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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Age and early maternal smoking contribute to epithelial cell IL-13 responsiveness in a pediatric asthma population

To the Editor

Many asthma phenotypes are associated with excessive T helper type 2-like responses, and IL-13 is sufficient to induce pathological changes observed in asthmatic airways.¹ IL-13 signaling through a heterodimer of IL-13R α 1 and IL-4R α induces the phosphorylation/homodimerization of the transcription factor STAT6.¹ IL-13/IL-13R α 1/STAT6 pathway activation in respiratory epithelial cells is required for the development of experimental asthma,² and polymorphisms in IL-13, IL-13R α 1, or IL-4R α influence asthma susceptibility and/or severity.³ As IL-13 “strength-of-signal” may influence allergic asthma development and/or severity, the goal of this study was to determine whether primary nasal epithelial cells (NECs) from children with asthma displayed cell-intrinsic variations in IL-13 responsiveness and to identify factors influencing the magnitude of IL-13-induced signaling.

Following Cincinnati Children’s Hospital Medical Center (CCHMC) IRB approval, children (5–19 years old) with physician-diagnosed asthma were enrolled in the Pediatric Environmental Exposure Study (PEES).⁴ Children and parents completed surveys to assess current secondhand smoke (SHS) exposure, early maternal smoking, asthma symptom frequency over the past 4 weeks (wheeze, shortness of breath, cough, and chest tightness), asthma control (childhood Asthma Control Test [ACT]), medication usage/treatment step, and exposure to air pollutants based on estimated mean concentration of elemental carbon attributable to traffic (ECAT) exposure using birth and current address.⁴ Spirometry was performed, and asthma severity (mild, moderate, severe) was determined according to ATS guidelines. Children were currently taking asthma medication. Participants taking nasal steroids in the past 30 days were ineligible. Other medication usage was not restricted. Skin prick testing was not performed. NECs were collected from beneath the inferior turbinate using a cytology brush. NECs were passaged twice, treated with media or IL-13 (100 ng/mL) for 15 minutes, and levels of total and phosphorylated STAT6 (Tyr641) in cellular lysates were assessed by Western blot (Figure S1). IL-13-induced STAT6 activation (IL-13-stimulated pSTAT/STAT6 ratio, divided by medium-stimulated pSTAT/STAT6 ratio) was natural-log-transformed and used as the primary outcome variable in statistical analyses (SAS 9.4).

To determine whether IL-13 responsiveness varied with asthma severity in primary NECs, IL-13-induced STAT6 activation was assessed in nasal cells from children with mild ($n = 12$), moderate ($n = 18$), or severe asthma ($n = 17$). Asthma severity did not vary with age, sex, race, % predicted FEV1, environmental exposures, and total STAT6 expression (Table S1). Total IgE levels were significantly elevated in moderate (median: 318 IU/mL [IQR: 90–767]) and severe (384 IU/mL [116–598]) asthmatics compared with mild asthmatics (68 IU/mL; [29–148]) ($P = 0.017$) (Table S1). NECs responded to IL-13 (8- to >500-fold IL-13-induced STAT6 activation; Figure 1A); however, due to technical problems, samples from one participant failed to demonstrate IL-13-responsiveness and were excluded from subsequent analyses.

We performed a univariate analysis to examine the individual impact of potential factors on IL-13-induced STAT6 activation using Pearson correlation (continuous factors) and t test or ANOVA (categorical factors). Although not statistically significant, cells from children with moderate or severe asthma trended toward greater IL-13-induced STAT6 activation compared to children with mild asthma ($P = 0.25$; Table 1). Combining moderate and severe asthmatics, which demonstrated similar IL-13-induced STAT6 activation, did not improve this association ($P = 0.105$; Figure 1B). However, children who had experienced asthma symptoms within the past 4 weeks (ie, Maximum Respiratory Symptom Score (MRSS) > 0; 89.4% of participants) had significantly more IL-13-induced STAT6 activation compared to those without recent symptoms ($P = 0.040$; Figure 1C, Table 1). IL-13-induced STAT6 activation was also marginally associated, albeit not significantly, with age and peripheral eosinophil count (Table 1). Interestingly, IL-13-induced STAT6 activation was significantly elevated in children exposed to early maternal smoking (25.5% of participants; $P = 0.004$; Figure 1D, Table 1). No associations were observed with primary smoking, current exposure to SHS, or ECAT exposure (current or pregnancy) (Table 1).

The univariate analysis suggested potential impacts of age, peripheral eosinophil count, and early maternal smoking; therefore, these factors were tested as co-variables using multiple linear regression (SAS GENMOD). In this model, eosinophil count did not

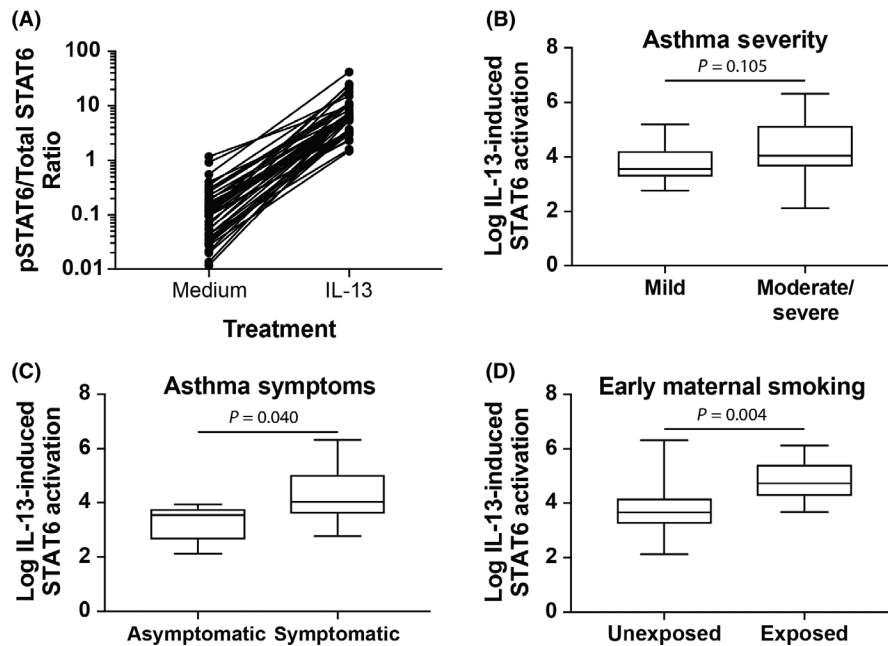


FIGURE 1 IL-13-induced STAT6 activation is associated with respiratory symptom frequency and early maternal smoking in a pediatric asthma population. Nasal epithelial cells from children with mild ($n = 12$), moderate ($n = 18$), or severe ($n = 17$) asthma were stimulated with IL-13, and STAT6 activation was assessed by Western blot (A). Associations between IL-13-induced STAT6 activation and physician-determined asthma severity (B), presence of asthma symptoms (cough, wheeze, shortness of breath, chest tightness) in the past 4 wk (42 of 47 participants) (C) and maternal smoking during pregnancy and/or in the first 5 y of life (12 of 47 participants) (D) were determined

show statistical significance ($P > 0.05$) likely due to its correlation with age and early maternal smoking (Pearson $r = -0.19$ and 0.30 , respectively) and was excluded from further analyses. Therefore, in the final model, we tested the association of IL-13-induced STAT6 activation with asthma severity controlling only for age and early maternal smoking. Early maternal smoking ($P = 0.002$) and age ($P = 0.041$) were significantly associated with IL-13-induced STAT6 activation; asthma severity was not ($P = 0.54$). In children who reported early maternal smoking, the log IL-13-induced STAT6 activation was 1.03 units higher for all ages. When age increased by 5 years, the log IL-13-induced STAT6 activation was lower by 0.38 units. Age did not modify the effect of early maternal smoking on IL-13-induced STAT6, and vice versa (Figure S2).

As an exploratory measure, we also tested the associations between IL-13-induced STAT6 activation and current respiratory symptoms, controlling for age and early maternal smoking. A marginal association was detected between report of asthma symptoms in the past 4 weeks and IL-13-induced STAT6 activation ($P = 0.06$). Associations with age ($P = 0.017$) and early maternal smoking ($P = 0.003$) remained significant with similar effects (data not shown).

This study represents the first assessment of epithelial cell IL-13 responsiveness in a pediatric asthma population. The strong association between IL-13 responsiveness and early maternal smoking, but not current SHS, pregnancy, or current ECAT exposure is unexpected. However, our observations may explain previous results from mouse models demonstrating that pre-

but not postnatal, exposure to cigarette smoke enhanced allergen-induced STAT6 activation in the lung.⁵ Similarly, maternal smoking during pregnancy is associated with persistent asthma in children with specific IL-13 SNPs,⁶ and cigarette smoke exposures alter IL-13-induced gene expression in human epithelial cells.⁷ The mechanisms related to this effect remain unclear, but may involve epigenetics as smoke exposure induces epithelial cell epigenetic changes. Importantly, methylation of CpG sites in the IL-4R α promoter has been linked to altered asthma risk, although it is unclear whether such effects are related to altered IL-13 production, altered IL-13 responsiveness, or other poorly appreciated influences.^{8,9}

Although samples from approximately equal numbers of children with mild, moderate, and severe asthma were included here, the groups were small. However, our rigorous phenotyping likely increased our ability to detect relevant associations. Indeed, IL-13-induced STAT6 activation was marginally associated with the presence of respiratory symptoms and strongly associated with early maternal smoking and age, even after adjustment for other confounding factors. The ongoing assessment of IL-13-induced STAT6 activation in the entire PEES population will further increase our power to detect associations between asthma severity and IL-13-induced STAT6 activation. Collectively, these data may explain some previously noted, though poorly understood, influences on asthma pathogenesis by suggesting an underappreciated role of early maternal smoking and age on responsiveness to a critical asthma-inducing cytokine.

TABLE 1 Unadjusted associations with IL-13 responsiveness in primary human nasal epithelial cells^a

	Parameters (95% CI)	P-Value
Age (years)	-0.24 (-0.50, 0.05)	0.11
Sex		
Male	4.29 (3.93, 4.66)	0.16
Female	3.84 (3.29, 4.39)	
Race		
White	4.34 (3.62, 5.07)	0.47
Black	4.19 (3.82, 4.57)	
Mixed/Biracial	3.76 (3.03, 4.48)	
Asthma severity ^b		
Mild	3.74 (3.15, 4.32)	0.25
Moderate	4.36 (3.88, 4.85)	
Severe	4.21 (3.72, 4.70)	
% Predicted FEV1	0.01 (-0.28, 0.30)	0.96
Maximum respiratory symptom score (MRSS) ^c		
0	3.27 (2.40, 4.15)	0.040
≥1	4.25 (3.94, 4.57)	
Asthma control score ^d		
≤19	4.28 (3.76, 4.79)	0.48
>19	4.06 (3.67, 4.45)	
Log IgE (IU/L) ^e	0.08 (-0.22, 0.37)	0.59
Blood eosinophils (%)	0.27 (-0.03, 0.52)	0.07
Early maternal smoking ^f		
No	3.88 (3.53, 4.23)	0.004
Yes	4.83 (4.34, 5.32)	
Current SHS ^g		
Yes	4.13 (3.74, 4.53)	0.93
No	4.16 (3.65, 4.67)	
Primary smoker		
Yes	4.09 (3.19, 4.98)	0.87
No	4.15 (3.82, 4.49)	
Pregnancy ECAT ^h	-0.02 (-0.34, 0.31)	0.92
Current ECAT ⁱ	-0.15 (-0.42, 0.14)	0.31

Bold number indicate p values of statistical significance ($P < 0.05$).

^aTransformation of IL-13-induced STAT6 activation was performed to approximate a normal distribution. Pearson correlation coefficients (continuous variables) or means (tested with t test or ANOVA; categorical variables) are shown.

^bAsthma severity was physician-determined using ATS guidelines.

^cMRSS is the maximum respiratory symptom queried: Frequency of cough, wheeze, shortness of breath or chest tightness in the last 4 wk: 0 = never; 1 = <1 d/wk; 2 = 1-2 d/wk; 3 = 3-5 d/wk; 4 = 6-7 d/wk.

^dAsthma control assessed by the child asthma control test (ACT).

^eIgE was log-transformed to approximate a normal distribution.

^fEarly maternal smoking was defined as maternal smoking during pregnancy or during the first 5 y of life.

^gCurrent SHS was defined as presence of mother, father, or others in the same household described as anything other than a nonsmoker.

^hPregnancy ECAT determined using geocode of home address during pregnancy.

ⁱCurrent ECAT determined using geocode of the children's current address.

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CONFLICTS OF INTEREST

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SUPPORTING INFORMATION

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Mepolizumab does not alter the blood basophil count in severe asthma

To the Editor:

Mepolizumab (anti-IL-5) depletes blood and airway eosinophils, and, clinically, allows down-titration of oral corticosteroid and a reduction in the frequency of eosinophil-dependent exacerbations.¹ Basophils also express IL-5R α , participate in T2-mediated inflammatory pathways² and have been associated with exacerbation frequency.³ Whilst basophil progenitors are unlikely to depend on IL-5 for development,⁴ blood basophil counts measured in routine clinical laboratories suggest they decrease following mepolizumab treatment.⁵⁻⁸

Our primary objective was to determine whether anti-IL-5 monoclonal antibody treatment reduces blood basophil levels as an additional potential efficacy mechanism. To achieve this, we measured blood basophils, eosinophils and other type 2 inflammatory cells, before and after 16 weeks of mepolizumab ("Nucala," GlaxoSmithKline) by flow cytometry. Patient eligibility criteria are in the online supplement and the study schedule in Figure S1.

Blood samples were obtained from 26 severe asthma subjects, attending a difficult asthma clinic at a single UK centre, at baseline and following a median (IQR) of 16 (16-17) weeks of mepolizumab,

administered as a 100 mg subcutaneous injection every 4 weeks. In 2 cases, it was not possible to obtain post-treatment samples ($n = 1$, withdrew consent; $n = 1$, discontinued), totalling 24 (Figure S1 and Table S1). We also recruited 15 nonasthmatic healthy controls (Table S1) to obtain samples at parallel time points but without an intervention (Figure S1). Flow cytometric measurements were compared with data derived through the routine pathology service, which utilizes an ADVIA 2120/2120i analyser (Siemens, UK). A detailed description of the methodology for both approaches is described in the online supplement, and for flow cytometry, the gating strategy is shown in Figure S2. Our criteria for identifying cell subsets were as follows: eosinophils (CD45⁺CD3⁻CD193⁺CD294⁺SSC^{hi}CD123^{-/+}), basophils (CD45⁺CD3⁻CD193⁺CD294⁺SSC^{lo}CD123⁺), cTH2 and pTH2 (both CD4⁺CD294⁺ but CD161⁻ or ⁺, respectively), cTC2 (CD8⁺CD294⁺) and ILC2s (Lineage⁻CD294⁺CD161⁺).

For methodological comparisons, data from asthma and healthy subjects ($n = 39$) were pooled. A good correlation was observed between flow cytometry and the ADVIA 2120i for total cells ($r^2 = 0.24$, $P = 0.0014$) and eosinophils ($r^2 = 0.75$, $P < 0.0001$), but not basophils ($r^2 = 0.06$, $P = 0.13$ Figure S3). In addition, the change in cell concentration between baseline and follow-up showed a good correlation

Siddiqui, Cousins and Brightling are co-senior authors.

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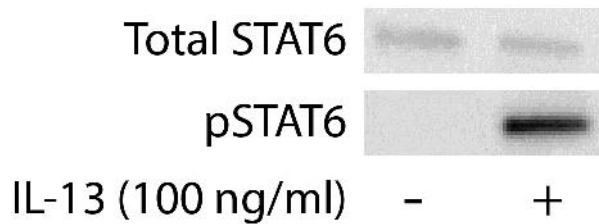
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1 **Supplementary Figures:**

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3 **Supplementary Figure 1:**

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5 **McAlees et al, Supplementary Fig 1**

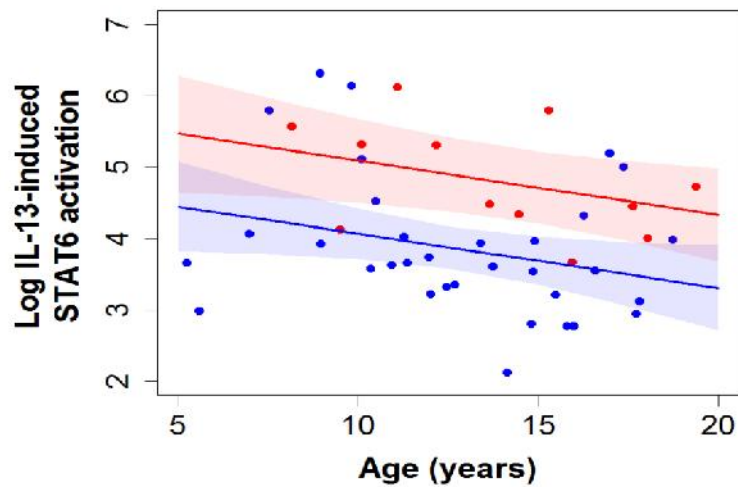
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7 **Supplementary Figure 1: IL-13-induced STAT6 phosphorylation in primary nasal**
8 **epithelial cells.** Primary nasal epithelial cells from asthmatic children were
9 cultured in the presence of medium alone, or IL-13 (100 ng/ml). 15 minutes later,
10 cells were lysed and amount of total (top panel) and phosphorylated (Tyr641;
11 bottom panel) STAT6 was assessed by Western blot. One representative blot
12 shown.

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14 **Supplementary Figure 2:**

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17 Supplementary Figure 2: Age and early maternal smoking independently
18 influence IL-13-induced STAT6 activation. The influence of age and early
19 maternal smoking (exposed – red; unexposed – blue) on IL-13-induced STAT6
20 activation was assessed. Dots and lines indicate observed and model predicted
21 values, respectively. Shaded areas indicate 95% CI.

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23 **Supplementary Tables:**

Supplementary Table 1: Demographics and Characteristics of Study Participants

	Mild (N=12)	Moderate (N=18)	Severe (N=17)
[†] Age (year)	14.1 (3.5)	12.8 (3.1)	12.7 (4.3)
[‡] Male	9 (75%)	13 (72%)	10 (59%)
[‡] Race			
Black	6 (50%)	12 (67%)	12 (71%)
White	4 (33%)	3 (17%)	2 (12%)
Mixed/Biracial	2 (17%)	3 (17%)	3 (18%)
[†] % predicted FEV1	95.5 (14.4)	92.4 (14.6)	84.1 (23.1)
[§] IgE (IU/ml)*	68 (29-148)	318 (90-767)	384 (116-598)
[§] Blood Eosinophils (%)	3.00 (3.00-6.00)	6.00 (3.00-7.00)	5.00 (4.00-8.00)
[‡] Early SHS exposure	2 (17%)	7 (41%)	3 (18%)
[‡] Current SHS exposure	6 (50%)	12 (67%)	10 (59%)
[‡] Primary Smoker	2 (17%)	4 (22%)	2 (12%)
[§] Total STAT6 levels	5.7 (3.7 - 9.9)	4.2 (2.9 - 6.3)	5.8 (4.1 - 9.4)

Note: [†]shown as mean (SD) and compared using ANOVA; [‡]shown as frequency (%) and compared using Fisher's exact test; [§]shown as median (IQR) and compared using Kruskal-Wallis test. FEV1-forced expiratory volume in 1 minute. SHS-second hand smoke exposure. *p-value < 0.05.

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