# Sputum and bronchial submucosal IL-13 expression in asthma and eosinophilic bronchitis

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**Background: Nonasthmatic eosinophilic bronchitis is a condition characterized by the presence of eosinophilic airway inflammation in the absence of airflow obstruction or airway hyperresponsiveness. In asthma, the $T_H2$-type cytokine IL-13 has been implicated in the development of airway inflammation and hyperresponsiveness. Whether the expression of IL-13 is different between these 2 conditions is unknown.**

**Objective: We sought to investigate whether IL-13 expression is increased in asthma compared with eosinophilic bronchitis.**

**Methods: Sputum samples from subjects with mild asthma (n = 30) and eosinophilic bronchitis (n = 15) and normal controls (n = 16) were dialyzed, and IL-13 concentration was measured by ELISA. In a subgroup of these patients, IL-13 protein expression in bronchial biopsies was assessed by immunohistochemistry.**

**Results: The concentration of sputum IL-13 was higher in patients with mild asthma than in normal controls ($P = .03$) and in patients with eosinophilic bronchitis ($P = .03$). The median (interquartile range) number of IL-13$^+$ cells/mm$^2$ submucosa was significantly higher in asthma 4 (8) than eosinophilic bronchitis 1.7 (1.9) and normal controls 0.5 (1.1; $P = .004$). Eighty-three percent of the cells expressing IL-13 in the submucosa were eosinophils, and 8% were mast cells. The median (interquartile range) proportion of eosinophils that expressed IL-13 was higher in the subjects with asthma, 16 (10)%, than those with eosinophilic bronchitis, 7 (3)% ($P = .02$).**

**Conclusion: The increased expression of IL-13 in asthma compared with eosinophilic bronchitis supports the concept that IL-13 may play a critical role in the pathophysiology of asthma. (J Allergy Clin Immunol 2004;114:1106-9.)**

***Key words:*** *Asthma, IL-13, eosinophilic bronchitis, eosinophils, mast cells*

Asthma is characterized by the presence of variable airflow obstruction, airway hyperresponsiveness, and an airway inflammatory response usually with eosinophilic airway inflammation.[1] There is increasing evidence that the development and maintenance of airway inflammation in asthma is a result of the production of $T_H2$ cytokines such as IL-4, IL-5, and IL-13.[2]

*Abbreviations used*
MBP: Major basic protein
UK: United Kingdom

Eosinophilic bronchitis, like asthma, is characterized by eosinophilic airway inflammation, but unlike asthma, there is no airway hyperresponsiveness or bronchoconstriction. The airway immunopathology of asthma and eosinophilic bronchitis are almost identical.[3-6] However, one fundamental difference between asthma and eosinophilic bronchitis is that in asthma, mast cells expressing IL-4 and IL-13 infiltrate the airway smooth muscle.[3,7] It is unknown whether the difference in IL-13 expression between these 2 conditions is reserved to mast cells within the airway smooth muscle bundle or there is a more generalized upregulation of IL-13 in asthma compared with eosinophilic bronchitis.

We hypothesized that there is differential expression of IL-13 in asthma and eosinophilic bronchitis. To test our hypotheses, we measured the sputum IL-13 concentration and the number of bronchial submucosal cells that express IL-13 in subjects with asthma and eosinophilic bronchitis and normal controls.

## METHODS

### Subjects

Patients with eosinophilic bronchitis, patients with asthma, and healthy volunteers were recruited from respiratory clinics and from staff at Glenfield Hospital. Subjects with asthma (n = 30) gave a suggestive history and had objective evidence of variable airflow obstruction as indicated by one or more of the following: (1) methacholine airway hyperresponsiveness ($PC_{20}$ $FEV_1$ < 8 mg/mL), (2) >15% improvement in $FEV_1$ 10 minutes after 200 μg inhaled salbutamol, or (3) peak expiratory flow (>20% maximum within-day amplitude from twice-daily peak expiratory flow measurements over a period of 14 days). The subjects with eosinophilic bronchitis (n = 15) had an isolated cough, no symptoms or objective evidence of variable airflow obstruction, and a sputum eosinophilia (>3% nonsquamous cell). None of the subjects with asthma or eosinophilic bronchitis had been treated with corticosteroids for at least 6 weeks

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**TABLE I.** Subjects' clinical characteristics

| | Normal | Eosinophilic bronchitis | Mild asthma |
|---|---|---|---|
| Number | 16 | 15 | 30 |
| Age† | 38.1 (5.6) | 53.5 (2.4) | 54.8 (4.7) |
| Male | 7 | 11 | 14 |
| Atopy | 2 | 6 | 13 |
| $PC_{20}$ $FEV_1$ (mg/mL)* | >16 | >16 | 1.75 (0.23-7.4) |
| $FEV_1$ % predicted† | 107.1 (4.8) | 101 (3.1) | 84.1 (4.2) |
| Bronchodilator reversibility (%)† | 1.3 (0.9) | 0.8 (0.6) | 5.3 (2.4) |
| $FEV_1$/forced vital capacity %† | 84.3 (2.3) | 81.7 (1.1) | 71.9 (2.2) |
| Total cell count ($\times 10^6$/g sputum)† | 1.7 (0.4) | 3.1 (0.9) | 2.5 (0.7) |
| Eosinophil %* | 0.4 (0-2.5) | 12.8 (3-68) | 4.2 (0-40) |
| Neutrophil %† | 32 (4.3) | 50.6 (5.9) | 60.7 (5.3) |
| Macrophage %† | 62.4 (4.2) | 25.2 (4.9) | 26.9 (4.3) |
| Lymphocyte %† | 2.7 (0.9) | 0.5 (0.2) | 1.0 (0.2) |
| Epithelial cells %† | 3.7 (1.5) | 1.8 (0.8) | 3.1 (0.8) |
| Squamous cell contamination %† | 4.6 (1.7) | 5.7 (1.6) | 8.8 (1.7) |
| Viability %† | 63.4 (6.5) | 73.1 (3.6) | 64.6 (3.9) |

*Median (range).
†Mean (SEM).

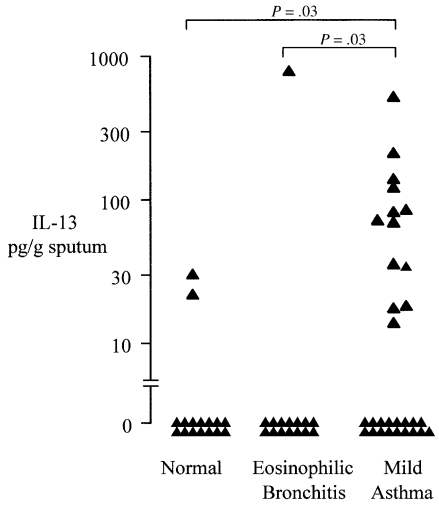


**FIG 1.** Sputum IL-13 concentration for subjects with eosinophilic bronchitis, normal controls, or subjects with asthma.

before recruitment. The healthy subjects (n = 16) gave no history of respiratory diseases, had normal spirometry and normal airway responsiveness ($PC_{20} > 16$ mg/mL). None of the subjects had smoked, except for 5 subjects with asthma, all with <5 pack-year history. Ten subjects with eosinophilic bronchitis, mild asthma, and normal controls had been included in an earlier study[5] but were recharacterized for this study. The protocol was approved by the Leicestershire ethics committee.

## Protocol and clinical measurements

Subjects had spirometry; allergen skin prick tests to *Dermatophagoides pteronyssinus*, cat fur, grass pollen, and *Aspergillus fumigatus*; and a methacholine inhalation test,[8] followed on recovery by sputum induction.[9]

One week later, a subgroup of patients with mild asthma (n = 8) or eosinophilic bronchitis (n = 7) and normal controls (n = 7) underwent bronchoscopy.[10] Bronchial biopsies were taken from the right middle and lower lobe carinae. Mucosal biopsies were processed into glycolmethacrylate (Polysciences, Northampton, United Kingdom [UK]).

## Immunohistochemistry

The technique of immunostaining applied to glycolmethacrylate-embedded tissue has been described previously.[11] The mouse monoclonal antibodies used were CD3 for T lymphocytes (Dako, High Wycombe, UK), AA1 to mast cell tryptase (Dako), major basic protein (MBP) for eosinophils (Caltag-Medsystems, Silverstone, UK), and IL-13 (R&D, Abingdon, UK). Sequential sections were stained for IL-13 and tryptase or MBP to assess colocalization as described previously.[7]

## Validation of IL-13 ELISA

*Effect of dithiothreitol on recovery of IL-13 standard.* IL-13 was measured by ELISA (Caltag-Medsystems and R&D). The effect of dithiothreitol on the recovery of IL-13 was first assessed by comparing the concentrations of a standard curve generated with IL-13 reconstituted with or without dithiothreitol (0.045% final concentration; n = 2).

*Spiking before removal of dithiothreitol by ultrafiltration.* The standards were reconstituted with and without dithiothreitol (0.045%), frozen at −80°C overnight, and defrosted the next day before analysis to mimic the sputum processing protocol. The standards were dialyzed for 18 hours against PBS by using a 10-kd dialyzer (Sigma, Poole, Dorset, UK) and then measured by ELISA. Recovery of standards spiked with dithiothreitol was compared with standards without dithiothreitol (n = 2).

*Spiking of dithiothreitol processed sputum sample.* Sputum supernatants processed with dithiothreitol from 8 subjects were divided into an aliquot spiked or not spiked with exogenous IL-13 of a known concentration. The samples and standards were dialyzed as described, and the recovery of spiked IL-13 was determined.

## IL-13 ELISA

Sputum IL-13 was measured by ELISA (Caltag-Medsystems). The inter-assay and intra-assay variability was between 5% and 10%. The limit of detection was 10 pg/g of sputum for IL-13.

## Analysis

Sputum supernatants with undetectable levels of cytokine were assigned the concentration of zero. Nonparametric data were compared across the groups by the Kruskal-Wallis test and between groups by the Mann-Whitney test. IL-13 was not measurable in more than half of the subjects in each group; thus, differences between groups were also compared by $\chi^2$ test. Significance was accepted at the level of 95%.

# RESULTS

The mucolytic dithiothreitol affected recovery of IL-13 by using both commercial ELISA kits (Caltag-Medsystems and R&D). The percentage recovery (coefficient of variation) of standards spiked with dithiothreitol compared with standards without dithiothreitol was similar with both assays: 38% (14%) and 36% (29%) for the Caltag-Medsystems and R&D ELISA, respec-

**TABLE II.** Median (IQR) number of eosinophils ($MBP^+$), mast cells ($tryptase^+$), T lymphocytes ($CD3^+$), and $IL\text{-}13^+$ cells/$mm^2$ submucosa in subjects with asthma, subjects with eosinophilic bronchitis, or normal controls

| | Asthma (n = 8) | Eosinophilic bronchitis (n = 7) | Normal controls (n = 7) |
|---|---|---|---|
| $MBP^+$ cells/$mm^2$ | 20 (23)* | 28 (34)* | 7 (10) |
| $Tryptase^+$ cells/$mm^2$ | 21 (12) | 26 (13) | 22 (14) |
| $CD3^+$ cells/$mm^2$ | 42 (59) | 41 (24) | 59 (110) |
| $IL\text{-}13^+$ cells/$mm^2$ | 4 (8)* | 1.7 (1.9) | 0.5 (1.1) |

*$P < .05$.

tively. The Caltag-Medsytems ELISA was more sensitive and therefore was further validated. The recovery of IL-13 standard after dialysis was 90% (13% coefficient of variation), confirming that dithiothreitol added to the standard did not markedly affect IL-13 recovery after dialysis. The recovery of the exogenous IL-13 spike added to sputum samples was 95% (6.5% coefficient of variation), demonstrating that our assay was valid. Therefore, sputum IL-13 concentration was measured in all of the samples and standards after dialysis.

The clinical characteristics and sputum cell counts were as shown (Table I). The sputum eosinophil count was higher in the subjects with eosinophilic bronchitis and asthma compared with the normal controls ($P < .001$). The individual values for induced sputum IL-13 concentration were as shown (Fig 1). The sputum IL-13 concentrations were significantly different comparing across the groups ($P = .014$). In the subjects with corticosteroid-naive asthma the concentration of sputum IL-13 was higher than that of normal controls ($P = .03$) and subjects with eosinophilic bronchitis ($P = .03$). Similarly, the number of subjects with measurable sputum IL-13 was higher in those subjects with asthma (13/30) compared with eosinophilic bronchitis (1/15) and normal controls (2/16; $P = .011$). There were no significant differences in age or sex between the subjects with measurable sputum IL-13 versus those with immeasurable sputum IL-13, and in the patients with asthma, there was no difference in atopic status or airway hyperresponsiveness between subjects with and without measurable sputum IL-13.

The number of $IL\text{-}13^+$ cells/$mm^2$ of submucosa was significantly higher in the subjects with asthma than in normal controls ($P = .003$) or subjects with eosinophilic bronchitis ($P = .03$; Table II). The median (interquartile range [IQR]) proportion of $IL\text{-}13^+$ cells that were colocalized to mast cells was 8 (38)% and to eosinophils was 83 (29)% (Fig 2, *A* and *B*). There was a similar increase in the number of submucosal eosinophils in the subjects with asthma and eosinophilic bronchitis, which was increased compared with the normal controls ($P = .008$), but there was no difference in the number of submucosal T cells or mast cells between groups (Table II). Therefore, the median (IQR) proportion of eosinophils that expressed IL-13 was higher in the subjects with asthma 16 (10)% than those with eosinophilic bronchitis 7 (3)% ($P = .02$).

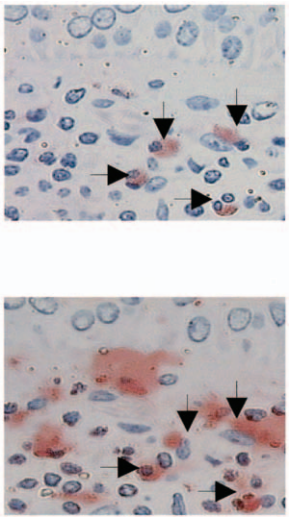

**FIG 2. (A)** Photomicrograph of $IL\text{-}13^+$ cells highlighted by the *arrows* (×400) colocalized with $MBP^+$ eosinophils on the corresponding sequential section **(B)**.

## DISCUSSION

This is the first study to fully validate the measurement of IL-13 in induced sputum. In addition, this study has made two important and novel observations. First, we have shown that the expression of IL-13 protein in the bronchial submucosa and the sputum concentration of IL-13 were increased in asthma compared with eosinophilic bronchitis, supporting the concept that IL-13 plays a critical role in the pathophysiology of asthma. Second, we identified that most of the cells in the bronchial submucosa that expressed IL-13 protein were eosinophils. The number of eosinophils in the submucosa was similar in asthma and eosinophilic bronchitis, suggesting that expression of IL-13 by eosinophils is different between these two conditions.

The validity of the measurement of any cytokine in induced sputum samples is dependent on a robust assay. The recovery of some cytokines, such as IL-5, has been improved by the addition of protease inhibitors.[12] The use of dithiothreitol as a mucolytic to aid dispersion of the sputum sample has proven particularly problematic in the measurement of some cytokines and chemokines. A recent study reported that the recovery of CCL11 was markedly affected by dithiothreitol, and good recovery was achieved only by omitting dithiothreitol in the processing of the sample.[13] We found that the recovery of IL-13 was also affected by the addition of the mucolytic dithiothreitol. This problem was overcome by dialyzing the samples to remove the dithiothreitol. We are confident that our IL-13 assay was valid, because the recovery of exogenous spiked IL-13 was almost 100%. Whether dialysis of sputum supernatant may improve the recovery of other cytokines and mediators affected by the addition of dithiothreitol needs to be further explored.

In asthma, IL-13 is elevated in BAL, bronchial biopsies, and sputum.[14-16] In addition, IL-13, but not IL-4, has been shown to attenuate relaxation to β-agonists and to augment contractility to acetylcholine, suggesting that one of the main effects of IL-13 may be the induction of airway hyperresponsiveness.[17,18] In support of this view, we found that the sputum IL-13 concentration and IL-13 expression in the bronchial submucosa was increased in asthma compared with eosinophilic bronchitis, a condition with a similar immunopathology to asthma, but with normal airway responsiveness.[3-5] One important question is why there is differential expression of IL-13 in asthma and eosinophilic bronchitis. Several cell types have been described as important sources of IL-13, including T-lymphocytes,[14,15,19] mast cells,[20] eosinophils,[21] and basophils.[22] We cannot be certain of the cellular source of IL-13 in the sputum; however, we found that most of the cells that expressed IL-13 protein in the bronchial submucosa were eosinophils, with a smaller proportion that were mast cells. There was a similar degree of eosinophilic inflammation in asthma and eosinophilic bronchitis. Thus, the proportion of eosinophils that expressed IL-13 was increased in asthma compared with eosinophilic bronchitis, suggesting that there is a fundamental difference in the production of IL-13 by airway eosinophils between these two conditions. Further work is required to investigate this difference in eosinophil function between eosinophilic bronchitis and asthma.

In summary, the increased expression of IL-13 in asthma compared with eosinophilic bronchitis supports the hypothesis that IL-13 plays a role in the pathophysiology of asthma. Specific therapies directed at IL-13 may offer additional benefit in the treatment of asthma.

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