁰ Increased CCL24 and CCL26 mRNA levels were recently documented in airway epithelial brushings of asthmatic patients.³¹ This was associated with lower lung function, increased sputum eosinophil counts, and asthma exacerbation events. Given that IL-13 induces production of CCL26 by bronchial epithelial cells (BECs)³² and that IL-13 levels in the bronchial mucosa and sputum of patients with severe asthma are increased,³³ we postulated that IL-13 was promoting the release of eotaxins by human BECs.

Consequently, we investigated the effect of IL-13 on CC chemokine expression by BECs obtained from healthy subjects and from patients with mild or severe eosinophilic asthma. We also assessed whether CCL26 levels were increased in the bronchial tissue and airway lumen of asthmatic patients.

METHODS

Reagents

Human recombinant IL-13 was purchased from PeproTech (Rocky Hill, NJ); polyclonal antibody CCL26, human CCL26/eotaxin-3 DuoSet ELISA

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Abbreviations used

BEC: Bronchial epithelial cell
 JAK: Janus kinase
 MBP: Major basic protein
 qPCR: Quantitative PCR
 SOCS: Suppressor of cytokine signaling
 STAT6: Signal transducer and activator of transcription 6

kit, human CCL26/eotaxin-3 Quantikine ELISA Kit, anti-goat HRP-AEC staining kit, and Antigen Retrieval Reagent-Basic were from R&D Systems (Minneapolis, Minn); DMEM/F12 medium and FBS were from Wisent (St-Bruno, Quebec, Canada); anti-human eosinophil major basic protein (MBP) clone BMK-13 was from Meridian Life Science (Memphis, Tenn); RLT Plus, RNeasy Plus MiniKit, Human CCL26 QuantiTect Primers, RT² First Strand Kit, RT² Profiler qPCR Multiplex Array Kit, RT² SYBR Green ROX qPCR Master Mix, RT² Profiler PCR array 386-well plates, and Human RRN18S QuantiTect primer assay were from Qiagen (Mississauga, Ontario, Canada); the qScript cDNA Synthesis Kit and BR-SYBR Green SuperMix were from Quanta Biosciences (Gaithersburg, Md); Mayer hematoxylin and the Giemsa Stain Kit (Jenner-Wright) Artisan, were from Dako Canada (Burlington, Ontario, Canada); aqueous-based permanent CC/Mount medium was from Sigma-Aldrich Canada (Oakville, Ontario, Canada); and the anti-mouse EnVision + System-HRP (AEC) kit was from Dako Canada.

Clinical criteria for subject selection

Never-smoking asthmatic patients and healthy subjects without recent airway infection (8 weeks) were recruited for bronchial biopsy. Asthmatic patients had a history of asthma for at least 6 months and were classified by using American Thoracic Society criteria.³⁴ Patients with mild asthma only used β_2 -agonists on demand as therapy and had normal FEV₁. Patients with moderate asthma were taking a low-to-moderate dose of inhaled corticosteroids (≤ 500 μ g of fluticasone propionate or equivalent). Patients with severe eosinophilic asthma required a high dose of inhaled corticosteroids (>1000 μ g of fluticasone propionate or equivalent in combination with a long-acting β_2 -agonist) and demonstrated a high ($>5\%$, severe eosinophilic) or low ($\leq 2\%$, severe noneosinophilic) induced sputum eosinophil count, with the sputum neutrophil count being 64% or less. Healthy volunteers had no history of allergy, asthma, or any other inflammatory disease and had a PC₂₀ value of greater than 16 mg/mL. Approval from the local ethics committee was obtained, and all volunteers signed an informed consent form.

Human primary BEC line culture and stimulation

BEC lines from healthy subjects and patients with mild or severe eosinophilic asthma were obtained, as described previously.^{35,36} BECs were cultured in DMEM-F12 medium supplemented with 10% FBS. Cells were grown in 12-well plates (3×10^5 cells/well) until they reached 80% confluence. Then, cells were stimulated with vehicle or increasing concentrations of IL-13 in DMEM-F12 medium supplemented with 1% FBS and incubated for up to 24 hours. Incubations were stopped by harvesting the culture medium and processing the cells for mRNA extraction. Culture media were immediately spun to remove cell debris, and the resulting supernatants were kept at -80°C until chemokine quantitation by means of ELISA.

Quantitative PCR analyses

After mRNA extraction with the RNeasy Plus MiniKit, RNA was quantified by using UV with the Synergy H1 Hybrid Multi-Mode microplate reader (BioTek, Winooski, Vt). For the analysis of CCL26 mRNA by quantitative PCR (qPCR), total RNA was converted into cDNA by using the qScript cDNA Synthesis Kit with the DNA engine PTC-200 (MJ Research, Watertown, NY). Amplification was performed with the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, Ontario, Canada) and using the

BR-SYBR Green SuperMix and human CCL26 QuantiTect Primers (NM_006072). For CC chemokine mRNA analysis, cDNA synthesis was performed with the RT² First Strand Kit, and qPCR reactions were performed with the 7900HT Fast Real-Time PCR system (Applied Biosystems, Burlington, Ontario, Canada) with a custom-made RT² Profiler qPCR Multiplex Array Kit and the RT² SYBR Green ROX qPCR Mastermix. The RT² Profiler PCR array 386 wells plates were filled by using the epMotion 5075 LH system (Eppendorf, Hamburg, Germany). External control mix for the efficiency of reverse transcriptase, amplification reactions, and detection of genomic DNA contamination were also included. PCR amplifications were then performed by using the following protocol: a denaturation step at 95°C for 5 minutes, followed by 40 cycles (1 minute of denaturation at 95°C , 30 seconds of annealing at 60°C , and 1 minute of elongation at 72°C). Data acquisition and analyses were performed with the CFX Manager Software 3.1 from Bio-Rad Laboratories, Mississauga, Ontario, Canada. Gene expression was calculated by using relative quantification normalized to 18S rRNA (human RRN18S QuantiTect primer assay, NM_X03205) as the reference gene, with the $2^{-\Delta\Delta\text{CT}}$ method related to the Pfaffl method.³⁷⁻³⁹ Negative controls were also performed as described above in the absence of cDNA templates.

Bronchial biopsy specimen processing and immunohistochemistry

Bronchial biopsy specimens of healthy subjects and asthmatic patients were taken from the IUCPQ Tissue Bank, the site of the Respiratory Health Network Tissue Bank of the Fonds de la recherche du Québec-Santé. All biopsy specimens were processed by using the same standardized methodology. For CCL26 staining, tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into $4\text{-}\mu\text{m}$ sections by using a microtome (Surgipath Medical Industries, Richmond, Ill). Then, tissue slides were deparaffinized with xylene, followed by numerous washes with decreasing concentrations of ethanol. Immunostaining of biopsy sections was done with the anti-goat HRP-AEC staining kit and the Antigen Retrieval Reagent-Basic, according to the manufacturer's instructions. For MBP staining, biopsy specimens were embedded in OCT compound (Tissue-Tek O.C.T. compound; Sakura, Torrance, Calif), frozen (-80°C), and then cut into $6\text{-}\mu\text{m}$ sections and kept frozen (-80°C) until analysis. Tissue slides were fixed and permeabilized with acetone/methanol and rehydrated in PBS. Immunostaining of biopsy sections was done with the anti-mouse EnVision + System-HRP (AEC) staining kit, according to the manufacturer's instructions. Tissue slides were incubated with the primary antibodies (CCL26 or MBP), counterstained with Mayer hematoxylin, and mounted with the aqueous-based permanent CC/Mount medium. CCL26 and MBP stainings were pictured with the digital slide scanner NANOZOOMER-XR C12000 (Hamamatsu, Middlesex, NJ). Chemokine staining intensity on each biopsy specimen was blindly scored by 2 of the authors (J.C. and P.J.), where a score of 0 corresponds to no staining, a score of 1 corresponds to focal staining with less than 10% of positive cells, a score of 2 corresponds to moderate staining with 10% to 75% of positive cells, and a score of 3 corresponds to strong staining with greater than 75% positive cells. Eosinophil numbering was determined as the number of MBP⁺ cells per square millimeter of bronchial tissue, excluding mucus glands, or by using the Giemsa Stain Kit (Jenner-Wright) Artisan. Measures of total bronchial tissue surface were achieved with Image-Pro Plus software (Media Cybernetics, Rockville, Md).

Quantitation of chemokines in induced sputum

Sputum samples were obtained with the collaboration of Dr Louis-Philippe Boulet, who supervises the induced sputum laboratory of our institution. Samples were processed (within 2 hours), as described previously.^{40,41} In brief, mucus plugs were isolated from saliva and then treated with a volume (in milliliters) of dithiothreitol representing 4 times the mucus weight (in grams). Then, an equal volume of Dulbecco PBS was added to all samples before filtration with a $0.45\text{-}\mu\text{m}$ nylon filter. The resulting supernatants were collected for chemokine quantitation. Cell pellets were assessed for cell viability and differential counts by using trypan blue exclusion and

TABLE I. Clinical characteristics of subjects who provided the primary BEC lines

Subjects	Age (y)	Sex (M/F)	Atopy (yes/no)	FEV ₁ (% predicted)	FVC (% predicted)	Sputum eosinophils (%)
Healthy control subjects	29.8 ± 3.4	4/5	0/9	98.8 ± 4.8	NA	NA
Patients with mild asthma	23.6 ± 1.4	7/2	9/0	94.9 ± 5.9	NA	NA
Patients with severe eosinophilic asthma	62 ± 1.1	2/3	4/1	57 ± 6.6	73 ± 6.8	34.2 ± 16.5

Values are presented as means ± SEMs.

F, Female; FVC, forced vital capacity; M, male; NA, not available.

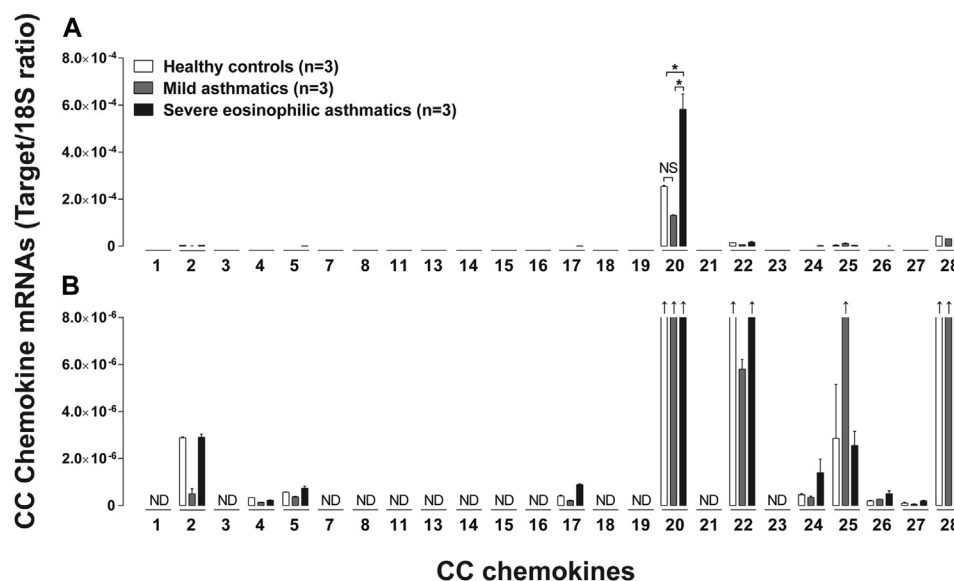


FIG 1. Relative expression of CC chemokines by human BECs. **A** and **B** represent the ratio between mRNAs of CC chemokines and 18S rRNA control. Fig 1, **B**, is a zoom of Fig 1, **A**. mRNAs were quantified by using a qPCR array, as described in the Methods section. Results are means ± SEMs of 3 independent experiments performed in duplicate. Two-way ANOVA with a Tukey multiple comparisons test was performed to obtain *P* values. **P* < .0001, patients with severe asthma versus healthy control subjects and patients with mild asthma. ND, Not detected; NS, not significant.

Diff-Quik staining, respectively. Aprotinin (10 µg/mL) was added to all samples to limit chemokine processing by proteases, such as mast cell tryptase.

Statistical analyses

Data are expressed as means ± SEMs and were analyzed by using 1- or 2-way ANOVA with a blocking factor, according to the protocols. Log transformation was investigated to stabilize variances. The normality assumption was verified with Shapiro-Wilk tests after Cholesky factorization. The Brown and Forsythe variation of the Levene test statistic was used to verify homogeneity of variances. Relationships between variables were expressed by using the Pearson correlation coefficient. Results were considered significant at *P* values of .05 or less. Analyses were performed with the statistical packages SAS, version 9.4 (SAS Institute, Cary, NC), and R, version 3.0.

RESULTS

IL-13 selectively increases expression of CCL26 by human primary BECs

IL-13 stimulates the production of CCL26 by epithelial cells.^{32,42-45} In this regard, we tested whether IL-13 would induce the expression of CCL26 and other CC chemokines by human primary BECs obtained from healthy control subjects and patients with mild or severe asthma. Clinical characteristics of these subjects are presented in Table I. Untreated cells from healthy control subjects and from patients with mild or severe eosinophilic asthma mainly

expressed the following: CCL20 >>> CCL28 ≈ CCL25 ≈ CCL22 ≈ CCL2 (Fig 1, A). Smaller and similar mRNA levels were also found for CCL4, CCL5, CCL17, CCL24, CCL26, and CCL27 (Fig 1, B). The mRNAs of other CC chemokines, including CCL11, were not detected. There was no difference between the 3 groups of volunteers, except for CCL20 (macrophage inflammatory protein 3α) for which an increased mRNA level was observed for patients with severe eosinophilic asthma compared with healthy control subjects, or patients with mild asthma (*P* < .0001). No difference was observed between CCL20 levels in BECs from healthy control subjects versus those in patients with mild asthma. When cells were treated with 30 ng/mL IL-13 for 24 hours, CCL26 mRNA levels were increased by up to 4 orders of magnitude (Fig 2). This increased CCL26 mRNA expression was greater in cells from patients with severe eosinophilic asthma compared with that in cells from either healthy control subjects or patients with mild asthma (*P* < .01). It is noteworthy that CCL26 was the only CC chemokine whose mRNA expression was significantly increased upon stimulation with IL-13 (*P* < .0001 compared with baseline).

Kinetic and concentration responses of IL-13-mediated CCL26 production by BECs

We next performed kinetic and concentration-response experiments in which we analyzed the effect of IL-13 on

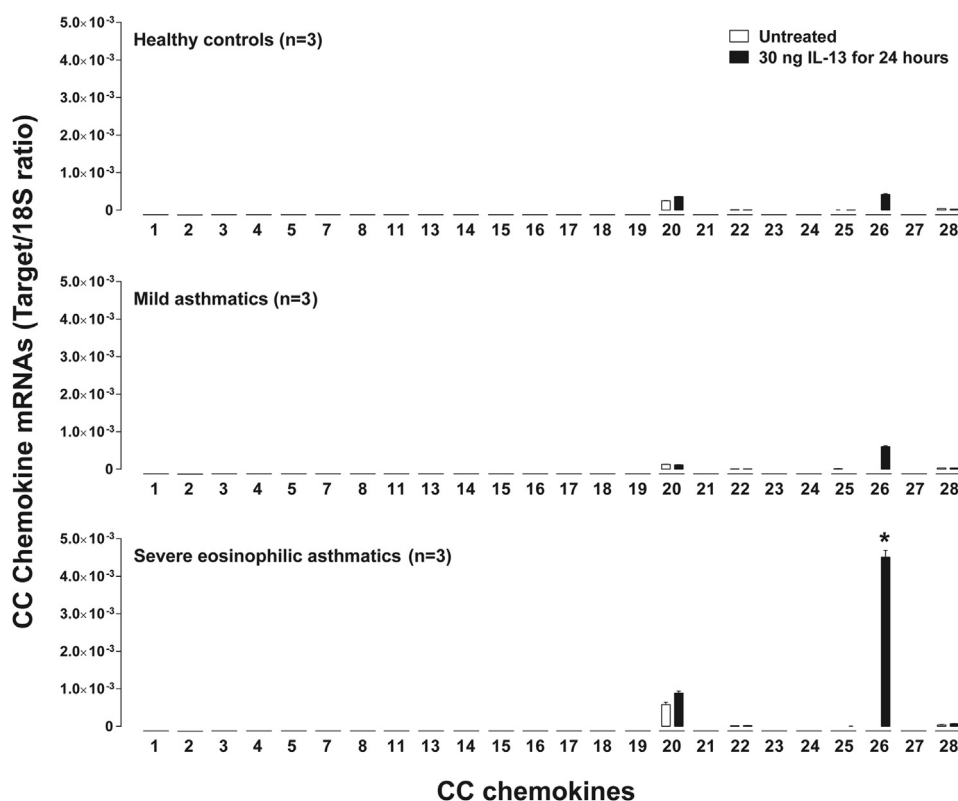


FIG 2. Effect of IL-13 on CC chemokine expression by human BECs. Cells were treated with vehicle or IL-13 for 24 hours. mRNAs were extracted, and CC chemokine expression was quantified by using a qPCR array, as described in the Methods section. Results represent means $\pm$ SEMs of 3 independent experiments performed in duplicate. Two-way ANOVA with a Tukey multiple comparisons test was performed to obtain *P* values. **P* < .01 compared with healthy control subjects and patients with mild asthma.

CCL26 expression and production. Treatment of BECs with 30 ng/mL IL-13 induced a time-dependent expression of CCL26 mRNA in all groups. CCL26 mRNA levels were detected after 4 hours of stimulation and increased up to 24 hours. In agreement with the qPCR array data presented in Fig 2, IL-13 induced an increase in CCL26 mRNA expression that was significantly higher in BECs from patients with severe eosinophilic asthma compared with those from healthy subjects or patients with mild asthma ($P < .01$; Fig 3, A). CCL26 was also released in the cell-culture supernatants in a time-dependent fashion and increased up to 24 hours after addition of IL-13 (Fig 3, B). At this time point, CCL26 protein levels in culture supernatants were significantly higher in the severe eosinophilic asthma group compared with those in the healthy control and mild asthma groups ($P \leq .03$; Fig 3, B), which is in agreement with the observed increased expression of CCL26 mRNA (Fig 3, A). It is noteworthy that we could not detect any CCL26 in the supernatants of untreated cells. Because no difference was found between cell lines from healthy control subjects and patients with mild asthma, we performed concentration-response experiments with 2 cell lines from both groups. These experiments showed that IL-13 stimulated CCL26 expression by BECs in a concentration-dependent manner (Fig 3, C).

CCL26 expression is increased in the bronchial epithelium of asthmatic patients

We next assessed whether CCL26 levels were increased in the bronchial epithelium of asthmatic patients by performing

immunohistochemistry analyses on bronchial biopsy specimens obtained from healthy control subjects, patients with mild asthma, and patients with severe eosinophilic asthma. The clinical characteristics of these subjects are presented in Table II. Typical CCL26 staining data are shown for each group in Fig 4. A weak epithelial expression of CCL26 was found in bronchial epithelium of healthy control subjects. This expression significantly increased in both patients with mild and those with severe eosinophilic asthma (asthmatic patients vs healthy subjects: $P = .027$; Fig 4, D). However, CCL26 staining was not different between the epithelium of patients with mild and those with severe eosinophilic asthma (Fig 4, E). These results indicate that the increased CCL26 expression in the bronchial epithelium of asthmatic patients could explain, in part, the recruitment of eosinophils into bronchial mucosa observed in patients with this disease. However, they contrast with the CCL26 production observed in IL-13-stimulated primary BECs, where higher CCL26 levels were found in patients with severe eosinophilic asthma (Fig 3).

Increased eosinophil numbers in bronchial biopsy specimens of patients with mild asthma but not those with severe eosinophilic asthma

We next determined the number of eosinophils by means of MBP staining in bronchial biopsy specimens of the same donors (Table II). In contrast to CCL26, eosinophil counts in the bronchial mucosa were different between the asthmatic groups

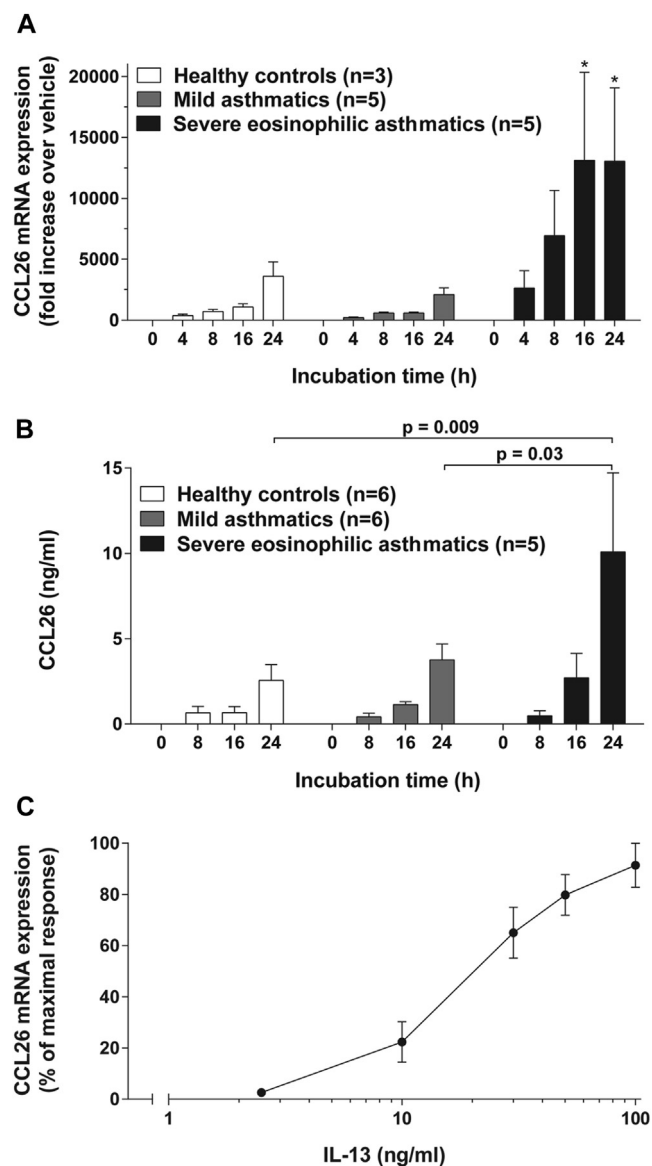


FIG 3. Kinetic and concentration responses of IL-13 on CCL26 expression and release by primary BECs. **A** and **B**, Cells were treated with 30 ng/mL IL-13 for up to 24 hours. **C**, Cells were treated with increasing concentrations of IL-13 for 8 hours. Fig 3, **A** and **C**, CCL26 and 18S rRNA expression was quantified by means of qPCR, as described in the Methods section. Fig 3, **B**, CCL26 protein in culture supernatants was analyzed by means of ELISA. Two-way ANOVA with a Tukey multiple comparisons test was performed in Fig 3, **A** and **B**. * $P < .01$ versus healthy control subjects and patients with mild asthma.

(Fig 5). The healthy control subjects had low eosinophil counts, and the patients with mild asthma had significantly greater eosinophil counts in the submucosa. The severe eosinophilic asthma group had low eosinophil counts comparable with those observed in healthy control subjects. This was surprising given that these patients with severe eosinophilic asthma had high eosinophil counts in their induced sputum (Table II). It is noteworthy that essentially similar results were obtained by enumerating eosinophils with the Giemsa Stain Kit (Jenner-Wright) Artisan, in which human eosinophils are bright pink (data not shown).

CCL26 protein levels correlate with eosinophil counts in the airway lumen

Given that BECs from patients with severe asthma release more CCL26 than those of healthy volunteers and patients with mild asthma *in vitro* (Fig 3), that the CCL26 staining in the bronchial epithelium was not associated with increased eosinophil counts in the bronchial mucosa (Figs 4 and 5), and that low eosinophil counts were obtained in bronchial mucosa from patients with severe eosinophilic asthma (Fig 5), we postulated that BECs released CCL26 in the airway lumen rather than in the submucosa and that induced sputum eosinophil counts would correlate with sputum CCL26 levels. In this respect, we measured CCL26 levels in the induced sputum of healthy control subjects and patients with asthma of different severities who had different eosinophil counts in their sputum. The clinical characteristics of these subjects are presented in Table III. We found a significant correlation between sputum CCL26 levels and sputum eosinophil counts and an inverse correlation between sputum CCL26 levels and sputum neutrophil counts (Fig 6), strongly supporting a crucial role of CCL26 in the recruitment of eosinophils into the airway lumen. It is noteworthy that high CCL26 levels were mainly found in the sputum samples of patients with severe eosinophilic asthma (Fig 6, A).

DISCUSSION

The current pharmacologic treatment of asthma includes daily use of inhaled corticosteroids to dampen airway inflammation. The corticosteroid dose is adjusted to control asthma symptoms and increases with disease severity. Sputum eosinophil counts are very useful to adjust the corticosteroid dose required to decrease airway eosinophilia and asthma symptoms.⁴⁶⁻⁴⁹ However, some patients with severe asthma remain symptomatic despite high doses of inhaled and/or oral corticosteroids, and many of these patients present with a persistent increased eosinophil count in their induced sputum.^{6,7} To study the severe asthma phenotype of BECs in terms of chemokine expression, we selected patients with severe asthma with persistent asthma symptoms and eosinophilic airway inflammation, as defined by a high eosinophil cell count in induced sputum (Tables I-III). This particular severe asthma phenotype represents a good opportunity to better clarify the mechanisms sustaining eosinophil recruitment into the airways.

Using primary BEC lines, we demonstrate that among all CC chemokines, BECs predominantly express CCL26 in response to IL-13 (Fig 2); that the induced CCL26 expression and release by IL-13-stimulated cells were significantly more important with cells of patients with severe eosinophilic asthma (Fig 3); that CCL26 staining is more prominent in bronchial epithelium of asthmatic patients compared with that of healthy control subjects (Fig 4); that eosinophil counts in the airway mucosa of patients with severe eosinophilic asthma are lower than those observed in patients with mild asthma (Fig 5); that CCL26 levels correlate with eosinophil counts in our sputum samples (Fig 6); and that increased sputum CCL26 levels are mainly found in patients with severe eosinophilic asthma (Fig 6). These results underscore the importance of BEC release of CCL26 in the airway recruitment of eosinophils and the pathogenesis of eosinophilic asthma and its severity. Moreover, they suggest that in patients with severe eosinophilic asthma, a great proportion of recruited eosinophils to the airway mucosa are further attracted

TABLE II. Clinical characteristics of subjects who provided bronchial biopsy specimens for CCL26 immunostaining and eosinophil (MBP⁺ cell) counts

Subjects	Age (y)	Sex (M/F)	Atopy (yes/no)	FEV ₁ (% predicted)	FVC (% predicted)	Sputum eosinophils (%)
Healthy control subjects	24.8 ± 1.9	5/3	0/8	100.4 ± 3.37	104.9 ± 5.2	NA
Patients with mild asthma	26 ± 3.6	3/5	7/1	90.4 ± 3.9	98.7 ± 4.1	NA
Patients with severe eosinophilic asthma	54.9 ± 2.8	6/4	7/3	64.9 ± 4.1	78.8 ± 4.0	26.5 ± 7.8

Values are presented as means ± SEMs.

F, Female; FVC, forced vital capacity; M, male; NA, not available.

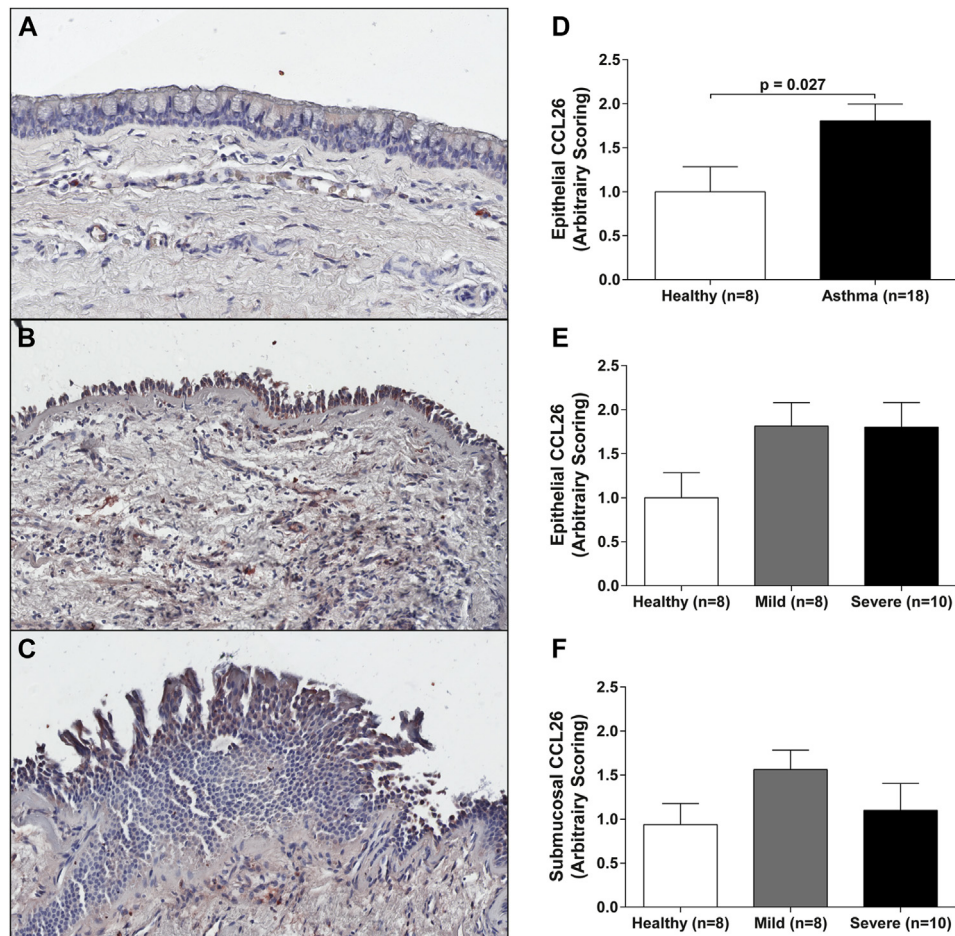


FIG 4. CCL26 protein expression by bronchial epithelium and submucosa. **A-C**, Representative images ($\times 200$ magnification) of CCL26 staining (red) from a healthy control subject (Fig 4, A), a patient with mild asthma (Fig 4, B), and a patient with severe eosinophilic asthma (Fig 4, C). **D**, Computed scores of CCL26 staining intensity in bronchial epithelium of asthmatic patients were compared with those of healthy control subjects. **E** and **F**, Computed scores of CCL26 staining intensity in bronchial epithelium and bronchial submucosa for healthy control subjects and patients with mild or severe eosinophilic asthma. Unpaired *t* tests were performed to obtain *P* values.

to the airway lumen, likely based on the effect of CCL26, thereby decreasing the number of eosinophils observed in the mucosa.

To our knowledge, this is the first study that evaluated the expression of all CC chemokines by primary BECs in asthmatic patients. Although a limited number of CC chemokines were expressed by unstimulated BECs, their treatment with IL-13 led to an important and selective upregulation of CCL26 expression by up to 4 orders of magnitude (Figs 2 and 3). This increase in CCL26 expression is in line with 2 recent studies. The first study

showed that human epithelial cells from patients with eosinophilic esophagitis robustly responded to IL-13 with an increase of CCL26 expression by approximately 4 orders of magnitude.⁵⁰ The second study showed that epithelial brushings from asthmatic patients showed increased expression of CCL24 and CCL26 mRNAs.³¹ Moreover, our observations further support the primordial role of CCL26 in the pathogenesis of eosinophilic diseases, adding severe eosinophilic asthma to atopic dermatitis, chronic rhinosinusitis, eosinophilic esophagitis, and Churg-Strauss vasculitis.²⁴⁻³⁰

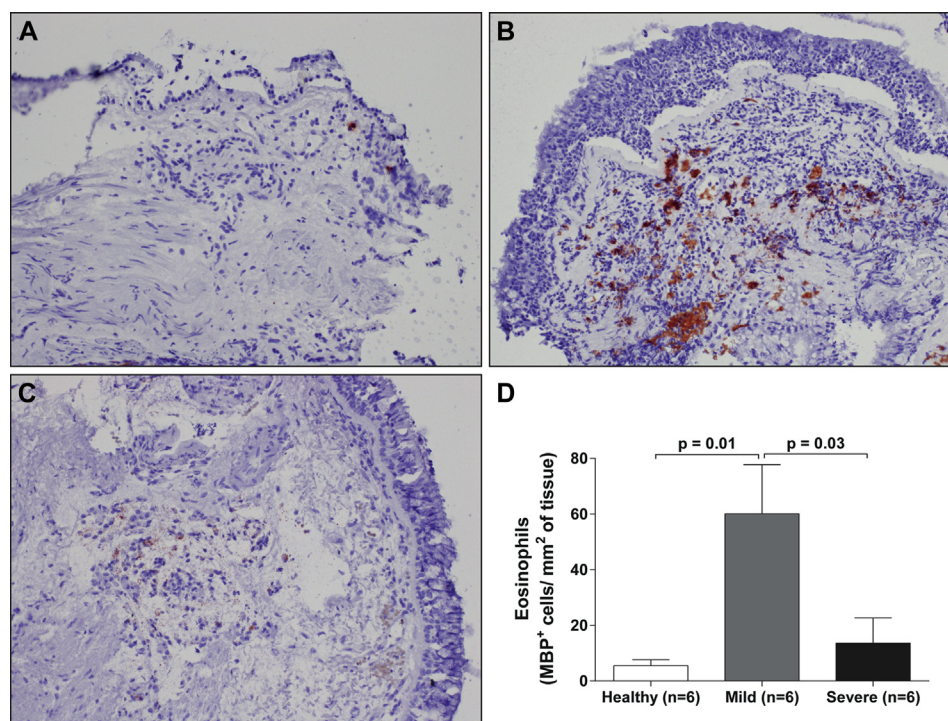


FIG 5. Eosinophil counts in bronchial mucosa in healthy subjects and asthmatic patients. **A-C**, Representative images ($\times 200$ magnification) of MBP staining (red) from a healthy control subjects (Fig 5, A), a patient with mild asthma (Fig 5, B), and a patient with severe eosinophilic asthma (Fig 5, C). **D**, Computed analyses of eosinophil counts in bronchial biopsy specimens of healthy control subjects and patients with mild or severe eosinophilic asthma. *P* values were obtained by using a 1-way ANOVA combined with a Tukey multiple comparisons test.

TABLE III. Clinical characteristics of subjects who provided induced sputum supernatants and cells

Subjects	Age (y)	Sex (M/F)	Atopy (yes/no)	FEV ₁ (% predicted)	FVC (% predicted)	Sputum eosinophils (%)
Healthy control subjects	62.1 $\pm$ 5.7	3/4	2/1	96 $\pm$ 4.6	102 $\pm$ 4.6	0.8 $\pm$ 0.4
Patients with moderate asthma	50.1 $\pm$ 5.0	7/4	7/2	80.4 $\pm$ 4.1	91.6 $\pm$ 2.6	2.4 $\pm$ 1.0
Patients with severe noneosinophilic asthma	47.3 $\pm$ 3.8	8/5	11/0	66.5 $\pm$ 3.2	87.2 $\pm$ 4.0	0.7 $\pm$ 0.2
Patients with severe eosinophilic asthma	52.7 $\pm$ 5.0	3/8	8/2	67.1 $\pm$ 6	89.5 $\pm$ 4.2	29.5 $\pm$ 6.7

Values are presented as means $\pm$ SEMs.

F, Female; FVC, forced vital capacity; M, male.

IL-13 levels are increased in bronchoalveolar lavage fluid, bronchial biopsy specimens, and sputum of asthmatic patients, notably those with severe asthma.⁵¹⁻⁵⁵ IL-13 is involved in several aspects of airway inflammation and remodeling, such as mucus production, IgE synthesis, recruitment of eosinophils, and proliferation of bronchial fibroblasts and smooth muscle cells.⁵⁶ Promising preclinical data from experimental models of asthma led to the development of various humanized anti-IL-13 mAbs.^{57,58} Among them, lebrikizumab elicited an improvement of lung function in patients with uncontrolled asthma characterized by high levels of T_H2 responses, such as circulating IgE and blood eosinophil counts.⁵⁹ Our data suggest that IL-13 might promote eosinophil recruitment and asthma severity by stimulating BECs to release CCL26 in the airways.

Some cellular mechanisms might explain the observed increased expression of CCL26 in BECs of patients with severe eosinophilic asthma. Airway epithelial cells express all IL-13 receptor subunits (IL-13R α 1, IL-13R α 2, IL-4R α and the

γ -chain).⁵⁶ It is possible that BECs express more receptors at their surface or that the downstream signaling triggered by their activation is enhanced. It is well established that the activation of IL-13 receptors involves numerous kinases, notably Janus kinase (JAK) 1, JAK2, JAK3, and tyrosine kinase 2. They rapidly phosphorylate the specific transcription factor signal transducer and activator of transcription 6 (STAT6), which then translocates to the nucleus and initiates transcription. The activation of STAT6 can be downregulated by the suppressor of cytokine signaling (SOCS) proteins, in particular SOCS1 and SOCS3.⁶⁰⁻⁶⁴ Thus it is possible that in BECs from patients with severe eosinophilic asthma, the JAK-STAT6 pathway is enhanced either because of an imbalance between JAK and SOCS proteins or that there is an increase in STAT6 expression. Moreover, decreased methylation of the CCL26 gene could also be involved in the increased CCL26 release we observed. In this respect, Lim and Rothenberg⁶⁵ demonstrated that increased CCL26 mRNA levels in patients with eosinophilic esophagitis were linked to lower

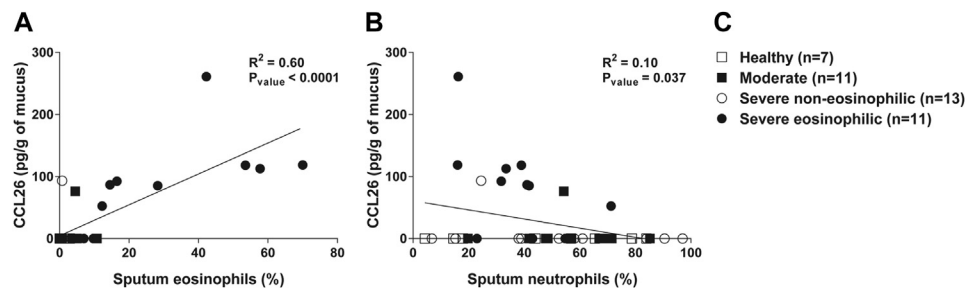


FIG 6. Correlation between CCL26 levels and eosinophil counts in sputum of healthy subjects and asthmatic patients. Induced sputum from healthy control subjects, patients with moderate asthma, patients with severe noneosinophilic asthma, and patients with severe eosinophilic asthma were isolated and processed, as described in the Methods section. **A** and **B**, CCL26 levels, as well as eosinophil (Fig 6, **A**) and neutrophil (Fig 6, **B**) counts, were obtained, as described in the Methods section. **C**, Legend for Fig 6, **A** and **B**. For analysis of the correlation, a Spearman rank correlation test was performed.

methylation of the CCL26 gene compared with that seen in healthy subjects. Further experiments are necessary to define whether those cellular mechanisms or a combination of these processes are involved.

Corticosteroids activate anti-inflammatory genes that encode for cytokines, chemokines, adhesion molecules, inflammatory enzymes and receptors, and suppress inflammatory genes.⁶⁶ However, patients with severe eosinophilic asthma do not respond to corticosteroid therapy, and several molecular mechanisms leading to corticosteroid resistance might be involved in severe asthma, including glucocorticoid receptor dysfunction with altered glucocorticoid receptor binding affinity or overexpression of the proinflammatory β subunit, increased proinflammatory transcription factor levels, abnormal histone acetylation/deacetylation leading to the repression of anti-inflammatory gene activation, and genetic susceptibility.⁶⁶ Additional experiments exploring these hypotheses will be an important step to increase our understanding of chemokine expression in patients with asthma and asthma severity.

In conclusion, we showed that CCL26 is the only CC chemokine to be highly expressed by BECs on treatment with IL-13. Importantly, BECs from patients with severe eosinophilic asthma released more CCL26, and a significant correlation was found between CCL26 levels and eosinophil counts in induced sputum, supporting a major role of CCL26 for eosinophil recruitment in asthmatic patients. The mechanism sustaining the increased sputum CCL26 levels observed in patients with severe eosinophilic asthma needs to be defined. These data suggest that sputum CCL26 could be a biomarker to identify a specific asthma phenotype in which IL-13 is a key player and could be a valuable target for the treatment of severe eosinophilic asthma. This report underscores the necessity to study the role of chemokines in the recruitment of eosinophils in human subjects and emphasizes the importance of better understanding the mechanisms of eosinophil recruitment in asthmatic patients to offer a more effective therapy to these patients, particularly those with severe asthma.

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Key messages

- IL-13 induced selective production of CCL26 by BECs.
- Sputum CCL26 levels correlate with sputum eosinophil counts and asthma severity.
- Targeting CCL26 might help in the treatment of patients with eosinophilic asthma whose symptoms are refractory to classic therapies.

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