

Pediatric severe asthma is characterized by eosinophilia and remodeling without T_H2 cytokines

Cara J. Bossley, MBChB,^{a,b} Louise Fleming, MD,^{a,b} Atul Gupta, MBBS,^{a,b} Nicolas Regamey, MD,^c Jennifer Frith, MBBS,^a Timothy Oates, BSc,^b Lemonia Tsartsali, MD,^a Clare M. Lloyd, PhD,^b Andrew Bush, MD,^a and Sejal Saglani, MD^b *London, United Kingdom, and Bern, Switzerland*

Background: The pathology of pediatric severe therapy-resistant asthma (STRA) is little understood.

Objectives: We hypothesized that STRA in children is characterized by airway eosinophilia and mast cell inflammation and is driven by the T_H2 cytokines IL-4, IL-5, and IL-13.

Methods: Sixty-nine children (mean age, 11.8 years; interquartile range, 5.6–17.3 years; patients with STRA, n = 53; control subjects, n = 16) underwent fiberoptic bronchoscopy, bronchoalveolar lavage (BAL), and endobronchial biopsy.

Airway inflammation, remodeling, and BAL fluid and biopsy specimen T_H2 cytokines were quantified. Children with STRA also underwent symptom assessment (Asthma Control Test), spirometry, exhaled nitric oxide and induced sputum evaluation.

Results: Children with STRA had significantly increased BAL fluid and biopsy specimen eosinophil counts compared with those found in control subjects (BAL fluid, $P < .001$; biopsy specimen, $P < .01$); within the STRA group, there was marked between-patient variability in eosinophilia. Submucosal mast cell, neutrophil, and lymphocyte counts were similar in both groups. Reticular basement membrane thickness and airway smooth muscle were increased in patients with STRA compared with those found in control subjects ($P < .0001$ and $P < .001$, respectively). There was no increase in BAL fluid IL-4, IL-5, or IL-13 levels in patients with STRA compared with control subjects, and these cytokines were rarely detected in induced sputum. Biopsy IL-5⁺ and IL-13⁺ cell counts were also not higher in patients with STRA compared with those seen in

control subjects. The subgroup (n = 15) of children with STRA with detectable BAL fluid T_H2 cytokines had significantly lower lung function than those with undetectable BAL fluid T_H2 cytokines.

Conclusions: STRA in children was characterized by remodeling and variable airway eosinophil counts. However, unlike in adults, there was no neutrophilia, and despite the wide range in eosinophil counts, the T_H2 mediators that are thought to drive allergic asthma were mostly absent. (*J Allergy Clin Immunol* 2012;■■■■:■■■-■■■.)

Key words: Pediatric asthma, eosinophilia, remodeling, severe therapy-resistant asthma, mediators

The 5% of patients with asthma classified as severe consume more than 50% of health care resources for the disease. Previous pediatric studies have investigated the pathology of “difficult asthma”^{1,2} after a steroid trial and also mild-to-moderate asthma,^{3,4} but the pathology of true severe therapy-resistant disease has been little studied. Children referred to us for evaluation of symptoms refractory to high-dose conventional asthma therapy are considered to have “problematic severe asthma.”⁵ This group comprises those with the wrong diagnosis, asthma with comorbidity, difficult asthma, and severe therapy-resistant asthma (STRA). They undergo detailed evaluation both at home and in the hospital.^{5,6} Those with difficult asthma in whom potentially reversible factors (persistent allergen exposure, poor adherence, and poor inhaler technique) contributing to poor asthma control are identified are excluded.⁵ Those remaining with genuine STRA undergo invasive testing with bronchoscopy, bronchoalveolar lavage (BAL), and endobronchial biopsy to characterize airway pathology and develop an individualized treatment plan.⁷

Previous studies have reported submucosal eosinophilia in pediatric patients with mild-to-moderate asthma^{3,4} but not in children with problematic severe asthma.² However, this included patients with difficult asthma and subjects in whom bronchoscopy was performed after a high-dose steroid trial. A further report in patients with difficult asthma has shown increased epithelial eosinophil and neutrophil counts and higher BAL levels of IFN- γ in children with persistent symptoms compared with those with good symptom control.⁸ However, there was no nonasthmatic control group and no assessment of basic asthma management.

In summary, the basic mechanisms and airway pathology in children with true STRA are unknown. Adult studies suggest that in many patients the pathobiology of severe asthma is mediated by immune pathways driven by T_H2-type CD4⁺ T cells, with signature cytokines including IL-4, IL-5, and IL-13.^{9,10} Hence biological modifiers of T_H2-type cytokines are a rational strategy

From ^aRespiratory Paediatrics, the Royal Brompton and Harefield NHS Trust, Sydney Street, London; ^bthe Leukocyte Biology Section, National Heart & Lung Institute, Imperial College London; and ^cthe Division of Paediatric Respiratory Medicine, Department of Paediatrics, Inselspital and University of Bern.

J.F. was funded by an Asthma UK Foundation Grant and a grant from the Royal Brompton & Harefield Biomedical Research Unit. S.S. is funded by the Wellcome Trust, UK, grant no. 083586/Z/07/Z. N.R. is the recipient of a European Respiratory Society Fellowship (no. 64) and a grant of the Swiss National Science Foundation (no. 1172/05b). The project was supported by the NIHR Respiratory Disease Biomedical Research Unit at the Royal Brompton and Harefield NHS Foundation Trust and Imperial College London.

Disclosure of potential conflict of interest: A. Gupta receives research support from the British Medical Association. N. Regamey is on advisory boards for Merck-Sharp and Dome and Novartis. C. M. Lloyd is a consultant for MedImmune. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication August 5, 2011; revised January 17, 2012; accepted for publication January 23, 2012.

Corresponding author: Sejal Saglani, MD, Respiratory Paediatrics, Imperial College London, 374 Sir Alexander Fleming Building, Exhibition Road, London SW7 2AZ, United Kingdom. E-mail: s.saglani@imperial.ac.uk.

0091-6749/\$36.00

© 2012 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2012.01.059

Abbreviations used

ASM:	Airway smooth muscle
BAL:	Bronchoalveolar lavage
BDR:	Bronchodilator reversibility
CV:	Coefficient of variation
DTT:	Dithiothreitol
H&E:	Hematoxylin and eosin
ICS:	Inhaled corticosteroid
IQR:	Interquartile range
RBM:	Reticular basement membrane
STRA:	Severe therapy-resistant asthma
Vs:	Subepithelial volume fraction of airway smooth muscle indexed to surface area of RBM
Vv:	Subepithelial volume fraction of airway smooth muscle indexed to subepithelial tissue

for developing new treatment approaches in adults.⁹ However, all studies to date, whether *in vivo* experimental models or *in vitro* models, have been conducted with adult animals¹¹ or samples,¹² respectively. Children with STRA are known to be markedly atopic,^{13,14} and therefore we hypothesized that these children would also have eosinophilic and mast cell inflammation and that childhood STRA would be characterized by increased levels of the T_H2 cytokines IL-4, IL-5, and IL-13. The aim of this study was to characterize the pathology and mediators of inflammation and remodeling in patients with STRA.

METHODS**Subjects**

Children aged 5 to 16 years referred to Royal Brompton Hospital with problematic severe asthma between April 2005 and June 2009 were included.⁶ Definitions of poor control and entry criteria were as follows:

1. persistent (most days for ≥ 3 months) chronic symptoms (which prompt use of short-acting β_2 -agonists ≥ 3 times per week) of airway obstruction despite high doses of inhaled corticosteroids (ICSs; ≥ 800 $\mu\text{g}/\text{d}$ budesonide equivalent) and/or regular oral corticosteroids, long-acting β_2 -agonists, and current (or previous failed trial of) montelukast OR
2. recurrent severe exacerbations requiring 1 or more admission to the intensive care unit or 2 or more hospital admissions requiring intravenous medications or 2 or more courses of oral corticosteroids in the past year despite therapy for persistent symptoms as described in (1) above OR
3. at least 1 very sudden (≤ 6 hours) severe attack (requiring hospitalization) without warning despite therapy for persistent symptoms, as described in (1) above.

Outpatient nurse-led assessments were performed to assess medication, dose, device and technique, atopic status, and asthma understanding. A home and school visit was undertaken to assess adherence, the environment, and any psychosocial issues. Evaluation of adherence included prescription records, accessibility of medications, and whether medications were in date and/or unwrapped; allergen sources, such as pets within the home; and environmental tobacco smoke exposure (including cotinine measurements).

After assessment,⁶ those reclassified as having difficult asthma⁵ were excluded (Fig 1). The remaining patients with STRA were further investigated. The study was approved by the local research ethics committee, and all procedures were performed after obtaining written informed parental consent and, where appropriate, child assent.

Nonasthmatic control subjects (aged 6–16 years) were either (1) undergoing a bronchoscopy to investigate upper airway symptoms and agreed to extra research samples being taken or (2) were undergoing general anesthesia for cardiac catheterization and agreed to have a research bronchoscopy at the same time. They had no history of wheeze or lower airway symptoms, and all

were deemed sufficiently stable that the extra few minutes to take research samples¹⁵ were permissible, as judged by an anesthetist independent of the study. The clinical characteristics of control subjects are shown in Table E1 in this article's Online Repository at www.jacionline.org.

Investigations

All investigations were performed on the same day. None of the children had an exacerbation at the time of bronchoscopy, and at least 2 weeks were required between the last exacerbation and investigations. Details of investigations are given in the **Methods** section in this article's Online Repository at www.jacionline.org.

Atopic status. Atopy was defined as 1 or more positive specific IgE RAST (≥ 0.34 kU/L) or 1 or more positive skin prick test results to aeroallergens.

Symptom control. Asthma control was assessed by using the Asthma Control Test^{16,17}; poor control was defined as a score of less than 20 of 25 (see Fig E1 in this article's Online Repository at www.jacionline.org).

Lung function. Spirometry and bronchodilator reversibility (BDR) defined as a greater than 12% change from baseline FEV₁ were measured according to American Thoracic Society/European Thoracic Society guidelines.^{18,19}

Exhaled nitric oxide. Exhaled nitric oxide measurements at a flow rate of 50 mL/s were made with a chemiluminescence analyzer (NIOX; Aerocrine AB, Solna, Sweden) in accordance with American Thoracic Society/European Thoracic Society guidelines.²⁰

Sputum induction and processing. Sputum induction was performed with 3.5% saline (0.9% if postbronchodilator FEV₁ was $< 65\%$ of predicted value).²¹ Samples were processed by adding 0.1% dithiothreitol (DTT). Supernatants were stored at -80°C . Samples were considered adequate if 80% or fewer squamous cells and 400 or more inflammatory cells were present and distinguishable.

Bronchoscopy, BAL, and endobronchial biopsy. Bronchoscopy, BAL, and endobronchial biopsy were performed under general anesthesia, as previously described.²² BAL fluid was processed for cytology by the hospital cytopathology department. Eosinophilia was defined as greater than 1.19%²³ and neutrophilia as greater than 3.5%²⁴ of the total cell count. Paraffin-embedded biopsy specimens were cut and stained with hematoxylin and eosin (H&E) to assess quality and adequate morphology and also to quantify airway remodeling (reticular basement membrane [RBM] thickness,²⁵ epithelial shedding,⁴ and airway smooth muscle [ASM] mass²⁶). Evaluable sections were also stained for eosinophils (Congo red dye), neutrophils (neutrophil elastase), mast cells (mast cell tryptase), and CD45⁺, CD4⁺, and CD8⁺ cells (for more information, see the **Methods** section in this article's Online Repository). All sections were coded, and inflammatory cells were expressed per square millimeter of tissue.²⁵

Induced sputum, BAL fluid, and biopsy specimen cytokine analysis.

Cytokines were quantified in BAL fluid and sputum supernatants by using the Luminex multiplex bead analysis system. The premade 21-plex Luminex plate (Bio-Rad Laboratories, Hercules, Calif) was used to assay levels of IL-1 β , IL-4, IL-5, IL-8, IL-13, eotaxin, and IFN- γ . In addition, the Cytometric Bead Array (CBA Human Inflammation kit; BD Biosciences, PharMingen, San Diego, Calif) was used to quantify IL-5 and IL-13 (for more information, see the **Methods** section in this article's Online Repository).²⁷ Endobronchial biopsy specimens were stained for IL-5⁺ and IL-13⁺ cells by using immunohistochemistry, and positive submucosal cells were quantified and expressed per square millimeter of tissue (for more information, see the **Methods** section in this article's Online Repository).

Additional investigations to exclude alternate diagnoses.

All children in whom BDR could not be demonstrated or who were nonatopic underwent high-resolution computed tomographic chest scans to exclude diagnoses, such as bronchiectasis or obliterative bronchiolitis. They also underwent blood tests to exclude rheumatologic diseases and pH studies to exclude gastroesophageal reflux.

Statistical analysis

There are insufficient data to inform a power calculation for pediatric biopsy studies; adult studies have reported that groups of 11 to 17 are

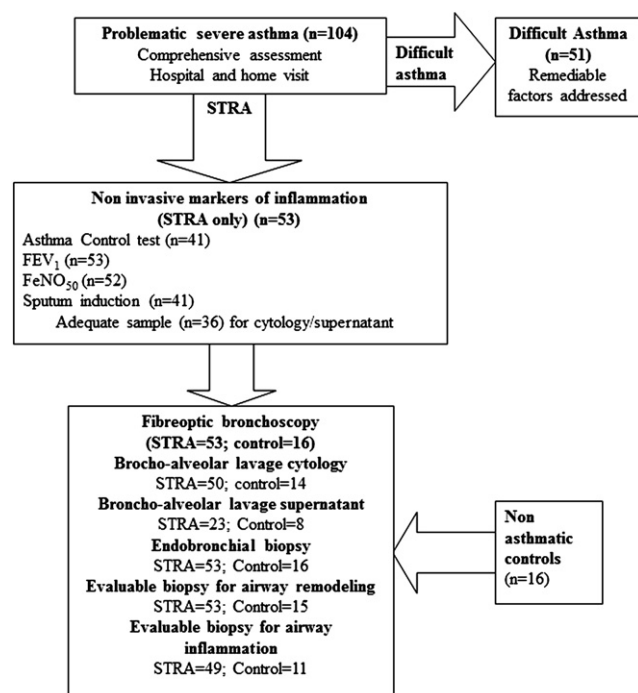


FIG 1. Flow diagram illustrating the number of children with problematic severe asthma who were assessed and how many patients with STRA underwent invasive investigations to assess airway pathology. FE_{NO50} , Fraction of exhaled nitric oxide at 50 mL/s.

sufficient.²⁵ The sample size for control subjects was opportunistic. Differences between groups were assessed by using the Mann-Whitney *U* test. Associations were assessed with Spearman correlation. Intraobserver repeatability was measured by calculating the coefficient of variation (CV) of 3 measurements of a section made on 3 occasions at least 3 days apart. Within-subject variability was assessed based on the CV of counts from 3 or more biopsy specimens from the same subject. Analysis was performed with the SPSS software, version 17 (SPSS, Inc, Chicago, Ill). A *P* value of less than .05 was considered significant for between-group comparisons, and a *P* value of less than .01 was considered significant for correlations.

RESULTS

Demographics

Of 104 patients referred with problematic severe asthma, 51 were classified as having difficult asthma (Fig 1). Fifty-three (51%) of 104 patients with true STRA were further investigated. The clinical characteristics of subjects are shown in Table I. Forty-five (85%) of 53 patients with STRA were atopic compared with 7 (47%) of 15 control subjects (*P* < .01). The median baseline FEV_1 in patients with STRA was 68.5% (interquartile range [IQR], 54.8% to 86.5%) of predicted value compared with 94% (IQR, 85% to 106%) in control subjects (*P* < .0001).

Bronchoscopic findings

There were no complications after bronchoscopy. Findings from nonasthmatic control subjects are listed in Table E1. Positive BAL fluid bacterial cultures were present in 10 (19%) of 53 patients with STRA, of whom 7 (70%) of 10 also had BAL fluid neutrophilia. Positive cultures and neutrophilia were present in 2 control subjects. BAL fluid supernatants from patients with a positive bacterial culture and neutrophilia were excluded from cytokine analysis.

TABLE I. Clinical characteristics of children with STRA and nonasthmatic control subjects

	Children with STRA (n = 53)	Control subjects (n = 16)
Age (y)	11.97 (9.97-14.4)	10.65 (7.54-12.96)
Male sex	33/53 (62%)	8/16 (50%)
Atopy	45/53 (85%)	7/15 (47%)*
Age at first symptoms (y)	1 (0.25-2.25)	—
Duration of symptoms (y)	10.1 (8.3-12.7)	—
Previous intubation for asthma	11/53 (21%)	—
Parental smoking	13/53 (25%)	2/9 (22%)
Percent predicted FEV_1	68.5 (54.8-86.5)	94 (85-106)†
BDR (%)	15.6 (5.5-23.4)	2.5 (0-8.5)
Persistent airflow limitation	13/53 (25%)	—
Total IgE (IU/mL)	386 (115-1286)	40 (14-130)
Medications		
ICS (mg/d)‡	1.6 (0.8-1.6)	—
Maintenance oral prednisolone	24/53 (45%)	—
Long-acting β -agonist	53/53 (100%)	—
Leukotriene receptor antagonist	31/53 (58%)	—
Theophylline	12/53 (23%)	—
ACT score (/25)	13 (9-17)	—
FE_{NO50} (ppb; normal, <24 ppb)	50.3 (29.3-69.7)	—
Sputum eosinophils (%; normal, <2.5%)	7.5 (3.2-30.4)	—

Values are presented as medians (IQRs).

ACT, Asthma Control Test; FE_{NO50} , fraction of exhaled nitric oxide at a flow rate of 50 mL/s.

**P* < .01.

†*P* < .05.

‡Budesonide equivalent dose.

Inflammation in BAL fluid and biopsy specimens

Although BAL fluid and biopsy specimens were taken from all subjects, not all samples were of sufficient quality for analysis. One hundred twenty-one biopsy specimens were taken from 69 subjects, and at least 1 specimen was suitable for assessment for all subjects, except 1 control child. Three patients with STRA and 4 control subjects only had evaluable H&E-stained sections and therefore only had quantification of airway remodeling. The CV for intraobserver repeatability for all biopsy specimen inflammatory cells and cytokines was less than 10% (see Table E2 in this article's Online Repository at www.jacionline.org). The intraobserver repeatability for RBM thickness, epithelial shedding, and oral corticosteroid subepithelial volume fraction of ASM indexed to subepithelial tissue (Vv) smooth muscle were 4%, 6.4%, and 11.6%, respectively. The within-subject between-biopsy variability for eosinophil counts was 54% (see Table E3 in this article's Online Repository at www.jacionline.org). However, the between-subject variation in biopsy specimen eosinophil counts for patients with STRA was 185%.

BAL fluid was available in 50 of 53 patients with STRA and 14 of 16 control subjects (Fig 1). Children with STRA had significantly increased BAL fluid eosinophil counts compared with those seen in control subjects (median, 2.7% [IQR, 1% to 7.7%] vs 0% [IQR, 0% to 0.9%], respectively; *P* < .001), although there was wide variability among patients with STRA (Fig 2, A). Subepithelial mucosal eosinophil counts were significantly higher in patients with STRA than in control subjects (median, 11.2/mm² [IQR, 0-33.7/mm²] vs 0/mm² [IQR, 0-25.1/mm²], respectively; *P* < .01; Fig 2, B-D).

There were no group differences in BAL fluid neutrophil or lymphocyte counts, submucosal neutrophil counts, mast cell

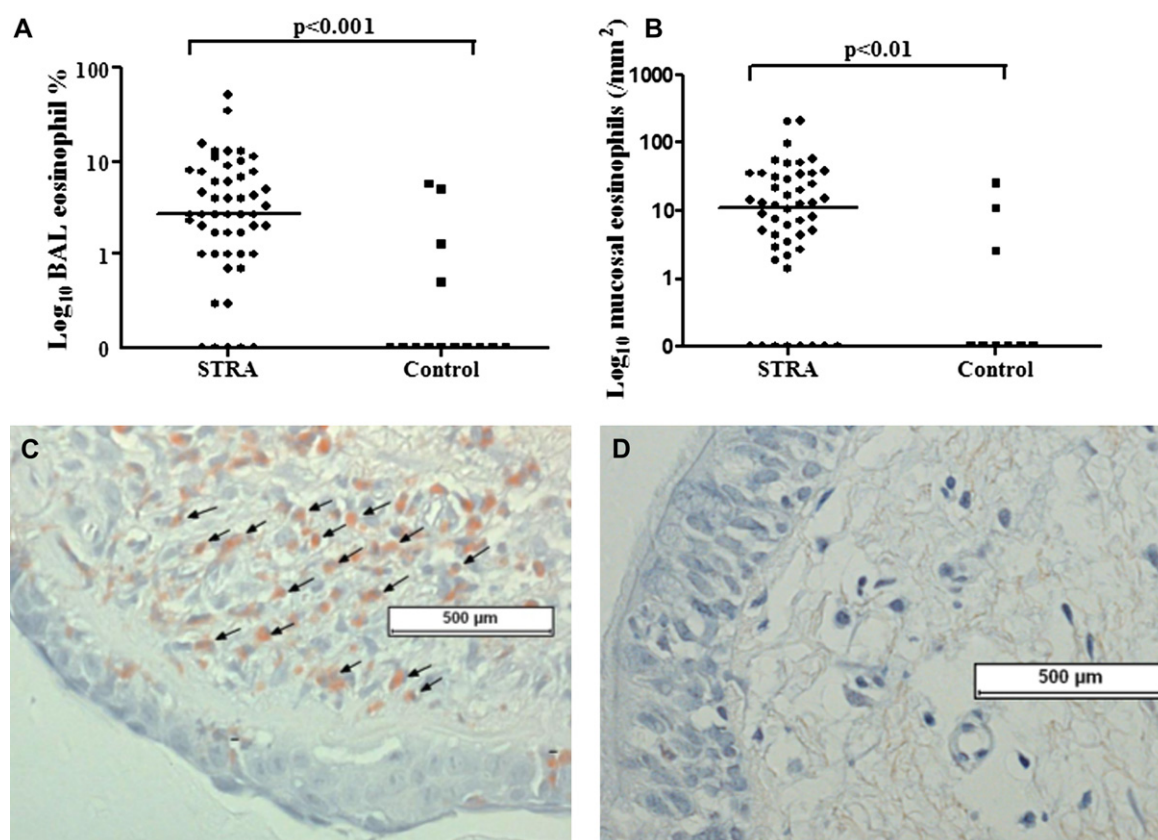


FIG 2. A and B, Increased BAL fluid eosinophil (Fig 2, A) and submucosal eosinophil (Fig 2, B) counts in children with STRA compared with those seen in control subjects. C, A 5- μm paraffin section stained with Congo red dye showing positively stained eosinophils (black arrows) in the submucosa of a patient with STRA compared with an absence of submucosal eosinophils in a control subject (D; original magnification $\times 400$).

counts, and $\text{CD}45^+$, $\text{CD}4^+$, or $\text{CD}8^+$ cell counts (Table II). Intraepithelial and smooth muscle inflammatory cells were similar between groups. No associations were found between mucosal inflammation and lung function, sex, intubation for asthma, persistent airflow limitation, gastroesophageal reflux, or serum IgE levels.

BAL fluid and sputum cytokine levels

There were no significant differences between patients with STRA and control subjects in levels of BAL fluid IL-1 β , IL-4, IL-5, IL-8, IL-13, IFN- γ , and eotaxin when measured with the Luminex multiplex assay (see Table E4 in this article's Online Repository at www.jacionline.org). A few children with STRA had detectable levels of BAL fluid IL-4 ($n = 10$), IL-5 ($n = 8$), and IL-13 ($n = 8$); however, the group median value was in the undetectable range (see Table E4). To confirm these findings, BAL fluid IL-5 and IL-13 levels were also quantified by using the Cytometric Bead Array, and again, there was no difference between patients with STRA and control subjects (see Table E4). The limits of detection for cytokines used in both assays are shown in Table E5 in this article's Online Repository at www.jacionline.org.

There was no difference in BAL fluid $\text{T}_\text{H}2$ cytokine levels between children with persistent symptoms and those with frequent exacerbations. However, children who had any detectable BAL fluid $\text{T}_\text{H}2$ cytokines (any of IL-4, IL-5, or IL-13) had significantly

lower FEV_1 and forced vital capacity (FVC) percent predicted values and greater BDR than those with undetectable $\text{T}_\text{H}2$ cytokines (Table III).

Forty-one children underwent sputum induction, and 36 had a sufficient sample. Children with STRA, as a group, had undetectable levels of IL-4, IL-13, and IFN- γ in sputum (see Table E4). Eight children with STRA had detectable levels of sputum IL-5 (median, 0.9 pg/mL [IQR, undetectable-1.0 pg/mL]).

Submucosal IL-5 $^+$ and IL-13 $^+$ cells

Tissue expression of IL-5 and IL-13 was assessed to further support findings from the BAL fluid and sputum cytokine quantification. The intraobserver repeatability of biopsy specimen cytokine-positive cells was less than 10%. The within-subject, between-specimen variability for biopsy specimen IL-5 expression was 39%, and the between-subject variability in biopsy specimen IL-5 expression for control subjects was 69%. There was a significantly higher number of IL-5 $^+$ submucosal cells in control subjects compared with numbers seen in children with STRA (median: STRA, 47.3/ mm^2 [IQR, 27.6-96.4/ mm^2] vs control subjects, 154.3/ mm^2 [IQR, 72.6-257.1/ mm^2]; $P < .05$; Fig 3, A). There were no significant differences in numbers of submucosal IL-13 $^+$ cells between patients with STRA and control subjects (median, 15.95/ mm^2 [IQR, 9.52-34.2/ mm^2] vs 8.58/ mm^2 [IQR, 2.08-102.3/ mm^2]), respectively (Fig 3, B).

TABLE II. Airway inflammation in children with STRA compared with nonasthmatic control subjects

Cell count	Patients with STRA (n = 53)	Control subjects (n = 16)	P value
BAL fluid neutrophils (%)	3.5 (2-7.2)	2.7 (1.2-7.5)	.37
Biopsy specimen neutrophils (/mm ²)	8.3 (0-26.4)	1.2 (0-20.0)	.32
Biopsy specimen mast cells (/mm ²)	45.7 (25.2-69.6)	60.5 (45.1-76.4)	.20
Biopsy specimen CD45 ⁺ cells (/mm ²)	149 (3.7-419.9)	209.7 (168.8-244)	.11
Biopsy specimen CD8 ⁺ cells (/mm ²)	51 (17.9-80.5)	54.8 (10.1-116.8)	.60
Biopsy specimen CD4 ⁺ cells (/mm ²)	145.4 (77.0-224.5)	176.5 (23.5-259)	.92
Biopsy specimen CD4/CD8 ratio	3.30 (1.77-5.89)	2.97 (1.86-12.43)	.93

Values are presented as medians (IQRs).

Relationship between IL-5 levels and eosinophilic inflammation

There was no difference in biopsy specimen, BAL fluid, or sputum eosinophil counts in subjects with STRA in whom IL-5 was detected compared with counts in those in whom IL-5 was undetectable (see Fig E3). Those children in whom IL-5 was detectable in sputum had a significantly lower FEV₁ than those with absent IL-5 ($P < .05$).

Airway remodeling

Children with STRA had increased RBM thickness compared with that seen in control subjects (7.12 μ m [IQR, 6.37-7.89 μ m] vs 4.89 μ m [IQR, 4.16-6.16 μ m], respectively; $P < .0001$; Fig 4, A). Epithelial shedding was similar in both groups (Fig 4, B). Vv was significantly increased in patients with STRA compared with control subjects (0.20 [IQR, 0-0.65] vs 0.09 [IQR, 0-0.16]; $P < .001$; Fig 4, C). The subepithelial volume fraction of ASM indexed to the surface area of RBM (Vs) was also increased in patients with STRA compared with that seen in control subjects (19.83 [IQR, 10.85-36.93] vs 9.14 [IQR, 4.55-15.88]; $P < .01$; Fig 4, D).

Influence of atopy on inflammation, remodeling, and T_H2 cytokines

When all subjects were compared, BAL fluid and biopsy eosinophil counts were higher in atopic compared with nonatopic subjects. RBM thickness, epithelial shedding, and ASM Vv were also greater in atopic compared with nonatopic children (Tables IV and V). However, atopic children with STRA had increased ASM mass compared with atopic control subjects (Tables IV and V).

Influence of steroids on inflammation, remodeling, and T_H2 cytokines

There was no relationship between dose of inhaled or maintenance oral steroids and any parameters of inflammation or detection of T_H2 cytokines in children with STRA (see Tables E6 and E7 in this article's Online Repository at www.jacionline.org).

TABLE III. Clinical characteristics of children with STRA with any detectable BAL fluid T_H2 cytokines (IL-4, IL-5, or IL-13) compared with those without detectable T_H2 cytokines

	Detectable BAL T _H 2 cytokines (n = 15)	No detectable BAL T _H 2 cytokines (n = 8)	P value
Age (y)	11.2 (9.4-14.4)	11.7 (10.0-12.3)	.78
Steroid dose:			
Maintenance (mg/d)	6.25 (5.0-7.5)	5.6 (5.0-7.2)	.80
ICS	1.6 (1.2-1.6)	1.4 (0.9-1.6)	.27
Exacerbations (in past year)	8 (4-8.5)	5 (4-10)	1.0
Admissions in past year	1.5 (0-5)	0 (0-10)	.70
Percent predicted FEV₁	65 (42.5-71)	91.5 (83.5-96.8)	<.001
Percent predicted FVC	90 (61-99)	106 (94.5-110)	.03
BDR (%)	19 (14-33)	8.1 (3.6-17.4)	.03
ACT score (/25)	10 (8-17)	13.5 (8-16.5)	.47
BMI (kg/m²)	17.6 (16.1-18.6)	23.8 (21-28)	.009
BMI percentile	66 (22-78)	99 (96-99.7)	.005

Values are presented as medians (IQRs). Boldface text indicates statistically significant results.

ACT, Asthma Control Test; BMI, body mass index; FVC, forced vital capacity.

However, there was a negative association between ASM mass and oral steroid dose (ASM Vv: $r = -0.63$, $P < .002$; ASM Vs: $r = -0.73$, $P < .001$).

DISCUSSION

In this large pediatric bronchoscopic study, an important novel facet is the use of prior detailed evaluation to exclude as far as possible all those who were nonadherent or had other confounding issues. We have shown that, unlike in adults,²⁸⁻³⁰ STRA in children is characterized by eosinophilic and not neutrophilic airway inflammation, there is no mast cell myositis,³¹ and the eosinophilic inflammation is not driven by signature T_H2 cytokines in most patients.⁹

Quantification of BAL fluid cytokines was performed by using 2 separate assays, and levels of T_H2 cytokines remained undetectable in patients with STRA and control subjects by using both techniques, despite the limits of detection for T_H2 cytokines with both assays being low. Indeed, the assays were sensitive enough to detect the levels of cytokines found in studies of severe eosinophilic asthma, with a comparable degree of airway eosinophilia.³² In addition, induced sputum supernatants were analyzed because these are less dilute with similar results. However, our sputum samples were processed with DTT, which can affect cytokine measurements.³³ Inspection of the calibration curves (see Fig E2 in this article's Online Repository at www.jacionline.org) showed that DTT did not affect IL-5 quantification but might have reduced the sensitivity for IL-4 and IL-13. DTT rendered sputum eotaxin completely undetectable. Finally, to support the BAL fluid and sputum findings, submucosal IL-5 and IL-13 expression were also quantified in biopsy specimens. Again, no differences were found in the expression of IL-13 between patients with STRA and control subjects, and if anything, IL-5⁺ cell counts were higher in control subjects. However, the spread of data is wide (Fig 3, A) and suggests the control subjects likely represent the normal range (because they include atopic subjects). We acknowledge that our control subjects were having a clinically indicated bronchoscopy and were therefore undergoing invasive procedures for significant medical problems, which might be a confounder. However, cytokine levels were

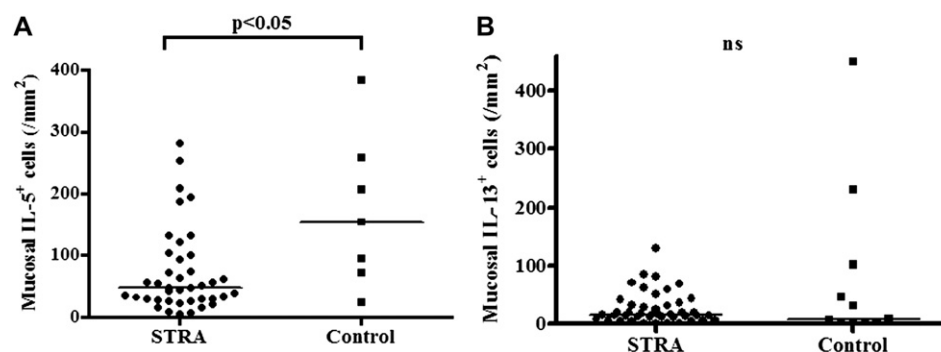


FIG 3. Submucosal IL-5⁺ (A) and IL-13⁺ (B) cell counts in biopsy specimens from children with STRA compared with control subjects. Submucosal IL-5⁺ cell counts were higher in control subjects compared with those seen in patients with STRA, and levels of IL-13⁺ cells were similar in both groups. Analyses were performed with the Mann-Whitney *U* test. *ns*, Not significant.

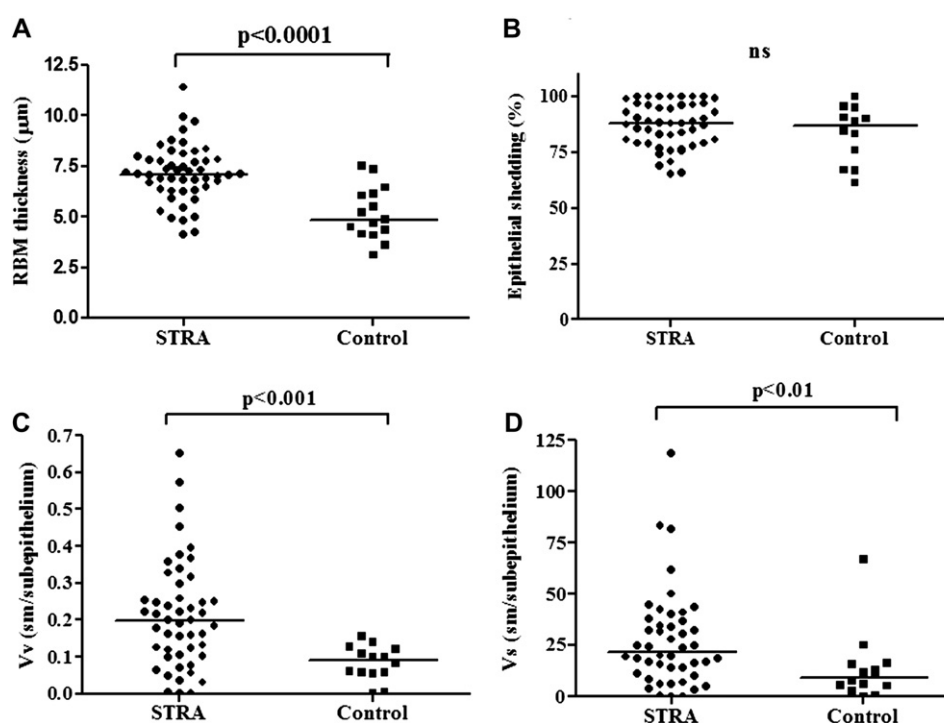


FIG 4. Markers of airway remodeling showing increased RBM thickness in patients with STRA compared with control subjects (A), similar epithelial shedding in patients with STRA and control subjects (B), increased volume of ASM indexed to subepithelium in patients with STRA compared with control subjects (C), and increased volume of ASM indexed to RBM in patients with STRA compared with control subjects (D). *ns*, Not significant.

predominantly undetectable in both groups, suggesting this confounder did not cause increases in false-positive results. Eight (15%) of 53 children with STRA had detectable levels of BAL fluid and sputum IL-5; however, even in these children, there was no relationship between sputum, BAL fluid, or biopsy eosinophil counts and IL-5 levels. We therefore believe that failure to detect increased T_H2 cytokine levels in our patients with STRA is real because only a very small number of children with STRA had any detectable levels in any compartment that we examined. Eight of 20 patients with STRA with BAL fluid and sputum samples had no detectable T_H2 cytokines in either. None of the patients with detectable T_H2 cytokines in sputum and BAL fluid had high tissue cytokine expression (defined as greater than the mean value for

control subjects in biopsy specimens). Even levels of IL-5, which could be reliably quantified in BAL fluid, sputum, and biopsy specimens and is most closely related to eosinophil recruitment, were not increased.

When we split the children with STRA into those with any detectable BAL fluid T_H2 cytokines (IL-4, IL-5, or IL-13) and undetectable T_H2 cytokines, the former had significantly lower spirometric results and greater BDR (Table III), suggesting more severe disease. Looking for the presence of any T_H2 cytokines in children with STRA might therefore be a biomarker that allows identification of the subgroup more likely to respond to specific antibody therapies directed against T_H2 cytokines. This suggests that pediatric STRA is heterogeneous and that treatment needs to

TABLE IV. Differences in airway inflammation and remodeling between atopic and nonatopic subjects

	All atopy (n = 52)	All without atopy (n = 16)	P value	STRA with atopy (n = 45)	STRA without atopy (n = 8)	P value	Control with atopy (n = 7)	Control without atopy (n = 8)	P value
Percent predicted FEV₁	68.5 (58.3-85.5)	86 (81-96)	.04	66 (55.8-83.1)	81.5 (52.3-92.3)	.53	94.8 (79.4-107.8)	92 (86-105)	.78
BDR (%)	14 (5.3-22.7)	16 (11-28.9)	.94	15.6 (6.9-23.2)	16 (1.1-28.9)	.78	2.5 (0-8.5)	*	*
FENO ₅₀ (ppb)				56.5 (31.4-70.6)	40.8 (15.3-49.5)	.11			
Serum IgE (IU/mL)	428 (150-1354)	28.5 (11.3-117)	<.001	440 (202-1452)	74.5 (16.8-166.7)	.001	97 (33-1101)	23 (4-43)	.07
Blood eosinophils (%)	4.1 (1-9)	1.4 (0-2)	.05	4.2 (0.95-9.2)	1.3 (0-6.7)	.29	3.1 (2.2-5.9)	1.5 (0.7-2)	.052
Sputum eosinophils (%)				7.5 (3.2-30.4)	9.4 (3.1-18)	.86			
Sputum neutrophils (%)				33 (12.5-71.1)	51.5 (24.1-74.0)	.42			
BAL fluid eosinophils (%)	2.7 (1-6)	0 (0-4)	.01	2.7 (1-7)	3.4 (0.3-8.8)	.83	0.9 (0-5.2)	0 (0)	.07
BAL fluid neutrophils (%)	3.3 (1.3-8.0)	3.7 (2-6.7)	.90	3.3 (1.2-7.3)	4.5 (3.4-8.8)	.43	4 (1.2-10.7)	2 (1-3.7)	.44
Biopsy specimen eosinophils (/mm²)	12 (2.6-34.7)	0 (0-8.3)	.01	12.9 (3.3-35.3)	4.13 (0-10.98)	.07	0 (0-17.9)	0 (0)	.79
Biopsy specimen neutrophils (/mm ²)	7.64 (0-25.5)	0.8 (0-28.8)	.50	9.61 (0-25.7)	3.1 (0-40.3)	.94	2.4 (0-30.9)	0 (0-17.8)	.56
Biopsy specimen mast cells (/mm ²)	46.5 (29.2-69.9)	55.8 (23.4-72.6)	.79	45.8 (29.2-68.3)	40.2 (17.2-87.4)	.70	62.7 (20.2-117.3)	63.7 (50.5-73.7)	1.0
Biopsy specimens CD4 ⁺ cells (/mm ²)	151.6 (92.3-225.4)	124.9 (24.2-239.1)	.49	151.6 (108-225.4)	74.5 (21.8-355.2)	.20	116.2 (23.5-286.1)	198.8 (89.01-249.1)	1.0
Biopsy specimen CD8 ⁺ cells (/mm ²)	31.0 (14.9-84.3)	60.4 (23.6-84.4)	.54	51 (16.8-84.3)	58.1 (21.6-84.8)	.77	30.8 (4.7-115.1)	60.4 (32.9-88.9)	.49
Biopsy specimen CD45 ⁺ cells (/mm ²)	164.4 (88.3-205.4)	201.1 (134.3-282.7)	.07	156.6 (88.3-202.4)	160.7 (107.1-373.2)	.40	190.9 (54.1-248.2)	228.9 (197.4-244)	.69
Biopsy specimen CD4/CD8 ratio	3.47 (1.8-5.5)	2.4 (0.3-12.0)	.59	3.47 (1.8-5.5)	2.1 (0.29-25.9)	.54	2.9 (2-9.4)	3.3 (1.3-9.7)	.89
RBM thickness (μm)	7.10 (6.30-7.84)	4.83 (4.22-6.69)	.001	7.26 (6.79-8.13)	6.29 (4.96-7.23)	.04	6.1 (4.9-6.5)	4.35 (3.63-4.71)	.07
Epithelial shedding (%)	88.8 (79.0-96.3)	81.9 (66.3-89.8)	.04	88.6 (79.8-96.5)	79.9 (68.3-87.3)	.06	90.1 (71.3-95.2)	83.9 (64.2-95.1)	.79
ASM Vv	0.18 (0.10-0.26)	0.1 (0.05-0.16)	.02	0.2 (0.1-0.35)	0.16 (0.10-0.25)	.52	0.10 (0.06-0.14)	0.06 (0.005-0.10)	.10
ASM Vs	19.8 (10.5-36.1)	8 (4-20.4)	.03	21.8 (10.9-37.4)	20.4 (11.9-39.1)	.87	14.7 (9.0-35.5)	5.4 (0.60-8.0)	.04

Values are presented as medians (IQRs). Boldface text indicates statistically significant results.

FENO₅₀, Fraction of exhaled nitric oxide at a flow rate of 50 mL/s.

*There were no patients in this group who had this investigation done, and therefore a *P* value could not be calculated.

be individualized. However, when children with STRA were split according to clinical criteria into those with persistent symptoms or those with frequent exacerbations, there was no difference in T_H2 cytokine levels between the 2 groups.

Another notable finding is the wide variation in BAL fluid and biopsy eosinophil counts between subjects. Although eosinophil counts were higher in children with STRA as a group compared with control subjects, within the group, eosinophil numbers were very different. This finding further underscores that STRA in children is a heterogeneous disease.

It is likely that T_H2 cytokines were undetectable because these children were receiving high-dose maintenance steroids. This might not have been found in previous studies because of a failure

to address adherence adequately. Importantly, the samples were taken when the children were receiving their usual prescribed medication but before a high-dose systemic steroid trial, which, if given before the bronchoscopy, might otherwise have modified the airway pathology. Although some subjects were prescribed low-dose maintenance oral steroids at baseline, *post hoc* analysis showed no difference between the children who were and were not prescribed oral steroids and no relationships between inhaled or oral steroid dose and airway pathology. We cannot exclude the possibility that T_H2 cytokines had initiated eosinophilic inflammation in patients with STRA, but we have presented clear evidence that these cytokines do not propagate established eosinophilic inflammation. We believe our data suggest that the

TABLE V. Differences in T_H2 cytokine levels between atopic and nonatopic subjects

	All atopy (n = 52)	All without atopy (n = 16)	P value	STRA with atopy (n = 45)	STRA without atopy (n = 8)	P value	Control with atopy (n = 7)	Control without atopy (n = 8)	P value
BAL fluid IL-4 (pg/mL)	U (U-0.52)	U (U-0.5)	.74	0.23 (0-0.56)	U (U-0.39)	.37	U	0.3 (U-0.69)	.49
BAL fluid IL-5 (pg/mL)	U (U-1.39)	U	.49	U (U-1.42)	U (U-1.1)	.79	U	U	1.0
Biopsy specimen IL-5 (/mm ²)	55.7 (26.7-132.7)	42.7 (31.7-75.2)	.70	51.8 (26.3-107.5)	36.1 (28.3-49.3)	.40	206.2 (48.1-320.6)	124.7*	*
BAL specimen IL-13 (pg/mL)	U (U-4.58)	U	.58	U (U-6.01)	U (U-16.7)	.97	U	U (U-17.0)	1.0
Biopsy specimen IL-13 (/mm ²)	16.0 (7.5-39.1)	12.2 (0-59.4)	.51	16.4 (11.7-33.7)	12.2 (0-59.4)	.40	6.0 (2.1-102.3)	19.8 (4.3-240.5)	.79

Values are presented as medians (IQRs).

U, Undetectable.

*There were only 2 patients in this group and therefore a P value and IQR could not be calculated.

classical T_H2 cytokines IL-4, IL-5, and IL-13 are steroid sensitive and therefore undetectable and that other steroid-resistant mediators are likely responsible for driving eosinophilic inflammation in patients with severe therapy-resistant disease. Although IL-17 might be a candidate,³⁴ the absence of neutrophilic inflammation and the fact that the source or role of IL-17³⁵ in this group is not known mean mechanisms need to be explored in experimental models³⁶ before being confirmed in pediatric airway samples because only limited samples (both size and volume) can be obtained from children.

Our data have important practical implications. The anti-IL-5 mAb mepolizumab has been shown to be beneficial in adults with persistent airway eosinophilia and multiple asthma exacerbations.³⁷⁻³⁹ Our findings suggest that there is little indication for anti-T_H2 mAb therapy in many children with STRA. In children, at least, the presence of sputum eosinophilia should not be uncritically assumed to be synonymous with T_H2-driven inflammation. There is a clear need to elicit the mechanism of this apparently non-T_H2-mediated airway eosinophilia to develop appropriate biological therapies in children with severe disease.

This study includes the largest cohort of children with genuine STRA that has been investigated invasively before a steroid trial. Previous pediatric reports have only included patients with difficult asthma and inflammatory data after a steroid trial.^{1,13} This is crucial because there is no reason to suppose *a priori* that airway pathology will be the same in a patients with steroid-resistant asthma as in a child who is nonadherent to ICSs.

There are a number of limitations. The study is cross-sectional, but this is inevitable because longitudinal invasive studies are unethical in children. Subjects with milder disease undergo bronchoscopy rarely, and therefore the data are only applicable to children with STRA. However, a previous study in children with mild-to-moderate asthma has shown increased tissue expression of IL-4 and IL-5,⁴⁰ which is in contrast to our findings. Also, because control subjects were age matched and isolated upper airway problems are less common in school-aged children than those less than 5 years old, recruitment was difficult, and thus numbers were small.

Atopy was not the main focus of the study because so few of the children with STRA were nonatopic. However, increases in biopsy specimen and BAL fluid eosinophil counts, epithelial shedding, RBM thickness, and ASM mass were present in atopic compared with the very few nonatopic subjects, regardless of disease status. These findings are in contrast to a previous report in children with

mild-to-moderate asthma, which showed similar pathologic changes in atopic and nonatopic subjects.⁴⁰ It is possible that there is an increased influence of atopy on pathology as disease severity increases. Importantly, the previous report did not include atopic nonasthmatic subjects, whereas 7 of 15 of our control subjects were atopic. When these subjects were compared with atopic patients with STRA, we found increased BAL fluid eosinophil counts, RBM thickness, and ASM mass in asthmatic patients.

In summary, we have shown that children with STRA have airway eosinophilia to varying degrees without neutrophilia or increased mast cell counts. There is considerable between-patient variability within this group. Importantly, signature T_H2 cytokines were absent in the majority of children. This report highlights that there might be different mechanisms driving pediatric as opposed to adult STRA and carries the implication that successful adult therapeutic strategies should not be uncritically extrapolated to children.

We thank all of the patients and parents for agreeing to take part in our study. We thank Winston Banya, statistician, for his advice, and Jackie Donovan for help with the multiplex assay. We are also grateful to our colleagues Mark Rosenthal, Ian Balfour-Lynn, Jane Davies, and Claire Hogg for allowing us to recruit their patients to this study.

Key messages

- Pediatric STRA is characterized by airway eosinophilia and remodeling but an absence of the classical T_H2 cytokines thought to drive allergic asthma.
- There was no neutrophilia or increase in mast cell inflammation in children with STRA, unlike in adults.
- A small subgroup of children with STRA had detectable T_H2 cytokines, suggesting phenotypic heterogeneity in this group.
- Treatments, such as anti-IL-5 antibody, might be ineffective in many children with STRA, and alternative mediators driving the disease need to be sought.

REFERENCES

1. Payne DN, Adcock IM, Wilson NM, Oates T, Scallan M, Bush A. Relationship between exhaled nitric oxide and mucosal eosinophilic inflammation in children with difficult asthma, after treatment with oral prednisolone. *Am J Respir Crit Care Med* 2001;164:1376-81.

2. Payne DN, Qiu Y, Zhu J, Peachey L, Scallan M, Bush A, et al. Airway inflammation in children with difficult asthma: relationships with airflow limitation and persistent symptoms. *Thorax* 2004;59:862-9.
3. Barbato A, Turato G, Baraldo S, Bazzan E, Calabrese F, Tura M, et al. Airway inflammation in childhood asthma. *Am J Respir Crit Care Med* 2003;168:798-803.
4. Barbato A, Turato G, Baraldo S, Bazzan E, Calabrese F, Panizzolo C, et al. Epithelial damage and angiogenesis in the airways of children with asthma. *Am J Respir Crit Care Med* 2006;174:975-81.
5. Bush A, Hedlin G, Carlsen KH, de Benedictis F, Lodrup-Carlsen K, Wilson N. Severe childhood asthma: a common international approach? *Lancet* 2008;372:1019-21.
6. Bracken M, Fleming L, Hall P, Van Stiphout N, Bossley C, Biggart E, et al. The importance of nurse-led home visits in the assessment of children with problematic asthma. *Arch Dis Child* 2009;94:780-4.
7. Bush A, Saglani S. Management of severe asthma in children. *Lancet* 2010;376:814-25.
8. Tillie-Leblond I, de Blic J, Jaubert F, Wallaert B, Scheinmann P, Gosset P. Airway remodeling is correlated with obstruction in children with severe asthma. *Allergy* 2008;63:533-41.
9. Levine SJ, Wenzel SE. Narrative review: the role of Th2 immune pathway modulation in the treatment of severe asthma and its phenotypes. *Ann Intern Med* 2010;152:232-7.
10. Slager RE, Hawkins GA, Ampleford EJ, Bowden A, Stevens LE, Morton MT, et al. IL-4 receptor alpha polymorphisms are predictors of a pharmacogenetic response to a novel IL-4/IL-13 antagonist. *J Allergy Clin Immunol* 2010;126:875-8.
11. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 2005;175:5-14.
12. Barnes PJ. Th2 cytokines and asthma: an introduction. *Respir Res* 2001;2:64-5.
13. Bossley CJ, Saglani S, Kavanagh C, Payne DN, Wilson N, Tsartsali L, et al. Corticosteroid responsiveness and clinical characteristics in childhood difficult asthma. *Eur Respir J* 2009;34:1052-9.
14. Fitzpatrick AM, Gaston BM, Erzurum SC, Teague WG. Features of severe asthma in school-age children: atopy and increased exhaled nitric oxide. *J Allergy Clin Immunol* 2006;118:1218-25.
15. Regamey N, Balfour-Lynn I, Rosenthal M, Hogg C, Bush A, Davies JC. Time required to obtain endobronchial biopsies in children during fiberoptic bronchoscopy. *Pediatr Pulmonol* 2009;44:76-9.
16. Nathan RA, Sorkness CA, Kosinski M, Schatz M, Li JT, Marcus P, et al. Development of the asthma control test: a survey for assessing asthma control. *J Allergy Clin Immunol* 2004;113:59-65.
17. Schatz M, Sorkness CA, Li JT, Marcus P, Murray JJ, Nathan RA, et al. Asthma Control Test: reliability, validity, and responsiveness in patients not previously followed by asthma specialists. *J Allergy Clin Immunol* 2006;117:549-56.
18. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. *Eur Respir J* 2005;26:319-38.
19. Pellegrino R, Viegi G, Brusasco V, Crapo RO, Burgos F, Casaburi R, et al. Interpretative strategies for lung function tests. *Eur Respir J* 2005;26:948-68.
20. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. *Am J Respir Crit Care Med* 2005;171:912-30.
21. Lex C, Payne DN, Zacharasiewicz A, Li AM, Wilson NM, Hansel TT, et al. Sputum induction in children with difficult asthma: safety, feasibility, and inflammatory cell pattern. *Pediatr Pulmonol* 2005;39:318-24.
22. Payne D, McKenzie SA, Stacey S, Misra D, Haxby E, Bush A. Safety and ethics of bronchoscopy and endobronchial biopsy in difficult asthma. *Arch Dis Child* 2001;84:423-6.
23. Fitch PS, Brown V, Schock BC, Taylor R, Ennis M, Shields MD. Chronic cough in children: bronchoalveolar lavage findings. *Eur Respir J* 2000;16:1109-14.
24. Ferreira FA, Filho LV, Rodrigues JC, Bush A, Haslam PL. Comparison of atopic and nonatopic children with chronic cough: bronchoalveolar lavage cell profile. *Pediatr Pulmonol* 2007;42:857-63.
25. Sullivan P, Stephens D, Ansari T, Costello J, Jeffery P. Variation in the measurements of basement membrane thickness and inflammatory cell number in bronchial biopsies. *Eur Respir J* 1998;12:811-5.
26. Regamey N, Ochs M, Hilliard TN, Muhlfeld C, Cornish N, Fleming L, et al. Increased airway smooth muscle mass in children with asthma, cystic fibrosis, and non-cystic fibrosis bronchiectasis. *Am J Respir Crit Care Med* 2008;177:837-43.
27. Collins DP, Luebering BJ, Shaut DM. T-lymphocyte functionality assessed by analysis of cytokine receptor expression, intracellular cytokine expression, and femtomolar detection of cytokine secretion by quantitative flow cytometry. *Cytometry* 1998;33:249-55.
28. Shaw DE, Berry MA, Hargadon B, McKenna S, Shelley MJ, Green RH, et al. Association between neutrophilic airway inflammation and airflow limitation in adults with asthma. *Chest* 2007;132:1871-5.
29. Simpson JL, Powell H, Boyle MJ, Scott RJ, Gibson PG. Clarithromycin targets neutrophilic airway inflammation in refractory asthma. *Am J Respir Crit Care Med* 2008;177:148-55.
30. Wenzel S. Severe asthma in adults. *Am J Respir Crit Care Med* 2005;172:149-60.
31. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med* 2002;346:1699-705.
32. Kaminska M, Foley S, Maghni K, Storness-Bliss C, Coxson H, Ghezzi H, et al. Airway remodeling in subjects with severe asthma with or without chronic persistent airflow obstruction. *J Allergy Clin Immunol* 2009;124:45-51.
33. Woolhouse IS, Bayley DL, Stockley RA. Effect of sputum processing with dithiothreitol on the detection of inflammatory mediators in chronic bronchitis and bronchiectasis. *Thorax* 2002;57:667-71.
34. Hansbro PM, Kaiko GE, Foster PS. Cytokine/anti-cytokine therapy—novel treatments for asthma? *Br J Pharmacol* 2011;163:81-95.
35. McAleer JP, Kolls JK. Mechanisms controlling Th17 cytokine expression and host defense. *J Leukoc Biol* 2011;90:263-70.
36. Saglani S, Mathie SA, Gregory LG, Bell MJ, Bush A, Lloyd CM. Pathophysiological features of asthma develop in parallel in house dust mite-exposed neonatal mice. *Am J Respir Cell Mol Biol* 2009;41:281-9.
37. Antoniu SA. Mepolizumab for difficult-to-control asthma with persistent sputum eosinophilia. *Expert Opin Investig Drugs* 2009;18:869-71.
38. Haldar P, Brightling CE, Hargadon B, Gupta S, Monteiro W, Sousa A, et al. Mepolizumab and exacerbations of refractory eosinophilic asthma. *N Engl J Med* 2009;360:973-84.
39. Nair P, Pizzichini MM, Kjarsgaard M, Inman MD, Efthimiadis A, Pizzichini E, et al. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N Engl J Med* 2009;360:985-93.
40. Turato G, Barbato A, Baraldo S, Zanin ME, Bazzan E, Lokar-Oliani K, et al. Non-atopic children with multitrigger wheezing have airway pathology comparable to atopic asthma. *Am J Respir Crit Care Med* 2008;178:476-82.

METHODS

Subjects

Children older than 5 years referred to the Royal Brompton Hospital with problematic severe asthma between April 2005 and June 2009 were included. Entry criteria included the following:

1. poor asthma control despite high doses of ICSs (≥ 800 $\mu\text{g}/\text{d}$ budesonide equivalent) or regular oral corticosteroids, a long-acting β agonist, and treatment with (or a failed trial of) montelukast OR
2. recurrent severe exacerbations requiring at least 1 admission to the intensive care unit or at least 2 hospital admissions requiring intravenous medications or 2 or more courses of oral corticosteroids in the past year despite therapy for persistent symptoms as described in (1) above OR
3. at least 1 very sudden (≤ 6 hours) and very severe attack (requiring hospitalization) without warning despite therapy for persistent symptoms as described in (1) above.

After assessment,^{E1} those reclassified as having difficult asthma^{E2} were excluded. The remaining subjects with STRA were further investigated.

Nonasthmatic control subjects were either (1) undergoing a bronchoscopy to investigate upper airway problems and agreed to extra research samples being taken during the bronchoscopy or (2) were having a general anesthetic for a cardiac catheterization and agreed to a research bronchoscopy at the same time. The clinical characteristics of the control subjects are shown in Table E1. The study was approved by the local research ethics committee, and all procedures were performed with informed parental consent and child assent, where appropriate.

Atopic status

Allergens tested included aeroallergens (cat, dog, grass, tree, *Aspergillus* species, *Alternaria* species, and occasionally cockroach and mice) and food allergens (milk, egg, and peanuts) plus any allergens that were thought to be relevant from the history.

Asthma control

Asthma control was assessed by using the Asthma Control Test (Fig E1). A score of 20 or greater was taken to represent adequate control.^{E3}

Spirometry and BDR

Spirometry was performed with a Compact Vitalograph 2120 (Vitalograph, Buckingham, United Kingdom) before and 15 minutes after administration of 1 mg of salbutamol through a large-volume spacer. BDR was defined as a greater than 12% change from baseline FEV₁.

Sputum induction and analysis

Sputum was induced by means of inhalation of an aerosol of nebulized hypertonic 3.5% saline if FEV₁ was 65% or greater of predicted value after pretreatment with 1 mg of salbutamol through a large-volume spacer device.^{E4} If the FEV₁ was less than this or the subject had previously been unable to tolerate hypertonic saline, then normal saline was used. Nebulizations of 5-minute intervals were performed up to 20 minutes or until an adequate sample was produced. Spirometry was performed after each expectoration to check that the FEV₁ had not decreased by 20% or greater from baseline, in which case the child would be given a further dose of salbutamol and the procedure was stopped.

Sputum processing and analysis

Sputum samples were processed within 2 hours of expectoration. Mucus plugs were selected from the expectorated sputum.^{E5} 0.1% DTT at a volume 4 times the volume of the selected sputum sample was added to the sputum and gently mixed by means of gentle aspiration with a wide-bore pipette and further on a mixing roller (Denley Spiramix 5; Denley Instruments, Colchester, United Kingdom) for 15 minutes. The liquefied sputum was filtered through a 48- μm -pore nylon gauze into a clean centrifuge tube. Four milliliters of PBS for every 1 g of selected sputum was used to wash out the remaining

contents of the original tube through the gauze filter. After centrifugation at 1500 rpm (400 g) at 4°C for 10 minutes (Sorvall RT6000D; Kendro, Bishops Cleeve, United Kingdom), supernatants were separated and stored at -80°C . Cell viability and total cells were established by using the Trypan blue exclusion method. A suspension of approximately 200,000 cells/mL was made up by means of dilution, where necessary, with PBS. Cytospin preparations were made with 100 to 300 μL of this suspension per slide and centrifuged at 450 rpm for 3 minutes. After air drying, slides were stained by using the Reastain Quik-Diff staining kit (Reagent Ltd, Toivola, Finland), fixed with methanol, and mounted.

Samples were presumed adequate if 80% or fewer squamous cells were present and 400 or greater inflammatory cells were present and distinguishable. Slides were coded so as to blind the investigator. A minimum of 400 cells was counted, and a percentage count was calculated.

Intraobserver variability of sputum eosinophil counts was 8.6%, 8.3%, and 1% for 3 subjects counted on 3 separate occasions. Because the sputum was processed with DTT and this can affect the standard curves,^{E6} we have analyzed the results using DTT and PBS to check whether there is any effect of the DTT. The standard curves for the cytokines we have analyzed by using each agent are shown in Fig E2. There is a significant effect of DTT processing on eotaxin recovery, such that this assay cannot be interpreted accurately, and the data have therefore not been used in the current article. There is a reduction in recovery of IL-4 and IL-13 with the use of DTT.

BAL fluid processing and analysis

BAL cytology was processed by the hospital cytology department. Eosinophilia was defined as greater than 1.19%^{E7} and neutrophilia as greater than 3.5%^{E8} of the total cell count. BAL fluid samples were also spun in a centrifuge at 1500 rpm (400 g) at 4°C for 10 minutes (Sorvall RT6000D; Kendro), and supernatants were separated and stored at -80°C for subsequent analysis.

Cytokine analysis

Luminex analysis. Sputum and BAL fluid supernatants were analyzed by using premade 21-plex Luminex plate (Bio-Rad Laboratories) to assay levels of IL-1 β , IL-4, IL-5, IL-8, IL-13, eotaxin, and IFN- γ . Samples and standards of known concentration were pipetted into the wells of a 96-well plate and incubated for 2 hours. After washing, the beads for cytokine-specific biotinylated antibodies were added to quantitate the amount of each cytokine captured on the bead. The detector antibody was incubated with the beads for an hour. After washing, streptavidin conjugated with a fluorescent protein, R-Phycoerythrin (streptavidin-RPE) bound to the detector antibodies associated with the immune complexes on the beads. A further wash was performed to remove any unbound beads, and the beads were then analyzed with the Luminex xMAP system, which incorporates the Luminex100 system plate analysis (Luminexcorp, Austin, Tex). The system has been validated for cytokine analysis in BAL fluid^{E9} and sputum.^{E10} The standard curves were run with DTT and PBS to check for the effect of DTT on the samples, and results are shown in Fig E2.

Cytometric Bead Array analysis. Sputum and BAL fluid supernatants were also analyzed by using the Cytometric Bead Array kit (CBA Inflammation kit, BD Biosciences) for IL-5 and IL-13.^{E11} Cytokine beads in solutions A1, A2, A3, A4, A5, and A6 provided in the kit were vortexed and added together in a universal tube. Assay diluent was added at the recommended ratio. The mixture was added at 50 μL per sample. TBP standard is diluted with a top concentration of 1:10 titrated 2-fold to generate a standard curve. R-phycoerythrin detection antibody was prepared and incubated for 3 hours in the dark. Wash buffer (1 mL) was added to each sample and spun at 200 g for 10 minutes. The supernatants were decanted, and beads were resuspended with 300 μL of wash buffer. One hundred microliters of cytometric beads were added to 3 tubes marked A, B, and C for calibration. All tubes contained wash buffer, with tube B containing fluorescein isothiocyanate and tube C containing phycoerythrin positive antibodies. The tubes were left for 30 minutes in the dark at room temperature. Samples were vortexed before analysis on a FACScalibur (Becton Dickinson FACScalibur) with CellQuest and Cytometric Bead Array Analysis software version 1.1 for Microsoft Excel 98 on a Macintosh platform.

Endobronchial biopsy specimen evaluation

During bronchoscopy, endobronchial biopsy specimens were taken from subsegmental carinae by using cupped forceps and fixed in paraffin blocks. Sections were cut and initially stained with H&E, assessed for morphology, and later assessed for measurements of airway remodeling. Suitable blocks were then cut and stained for inflammatory cells. Biopsy specimens for 8 subjects had sufficient material for 1 H&E-stained section, and no further stains were performed (4 patients with STRA and 4 control subjects).

Quantification of inflammatory cells

Tissue sections were stained for eosinophils (Congo red dye), neutrophils (neutrophil elastase), mast cells (mast cell tryptase), and CD45⁺, CD4⁺, and CD8⁺ cells, as well as cells expressing IL-5 and IL-13.

Staining for eosinophils

Eosinophils were stained with Congo red dye. This is a histochemical method that stains eosinophil granulocytes a red-brick color. The paraffin-embedded biopsy sections were initially dewaxed with Histoclear, 100%, 90%, and 70% water to alcohol. The sections were then counterstained with hematoxylin (20% in tap water and filtered) for 10 seconds. The counter-stained sections were then differentiated in tap water for 5 minutes. The sections were then placed in the Congo red stain for 20 minutes at room temperature. The sections were then rinsed in 70% ethanol until the background was clear (this usually took a few seconds). The slides were then dehydrated through 70%, 90%, and 100% alcohol and Histoclear, and they were mounted with DPX mounting medium. The result was a brick-red staining of eosinophils and amyloid.

Immunohistochemical staining of CD45⁺, CD4⁺, and CD8⁺ cells; neutrophils; mast cells; and IL-5– and IL-13–expressing cells

All of the histologic staining, including immunostaining, was performed Mr Timothy Oates. Identification of CD4⁺ (Abcam plc, Cambridge, United Kingdom), CD45⁺, and CD8⁺ (DAKO Ltd, Cambridge, United Kingdom) cells was performed by using the ABC Vectastain Elite kit (Vector Laboratories, Peterborough, United Kingdom) staining technique.^{E12} Monoclonal antibodies were applied, which had been raised from murine against human intracellular epitopes, neutrophil elastase (DAKO Ltd) for neutrophils, and mast cell tryptase (DAKO Ltd) for mast cells and against the membrane-associated epitopes CD4⁺ for CD4⁺ T cells, CD45⁺ for CD45⁺ cells, and CD8⁺ (DAKO Ltd) for CD8⁺ cells and murine primary IL-5 antibody for cells producing IL-5 and primary rabbit polyclonal human IL-13 for those producing IL-13.

Sections were dewaxed, rehydrated, and then heated (for antigen retrieval) in 10 mmol/L sodium citrate (solution at pH 6.0). However, antigen retrieval was not required for neutrophil elastase. All antigen retrieval sections were air dried and then, as with neutrophil sections, blocked with normal horse serum (Vector Laboratories) in buffer. Sections were then incubated with primary antibody for 1 hour. Sections were further incubated with a secondary biotinylated murine IgG (Vector Laboratories) with blocking serum. These were then further incubated with (avidin-biotin complex from) the ABC Vectastain Elite kit (Vector Laboratories). After a further wash, bound alkaline phosphatase was detected as a red product after incubation (with a solution prepared from Fast Red TR/Napthol AS-MX tablets; Sigma, St Louis, Mo).

Slides were counterstained with hematoxylin to provide nuclear and morphologic detail and mounted in mounting medium. Measurement of murine IgG with the primary antibody was used as a negative control procedure. Sections of resected human lymphoid (tonsillar) tissue were used as positive controls.

Measurements of airway remodeling

Quantification of airway remodeling was performed on H&E-stained sections by using computer-aided image analysis (Leica Qwin), as previously described.^{E13} Epithelial shedding was assessed as previously described,^{E14} and the length of incomplete epithelium was expressed as a percentage of

the total epithelial length at $\times 200$ magnification. Therefore if a patient has 100% shedding, this means that the whole length of the epithelium is denuded, and if they have 0% shedding, this means that the whole length is intact. Vv and Vs were measured by using point and line intersection counting, as previously described.^{E15} RBM thickness was measured by using computer-aided image analysis; the mean of 40 measurements taken 20 μ m apart was used to represent thickness for that biopsy specimen.^{E13,E16} The intraobserver CV, measured in the same section from 1 patient on at least 3 occasions a week apart, was 4.0% for RBM thickness, 7.4% for epithelial shedding, and 14.9% and 16% for Vv and Vs, respectively.

RESULTS

Nonasthmatic control subjects (aged 6–16 years) were either (1) undergoing a bronchoscopy to investigate upper airway symptoms and agreed to extra research samples being taken or (2) were undergoing general anesthesia for cardiac catheterization and agreed to have a research bronchoscopy at the same time. They had no history of wheeze or lower airway symptoms, and all were deemed fit enough for what was an elective general anesthetic and sufficiently stable that the extra few minutes to take research samples^{E17} were permissible, as judged by a consultant anesthetist independent of the study. The clinical characteristics of control subjects are shown in Table E1.

Inflammation in BAL fluid and biopsy specimens

Although BAL fluid and biopsy specimens were taken from all subjects, not all samples were of sufficient quality for analysis. The number of subjects in each group with each type of sample is therefore variable. A total of 121 biopsy specimens were taken from 69 subjects; of these, at least 1 biopsy specimen was suitable for assessment for all subjects except 1 control child. Three patients with STRA and 4 control subjects only had evaluable H&E-stained sections available and therefore only had quantification of airway remodeling. The intraobserver repeatability for all biopsy specimen inflammatory cells and cytokines was less than 10%. The intraobserver repeatability for RBM thickness, epithelial shedding, and Vv smooth muscle were 4%, 6.4%, and 11.6%, respectively (Table E2). The within-subject, between-biopsy repeatability for eosinophils was 54.8% (Table E3). However, the between-subject variation in biopsy specimen eosinophil counts was 185%.

BAL fluid and sputum cytokines

There were no significant differences between patients with STRA and control subjects in levels of BAL fluid IL-1 β , IL-4, IL-5, IL-8, IL-13, IFN- γ , and eotaxin when measured with the Luminex multiplex assay (Table E3). A few children with STRA had detectable levels of BAL fluid IL-4 ($n = 10$), IL-5 ($n = 8$), and IL-13 ($n = 8$); however, the group median value was in the undetectable range (Table E4). To confirm these findings, BAL fluid IL-5 and IL-13 were also quantified by using the Cytometric Bead Array, and again, there was no difference between patients with STRA as a group and control subjects (Table E4). The limits of detection for cytokines used in both assays are shown in Table E5.

Influence of steroids on inflammation, remodeling, and T_H2 cytokines

There was no relationship between dose of inhaled steroids and any parameters of inflammation, remodeling, or detection of T_H2

cytokines in children with STRA (Tables E6 and E7). For those receiving oral maintenance steroids, there was no relationship between steroid dose and either inflammation or T_H2 cytokine levels, but there was a negative association between ASM mass and steroid dose (ASM Vv: $r = -0.63$, $P < .002$; ASM Vs: $r = -0.73$, $P < .001$).

REFERENCES

- E1. Bracken M, Fleming L, Hall P, Van Stiphout N, Bossley C, Biggart E, et al. The importance of nurse-led home visits in the assessment of children with problematic asthma. *Arch Dis Child* 2009;94:780-4.
- E2. Bush A, Hedlin H, Carlsen KH, de Benedictis F, Lodrup-Carlsen K, Wilson N. Severe childhood asthma: a common international approach? *Lancet* 2008;372:1019-21.
- E3. Schatz M, Sorkness CA, Li JT, Marcus P, Murray JJ, Nathan RA, et al. Asthma Control Test: reliability, validity, and responsiveness in patients not previously followed by asthma specialists. *J Allergy Clin Immunol* 2006;117:549-56.
- E4. Lex C, Payne DN, Zacharasiewicz A, Li AM, Wilson NM, Hansel TT, et al. Sputum induction in children with difficult asthma: safety, feasibility, and inflammatory cell pattern. *Pediatr Pulmonol* 2005;39:318-24.
- E5. Pizzichini E, Pizzichini MM, Efthimiadis A, Evans S, Morris MM, Squillace D, et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Crit Care Med* 1996;154:308-17.
- E6. Woolhouse IS, Bayley DL, Stockley RA. Effect of sputum processing with dithiothreitol on the detection of inflammatory mediators in chronic bronchitis and bronchiectasis. *Thorax* 2002;57:667-71.
- E7. Fitch PS, Brown V, Schock BC, Taylor R, Ennis M, Shields MD. Chronic cough in children: bronchoalveolar lavage findings. *Eur Respir J* 2000;16:1109-14.
- E8. ERS Task Force on bronchoalveolar lavage in children. Bronchoalveolar lavage in children. *Eur Respir J* 2000;15:217-31.
- E9. Brasier AR, Victor S, Boetticher G, Ju H, Lee C, Bleecker ER, et al. Molecular phenotyping of severe asthma using pattern recognition of bronchoalveolar lavage-derived cytokines. *J Allergy Clin Immunol* 2008;121:30-7.
- E10. Kaminska M, Foley S, Maghni K, Storness-Bliss C, Coxson H, Ghezzi H, et al. Airway remodeling in subjects with severe asthma with or without chronic persistent airflow obstruction. *J Allergy Clin Immunol* 2009;124:45-51.
- E11. Collins DP, Luebering BJ, Shaut DM. T-lymphocyte functionality assessed by analysis of cytokine receptor expression, intracellular cytokine expression, and femtomolar detection of cytokine secretion by quantitative flow cytometry. *Cytometry* 1998;33:249-55.
- E12. Ormanns W, Schaffer R. An alkaline-phosphatase staining method in avidin-biotin immunohistochemistry. *Histochemistry* 1985;82:421-4.
- E13. Sullivan P, Stephens D, Ansari T, Costello J, Jeffery P. Variation in the measurements of basement membrane thickness and inflammatory cell number in bronchial biopsies. *Eur Respir J* 1998;12:811-5.
- E14. Barbato A, Turato G, Baraldo S, Bazzan E, Calabrese F, Panizzolo C, et al. Epithelial damage and angiogenesis in the airways of children with asthma. *Am J Respir Crit Care Med* 2006;174:975-81.
- E15. Regamey N, Ochs M, Hilliard TN, Muhlfeld C, Cornish N, Fleming L, et al. Increased airway smooth muscle mass in children with asthma, cystic fibrosis, and non-cystic fibrosis bronchiectasis. *Am J Respir Crit Care Med* 2008;177:837-43.
- E16. Payne DN, Rogers AV, Adelroth E, Bandi V, Guntupalli KK, Bush A, et al. Early thickening of the reticular basement membrane in children with difficult asthma. *Am J Respir Crit Care Med* 2003;167:78-82.
- E17. Regamey N, Balfour-Lynn I, Rosenthal M, Hogg C, Bush A, Davies JC. Time required to obtain endobronchial biopsies in children during fiberoptic bronchoscopy. *Pediatr Pulmonol* 2009;44:76-9.

In the past 4 weeks how much of the time did your asthma keep you from getting as much done at school or home?				
All of the time 1	Most of the time 2	Some of the time 3	A little of the time 4	None of the time 5
In the past 4 weeks how often have you had shortness of breath?				
More than once a day 1	Once a day 2	3-6 times a week 3	Once or twice a week 4	Not at all 5
During the past 4 weeks how often did your asthma symptoms (wheezing, coughing, shortness of breath, chest tightness or pain) wake you up at night or earlier than usual in the morning?				
4 or more nights a week 1	2 to 3 nights a week 2	Once a week 3	Once or twice 4	Not at all 5
In the past 4 weeks how often have you used your rescue inhaler or nebulizer solution?				
3 or more times per day 1	1 to 2 times per day 2	2 to 3 times per week 3	Once a week or less 4	Not at all 5
How would you rate your asthma control over the past 4 weeks?				
Not controlled at all 1	Poorly controlled 2	Somewhat controlled 3	Well controlled 4	Completely controlled 5
TOTAL SCORE /25				

FIG E1. The Asthma Control Test.

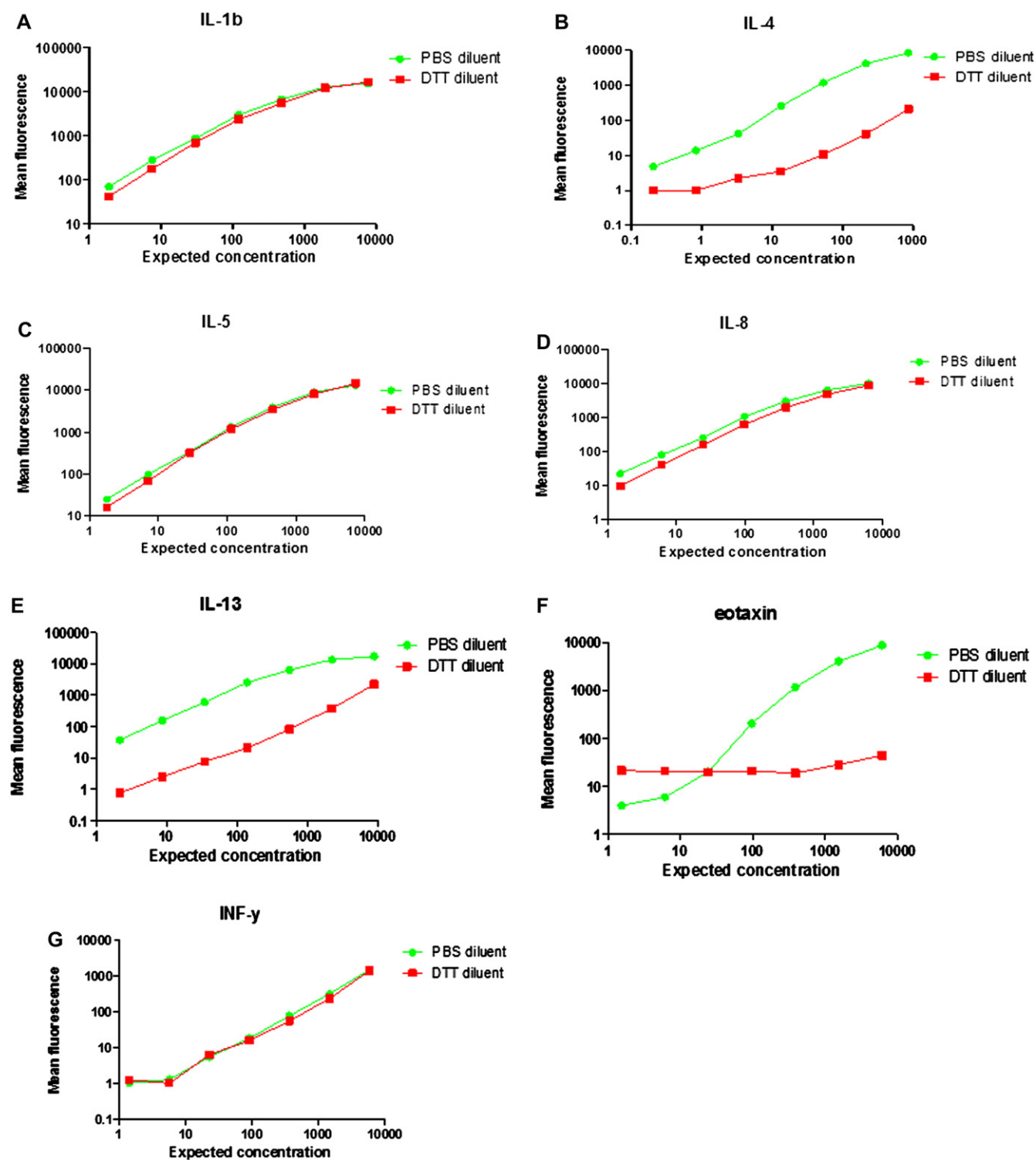


FIG E2. DTT effect on Luminex standard curves. **A**, IL-1 β ; **B**, IL-4; **C**, IL-5; **D**, IL-8; **E**, IL-13; **F**, eotaxin; and **G**, IFN- γ . There is a significant effect on eotaxin recovery, such that this assay is not useful. There is a reduction with DTT in the sensitivity of detection of IL-4 and IL-13.

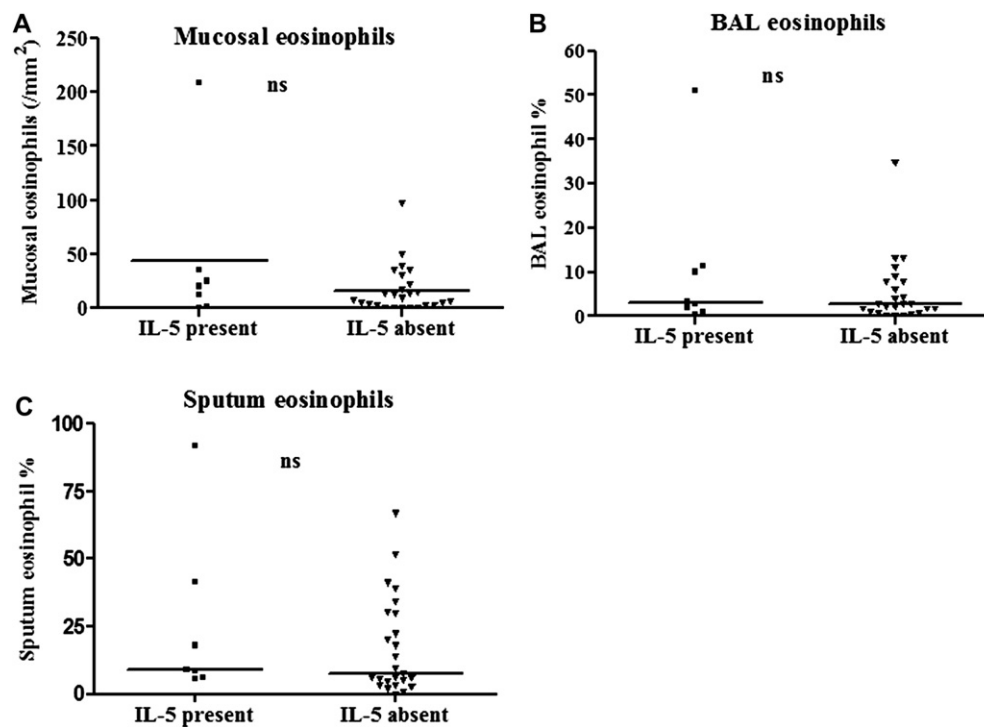


FIG E3. No relationship was found between endobronchial biopsy specimen (A), BAL fluid (B), and induced sputum (C) eosinophil counts and IL-5 levels in induced sputum. *ns*, Not significant.

TABLE E1. Clinical characteristics of nonasthmatic control subjects

Age (y)	Atopic status	Indication	Findings at FOB and outcome	BAL supernatant	Biopsy specimen quality
11.2	Nonatopic	Recurrent barking cough	Large adenoids; human metapneumovirus in BAL fluid	Yes	Adequate
16.4	Atopic	Change of pacemaker and v wire	Normal	Yes	Adequate
5.6	Atopic	Recurrent pneumonia	Upper airway bleeding; neutrophilia (11.7%) in BAL fluid	No	Adequate
6.4	Nonatopic	PDA repair	Normal, PDA repaired	Yes	Insufficient
9.1	Atopic	Recurrent croup	<i>Haemophilus influenzae</i> and <i>Streptococcus pneumoniae</i> in BAL fluid and neutrophilia (14%), given antibiotics	No	Adequate
11.0	Nonatopic	Recurrent croup and barking cough	Moderate adenoids, <i>Streptococcus pneumoniae</i> in BAL fluid and neutrophilia (2%); given antibiotics	Yes	Adequate
13	Nonatopic	Exertional stridor	Moderately large adenoids and tonsils	Yes	Adequate
12.4	Atopic	Stridor, cardiac history	Vascular ring, referred for cardiac surgery	Yes	Adequate
10.7	Atopic	Recurrent episodes of shortness of breath	Pharyngomalacia, tracheomalacia, laryngomalacia	Yes	Adequate
14.2	Nonatopic	Hemoptysis	No cause found; symptoms resolved	Yes	Adequate
16.4		Hemoptysis	Enlarged adenoids and tonsils otherwise normal, symptoms resolved	No	Adequate
7.5	Atopic	Recurrent croup	Pharyngomalacia, laryngomalacia	No	Adequate
5.9	Nonatopic	Recurrent croup	No cause found, symptoms resolved	No	H&E-stained section only
10.37	Nonatopic	Recurrent croup	No cause found, symptoms resolved	No	H&E-stained section only
10.13	Nonatopic	Hemoptysis	No cause found, symptoms resolved	No	H&E-stained section only
6.67	Atopic	Noisy breathing	Tracheomalacia	No	H&E-stained section only

FOB, Fiberoptic bronchoscopy; PDA, persistent ductus arteriosus.

TABLE E2. Intraobserver repeatability of all parameters of inflammation and remodeling

Measurement	CV 1 (%)	CV 2 (%)	CV 3 (%)	Mean CV (%)
Sputum eosinophils	8.6	8.3	1.0	6.0
Biopsy specimen eosinophils	15.0	6.9	7.1	9.7
Biopsy specimen neutrophils	9.2	11.9	6.1	9.0
Biopsy specimen mast cells	1.8	13.6	11.1	8.3
Biopsy specimen CD8 cells	7.5	11.5	7.0	8.7
Biopsy specimen CD45 cells	17.7	4.9	3.0	8.53
RBM thickness	3.5	1.1	7.4	4.0
Epithelial shedding	5.9	8.8	4.6	6.4
Vv (SM/subepithelium)	11	14.4	9.4	11.6
Vs (SM/IBM)	22	22	5.5	16.5

IBM, Intersections through basement membrane; *SM*, smooth muscle.

TABLE E3. Within-subject, between-biopsy variability

Measurement	Mean CV (%)	Median CV (%)
Biopsy specimen eosinophils	54.8	52.2
Biopsy specimen neutrophils	77.9	84.9
Biopsy specimen mast cells	47.7	45.3
Biopsy specimen CD8 cells	84.2	79.0
Biopsy specimen CD45 cells	44.7	41.6
RBM thickness	14.0	14.2
Epithelial shedding	12.4	10.2
Vv (SM/subepithelium)	59.7	65.7
Vs (SM/IBM)	81.1	85.9

IBM, Intersections through basement membrane; SM, smooth muscle.

TABLE E4. Cytokine levels measured in BAL fluid and induced sputum supernatants from children with STRA and control subjects

Concentration (pg/mL)	Patients with STRA (n = 53)	Control subjects (n = 16)	P value
Sputum IL-1 β	3.1 (U-10.8)	—	—
BAL fluid IL-4	U (U-0.52)	U (U-0.33)	.34
Sputum IL-4	U	—	—
BAL fluid IL-5	U (U-1.42)	U	.16
BAL fluid IL-5 measured by CBA	U (U-3.25)	U	.15
Sputum IL-5	U (U-1)	—	—
BAL fluid IL-8	23.3 (10.1-63.3)	12.8 (5.8-45.2)	.41
Sputum IL-8	111.5 (38.8-268.0)	—	—
BAL fluid IL-13	U (U-6.01)	U	.22
BAL fluid IL-13 measured by CBA	U	U	.62
Sputum IL-13	U	—	—
BAL fluid IFN- γ	U (12.9-26.7)	U (U-9.98)	.35
Sputum IFN- γ	U	—	—
BAL fluid eotaxin	40.6 (14.7-77.2)	30.3 (19.2-61.1)	.36

Values are presented as medians (IQRs). Levels were measured by using Luminex, unless otherwise stated.

CBA, Cytometric Bead Array; U, undetectable (less than lowest level of detection).

TABLE E5. Minimal detectable concentrations of cytokines with the methods used to analyze sputum and BAL fluid supernatants

Cytokine	Minimal detectable concentration using Luminex (pg/mL)	Minimal detectable concentration using CBA (pg/mL)
Sputum IL-1 β	1.9	—
BAL fluid IL-4	0.3	—
Sputum IL-4	0.2	—
BAL fluid IL-5	0.62	1.5
Sputum IL-5	1.8	—
BAL fluid IL-8	1.4	4
Sputum IL-8	1.8	—
BAL fluid IL-13	2.7	1.4
Sputum IL-13	2.2	—
BAL fluid IFN- γ	4.7	—
Sputum IFN- γ	1.4	—
BAL fluid eotaxin	8.0	—

All measurements are shown as picograms per milliliter.

CBA, Cytometric Bead Array.

TABLE E6. Relationship between doses of ICSs and maintenance oral steroids and lung function, inflammation, remodeling, and T_H2 cytokine levels

	ICS dose (n = 53)		Maintenance steroid dose (n = 24)	
	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
IgE (IU/mL)	−0.21	.12	0.15	.51
Percent predicted FEV ₁	−0.27	.051	−0.09	.70
FENO ₅₀ (ppb)	−0.16	.25	−0.29	.19
Blood eosinophils (%)	−0.32	.054	−0.31	.21
Sputum eosinophils	−0.19	.28	−0.35	.16
Sputum neutrophils	0.22	.19	0.18	.48
BAL fluid eosinophils	−0.009	.95	−0.09	.69
BAL fluid neutrophils	0.02	.88	0.08	.73
Biopsy specimen eosinophils	−0.18	.23	0.27	.24
Biopsy specimen neutrophils	0.33	.26	0.17	.47
Biopsy specimen mast cells	−0.04	.79	0.06	.81
Biopsy specimen CD4 ⁺ cells	−0.16	.27	−0.05	.83
Biopsy specimen CD8 ⁺ cells	0.006	.97	−0.05	.83
Biopsy specimen CD45 ⁺ cells	0.01	.94	0.24	.28
Biopsy specimen CD4/CD8 ratio	0.01	.93	−0.06	.80
RBM thickness	−0.15	.31	−0.21	.36
Epithelial shedding	0.004	.98	−0.15	.51
ASM Vv	−0.07	.63	−0.63	.002
ASM Vs	0.04	.81	−0.73	<.0001

Boldface text indicates statistically significant results.

FENO₅₀, Fraction of exhaled nitric oxide at a flow rate of 50 mL/s.

TABLE E7. Relationship between ICS dose, maintenance oral steroid dose, and cytokine levels

	ICS dose		Maintenance steroid dose	
	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
Sputum IL-4	∞	∞	∞	∞
BAL fluid IL-4	0.12	.58	0.24	.65
Sputum IL-5	0.15	.39	0.40	.15
BAL fluid IL-5	0.17	.45	∞	∞
Biopsy specimen IL-5	0.18	.26	−0.31	.22
Sputum IL-13	0.10	.56	∞	∞
BAL fluid IL-13	0.07	.74	0.57	.24
Biopsy specimen IL-13	0.07	.63	−0.005	.98

∞, All patients' levels were undetectable, and therefore a difference could not be calculated.