

The molecular phenotype of severe asthma in children

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Background: Although the clinical attributes of severe asthma in children have been well described, the differentiating features of the lower airway inflammatory response are less understood.

Objectives: We sought to discriminate severe from moderate asthma in children by applying linear discriminant analysis, a supervised method of high-dimensional data reduction, to cytokines and chemokines measured in the bronchoalveolar lavage (BAL) fluid and alveolar macrophage (AM) lysate.

Methods: Bronchoalveolar lavage fluid was available from 53 children with asthma (severe asthma, $n = 31$) undergoing bronchoscopy for clinical indications and 30 nonsmoking adults. Twenty-three cytokines and chemokines were measured by using bead-based multiplex assays. Linear discriminant analyses of the BAL fluid and AM analytes were performed to develop predictive models of severe asthma in children.

Results: Although univariate analysis of single analytes did not differentiate severe from moderate asthma in children, linear discriminant analyses allowed for near complete separation of the moderate and severe asthmatic groups. Significant correlations were also noted between several of the AM and BAL analytes measured. In the BAL fluid, IL-13 and IL-6 differentiated subjects with asthma from controls, whereas growth-related oncogene (CXCL1), RANTES (CCL5), IL-12, IFN- γ , and IL-10 best characterized severe versus moderate asthma in children. In the AM lysate, IL-6 was the strongest discriminator of all the groups.

Conclusion: Severe asthma in children is characterized by a distinct airway molecular phenotype that does not have a clear T_H1 or T_H2 pattern. Improved classification of children with severe asthma may assist with the development of targeted therapeutics for this group of children who are difficult to treat. (J Allergy Clin Immunol 2010;125:851-7.)

Key words: Asthma, macrophage, children, cytokine, chemokine, inflammation

Abbreviations used

AM: Airway macrophage
BAL: Bronchoalveolar lavage
GRO: Growth-related oncogene
ICS: Inhaled corticosteroid
IP-10: Human IFN-inducible protein 10
MCP: Monocyte chemoattractant protein
MDC: Macrophage-derived chemokine
MIP: Macrophage inflammatory protein

Severe or difficult-to-treat asthma in children is a complicated disorder characterized by ongoing symptoms and persistent airway inflammation and oxidant stress despite corticosteroid treatment.¹⁻⁴ Although severe asthma is likely a heterogeneous disorder,^{5,6} affected children share many similar clinical features, including gas trapping, bronchial hyperresponsiveness, and aero-allergen sensitization.^{1,2} However, the molecular and cellular patterns of inflammation in children with severe asthma are less uniform. Whereas some investigators have found increased eosinophils and T_H2 -derived cytokines in the airways of children with severe asthma,^{2,7} others have noted noneosinophilic patterns of airway inflammation with increased neutrophils and biomarkers of neutrophil activation.^{8,9} Given the heterogeneity of the inflammatory response in children with severe asthma,^{10,11} additional methods to distinguish severe from moderate asthma are needed.

Airway macrophages (AMs) are secretory cells that play a vital role in the regulation of airway inflammation and the immune response. Although AMs have traditionally received little attention in the field of asthma,¹² there are increasing reports of AM dysfunction in patients with severe asthma characterized by ongoing AM activation¹³ and abnormal secretion of inflammatory mediators^{14,15} despite corticosteroid treatment. We have further demonstrated impaired AM phagocytosis and increased AM apoptosis in children with severe versus moderate asthma after a microbial stimulus.¹⁶ Together, these data suggest that AM dysfunction may be an important regulator of the ongoing airway inflammation associated with severe asthma. To test this hypothesis, we applied a supervised classification method to the patterns of cytokine and chemokine expression measured in the bronchoalveolar lavage (BAL) fluid and AMs of children with severe asthma, children with moderate asthma, and healthy controls. Using linear discriminant analysis, predictive models of severe asthma were generated. Our findings provide the first preliminary evidence of the molecular phenotype of severe asthma in children, which is associated with AM dysfunction.

METHODS

Sample

A convenience sample of asthmatic children 5 to 17 years of age undergoing bronchoscopy for clinical indications was obtained. All children

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met criteria for persistent asthma¹⁷ and had a history of at least a 12% change in FEV₁ after bronchodilator administration.¹⁸ Severe asthma was diagnosed according to criteria developed by the Severe Asthma Research Program,^{1,5} which were adapted from the American Thoracic Society's Report (see this article's Table E1 in the Online Repository at www.jacionline.org).¹⁹ Thresholds for high-dose inhaled corticosteroids (ICSs) were defined as ≥ 440 μg fluticasone equivalent per day for children younger than 12 and ≥ 880 μg for children 12 to 17 years of age.¹⁷ The pharmacy records of all participants were reviewed, and children with fewer than 4 ICSs or oral corticosteroid refills in the previous 6 months were excluded. Other exclusion criteria included immunodeficiency, recent pneumonia, chronic aspiration syndromes, birth before 35 weeks of gestation, or a positive BAL culture. Informed consent and assent to share the BAL fluid between the clinical and research laboratories were obtained.

Children with severe asthma underwent flexible bronchoscopy with BAL as indicated for persistent asthma symptoms despite corticosteroid treatment.²⁰ Children with moderate asthma underwent bronchoscopy for suspected foreign body aspiration ($n = 3$), noisy breathing and suspected upper airway obstruction ($n = 3$), recurrent pneumonia ($n = 5$), suspected aspiration ($n = 5$), evaluation of chest mass/suspected endobronchial lesion ($n = 2$), and suspected congenital anomalies ($n = 4$). Participants were clinically stable. To enhance classification, nonsmoking, healthy adult volunteers were also recruited. Bronchoscopy with BAL was performed in these subjects for research purposes after informed consent.

Procedures

Spirometry was performed with a portable spirometer (KoKo Legend; Ferraris, Louisville, Colo)²¹ and interpreted with population reference standards.²² Fraction of exhaled nitric oxide was collected with a reservoir bag and analyzed offline by chemiluminescence (Sievers NOA 280-I; Ionic Instruments, Boulder, Colo).²³ Smoking status was verified in adults by using a urinary cassette test (Accutest; Jant Pharmacal, Encino, Calif) with a cotinine cutoff of 200 ng/mL.

Pediatric bronchoscopy was performed under general anesthesia by using a laryngeal mask airway and flexible bronchoscope (Olympus BF-3C160 or BF-P160; Olympus America Inc, Melville, NY). BAL fluid was collected from the right middle lobe with three 1-mL/kg (50 mL maximum) saline lavages. For adults, conscious sedation was administered, and a flexible bronchoscope (Olympus BF-1T20D) was passed transnasally into the right middle lobe. Three 50-mL saline aliquots were instilled. For children, the BAL fluid was pooled and divided between the research and clinical laboratories. The samples submitted to the clinical laboratories were subjected to standard culture and sensitivity testing.

Bronchoalveolar lavage was centrifuged at 1200 rpm for 7 minutes at 4°C within 1 hour of collection. The supernatant was divided into 250- μL aliquots and frozen with protease inhibitors at -80°C before analysis. Total cell counts were performed with a hemocytometer, and cellular differentials were determined from 300 consecutive cells after Wright staining. Cell pellets with $<90\%$ AM purity were excluded from further analysis. The AM pellets were frozen at -80°C with protease inhibitors. The protein content of the AM cell lysate was determined by using a Coomassie (Bradford) protein assay (Pierce Biotechnology, Rockford, Ill) with absorbance of 595 nm and a lower detection limit of 1 $\mu\text{g}/\text{mL}$.

Concentrations of the cytokines IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IFN- γ , GM-CSF, and TNF- α were determined by using a bead-based multiplex cytokine assay (HSCYTO-60SK; Millipore, Billerica, Mass). The chemokines growth-related oncogene (GRO [CXCL1]), IL-8 (CXCL8), human IFN-inducible protein 10 (IP-10 [CXCL10]), monocyte chemoattractant protein (MCP)-1 (CCL2), macrophage inflammatory protein (MIP)-1 α (CCL3), MIP-1 β (CCL4), RANTES (CCL5), MCP-3 (CCL7), eotaxin (CCL11), macrophage-derived chemokine (MDC [CCL22]), and fractalkine (CX3CL1) were quantified by using a similar bead-based multiplex kit (MPXHCYTO-60K; Millipore). These analytes were selected for their biological relevance to asthma and their associations with AM activation (see this article's Table E2 in the Online Repository at www.jacionline.org). For the assays, duplicate samples and serial dilutions

of the cytokine standards were added to 96-well plates and incubated overnight. Data were analyzed by using the BioRad Bio-Plex System (BioRad Laboratories, Hercules, Calif) with gates of 4335 and 10,000. Sample concentrations were determined using a 5-point logistic curve fitting algorithm (Bio-Plex Manager 3.0 Software; BioRad Laboratories). The sensitivity and prevision of each assay are listed in this article's Table E3 in the Online Repository at www.jacionline.org.

Statistical analysis

Data analysis was performed by using SPSS software (Version 15; SPSS Inc, Chicago, Ill). Wilks λ tests were used to test the equality of group means for each individual cytokine and chemokine. Because of the large number of comparisons, the threshold of significance was adjusted using a Bonferroni correction (for BAL analytes, $\alpha = 0.002$; for AM analytes, $\alpha = 0.005$). Variables that differed significantly between groups were further analyzed by ANOVA with Tukey post hoc tests using a 2-tailed α of 0.05.

Linear discriminant analysis was performed using the Fisher method,²⁴ which is relatively robust against departures from normality.²⁵ Linear discriminant analysis is similar to logistic regression and is useful for building a predictive model of group membership based on observed characteristics. The procedure yields a set of discriminant functions based on the linear combinations of variables that provide the best discrimination between groups. These functions can be plotted to visualize the proximity of that subject to others in the same group.

Linear discriminant analysis was used to generate 2 predictive models of severe asthma. The first model was generated from the linear combination of all 23 cytokines and chemokines measured in the raw BAL fluid without correction for the protein content or dilution of the BAL fluid. Because 12 of the 23 cytokines and chemokines measured were undetectable in the AM lysate, the second model was generated from the linear combination of only those 11 analytes expressed by the AMs, which included IL-6, TNF- α , GRO (CXCL1), IL-8 (CXCL8), IP-10 (CXCL10), MCP-1 (CCL2), MIP-1 α (CCL3), MIP-1 β (CCL4), RANTES (CCL5), MDC (CCL22), and fractalkine (CX3CL1). To account for differences in total AM cell counts, the AM cytokine and chemokine concentrations were adjusted for the protein content of the cell lysate and expressed as picograms per milligram of protein. Cross-validation of the models was performed by randomly selecting subjects with a random number generator and repeating the analyses. To determine further the strongest discriminators of severe asthma, stepwise linear discriminant analysis was used with an F-value entry probability of 0.05 and removal probability of 0.10. Additional statistical details are provided in this article's Online Repository at www.jacionline.org.

RESULTS

Sixty children with asthma (severe asthma, $n = 35$) and 30 healthy adult controls participated in this study. However, 7 children (severe asthma, $n = 4$) infected with *Streptococcus pneumoniae* or *Haemophilus influenzae* were excluded from data analysis (see this article's Table E4 in the Online Repository at www.jacionline.org). The resulting sample included 30 controls, 22 subjects with moderate asthma, and 31 subjects with severe asthma. Children with moderate asthma had no evidence of endobronchial lesions or chronic aspiration. Children with severe asthma were more likely to be of African American descent and had lower pulmonary function despite treatment with higher doses of ICS (Table I).

The BAL procedure was well tolerated in all participants. The BAL return volume, expressed as the percentage of saline recovered, was similar between controls and subjects with moderate asthma but tended to be lower in children with severe asthma (control vs moderate vs severe asthma, 48% vs 48% vs 39%; $P = .051$). However, there were no differences in the BAL fluid protein content between children with moderate and severe

TABLE I. Features of the sample

Feature	Adult control (n = 30)	Moderate asthma (n = 22)	Severe asthma (n = 31)
Age (y)	38 ± 10	10 ± 4*	11 ± 8*
Male	9 (30)	11 (50)	17 (55)
White	17 (56)	20 (91)*	10 (32)*†
Black	13 (43)	2 (9)*	21 (68)*†
ICS dose (fluticasone equivalents/d)	0	332 ± 235*	904 ± 244*†
Asthma medications			
Budesonide	0	4 (18)*	8 (26)*
Fluticasone	0	3 (14)*	1 (3)
Fluticasone/salmeterol	0	11 (50)*	21 (68)*
Montelukast	0	13 (59)*	30 (97)*†
Prednisone	0	0	15 (48)*†
Asthma medical history			
Emergency department visit (previous year)	0	4 (18)*†	28 (90)
Hospitalization (ever)	0	3 (14)*	26 (84)*†
Intensive care unit admission (ever)	0	0	14 (45)*†
Intubation (ever)	0	0	6 (19)*†
Pulmonary function			
FVC (% predicted)	94 ± 15	99 ± 16	86 ± 19†
FEV ₁ (% predicted)	97 ± 16	97 ± 18	74 ± 21*†
FEV ₁ :FVC	0.86 ± 0.08	0.87 ± 0.09	0.75 ± 0.14*†
FEF ₂₅₋₇₅ (% predicted)	115 ± 33	95 ± 36	57 ± 31*†
F _{ENO} (offline, ppb)	5 ± 3	12 ± 10*	13 ± 10*
BAL cellular differential (%)			
Macrophages/monocytes	90.2 ± 4.4	90.7 ± 5.3	88.1 ± 5.8
Neutrophils	2.6 ± 2.0	5.0 ± 3.3*	5.1 ± 3.8*
Eosinophils	0.5 ± 0.5	0.5 ± 0.7	1.9 ± 3.2*†
Lymphocytes	4.9 ± 1.8	3.8 ± 2.9	4.7 ± 3.1

F_{ENO}, Fraction of exhaled nitric oxide; FEF₂₅₋₇₅, forced expiratory flow at 25% to 75% of forced vital capacity; FVC, forced vital capacity.

Data represent the mean ± SD or the frequency (%).

**P* < .05 vs controls.

†*P* < .05 versus subjects with moderate asthma.

asthma (197 vs 204 μg/mL). Total cell counts were decreased in both groups of subjects with asthma compared with controls (7.98 vs 3.73 vs 3.30 × 10⁶ cells; *P* < .001) and were accompanied by a shift in the percentage of neutrophils and eosinophils (Table I).

BAL cytokines and chemokines

The concentrations of the cytokines and chemokines measured in the raw BAL fluid are shown in Tables II and III. Of the cytokines, only IL-6 and IL-13 differed between subjects with asthma and controls after correction for multiple comparisons. However, these cytokines were not significantly different between subjects with moderate and severe asthma. Similarly, whereas GRO (CXCL1), IL-8 (CXCL8), IP-10 (CXCL10), MIP-1β (CCL4), RANTES (CCL5), MCP-3 (CCL7), MDC (CCL22), and fractalkine (CX3CL1) were elevated in subjects with asthma versus controls, no differences were seen between the asthmatic groups. However, when linear discriminant analysis was performed, the model resulted in good separation of each of the 3 groups (Fig 1), with correct identification of 100% of the controls, 86% of the subjects with moderate asthma, and 91% of the children with severe asthma (see this article's Tables E5-E8 in the Online

Repository at www.jacionline.org). Similar separation was observed with the validation model, with an accurate prediction rate of 83.3% (Tables E7 and E8). To highlight further the differences between the moderate and severe asthma groups, discriminant analysis was repeated excluding the controls. This resulting model was also statistically significant (Wilks λ = 0.327; χ² = 34.682; *P* = .046) and provided correct classification of 96% of the children with moderate asthma and 82% of the children with severe asthma (see this article's Fig E1 in the Online Repository at www.jacionline.org).

To determine which cytokines and chemokines in the BAL fluid best discriminated controls, subjects with moderate asthma, and subjects with severe asthma, linear discriminant analysis was repeated using a stepwise approach. With this method, GRO (CXCL1), IL-13, IL-6, RANTES (CCL5), IL-12, IL-10, and IFN-γ were identified as the most important predictors to include in the model (see this article's Table E9 in the Online Repository at www.jacionline.org). These 7 markers explained 81.7% of the variance (χ² = 94.785; *P* < .001) in the first discriminant function (which separated subjects with asthma from controls) and 18.3% of the remaining variance (χ² = 24.124; *P* < .001) in the second discriminant function (which separated subjects with severe asthma from subjects with moderate asthma). Whereas IL-13 and IL-6 provided the strongest separation of subjects with asthma from controls in the stepwise analysis, GRO (CXCL1), RANTES (CCL5), IL-12, IFN-γ, and IL-10 best differentiated severe from moderate asthma in children (see this article's Table E10 in the Online Repository at www.jacionline.org). When the stepwise analysis was repeated excluding the healthy controls, only RANTES (CCL5) was identified as the strongest differentiating factor between subjects with moderate and severe asthma (Wilks λ = 0.891; χ² = 4.573; *P* = .032).

AM cytokines and chemokines

To determine whether AM activation might contribute to airway inflammation in severe asthma, these same cytokines and chemokines were measured in AM lysates from a subset of the original sample (control, *n* = 14; moderate asthma, *n* = 6; severe asthma, *n* = 6). Concentrations of these analytes are shown in Table IV. IL-1β, IL-2, IL-4, IL-5, IL-7, IL-10, IL-12p70, IL-13, IFN-γ, GM-CSF, eotaxin (CCL11), and MCP-3 (CCL7) were undetectable. IL-6 and IL-8 were again elevated in the AMs from children with moderate and severe asthma, but no significant differences were observed between the asthmatic groups. The concentrations of IL-6 and IL-8 measured in the AMs were highly correlated with the concentrations measured in the BAL fluid (IL-6: *r* = 0.815, *P* < .001; IL-8: *r* = 0.817, *P* < .001). Significant correlations between AM and BAL chemokine expression were also observed for GRO (CXCL1; *r* = 0.630; *P* = .003), IP-10 (CXCL10; *r* = 0.704; *P* = .001), RANTES (CCL5; *r* = 0.784; *P* < .001), and MDC (CCL22; *r* = 0.542; *P* = .014).

Linear discriminant analysis was performed by entering the 11 cytokines and chemokines detected in the AM lysate into the model. The resulting model again provided separation of the groups (Fig 2), with correct identification of 93% of the controls, 83% of the subjects with moderate asthma, and 100% of the subjects with severe asthma. The validation model further resulted in correct classification of 100% of the subjects in each group, although the sample size was small (see this article's Tables E11-E14 in the Online Repository at www.jacionline.org). IL-6 was

TABLE II. Cytokine concentrations (pg/mL) in the BAL fluid

Cytokine	Adult control (n = 30)	Moderate asthma (n = 22)	Severe asthma (n = 31)	Wilks λ *	P value†
IL-1 β	0 (0-0.01)	0.07 $\pm$ 0.07§ (0-0.23)	0.06 $\pm$ 0.07§ (0-0.36)	0.742	<.001‡
IL-2	0.05 $\pm$ 0.08 (0-0.37)	0.07 $\pm$ 0.05 (0-0.21)	0.08 $\pm$ 0.11 (0-0.62)	0.975	.386
IL-4	0.53 $\pm$ 0.73 (0-2.76)	0.65 $\pm$ 0.84 (0-2.40)	0.55 $\pm$ 0.74 (0-2.08)	0.950	.798
IL-5	0.06 $\pm$ 0.16 (0-0.75)	0.29 $\pm$ 0.56 (0-2.03)	0.39 $\pm$ 1.03 (0-4.53)	0.947	.225
IL-6	0.59 $\pm$ 0.48 (0.18-2.11)	5.60 $\pm$ 3.73§ (1.23-17.09)	8.27 $\pm$ 11.65§ (0.93-56.15)	0.744	.001‡
IL-7	0.76 $\pm$ 1.69 (0-8.52)	1.24 $\pm$ 1.30 (0.03-6.55)	0.94 $\pm$ 0.98 (0.01-4.53)	0.983	.459
IL-10	0.01 $\pm$ 0.03 (0-0.15)	0.81 $\pm$ 1.86 (0-8.19)	0.69 $\pm$ 1.46 (0-7.33)	0.934	.082
IL-12 (p70)	0.04 $\pm$ 0.06 (0-0.19)	0.10 $\pm$ 0.11 (0-0.39)	0.14 $\pm$ 0.18 (0-0.81)	0.893	.030
IL-13	0.02 $\pm$ 0.12 (0-0.55)	0.62 $\pm$ 0.52§ (0-2.32)	1.04 $\pm$ 1.40§ (0-5.96)	0.690	<.00‡
IFN- γ	0.03 $\pm$ 0.13 (0-0.60)	0.26 $\pm$ 0.38 (0-1.41)	0.14 $\pm$ 0.21 (0-0.91)	0.876	.008
GM-CSF	0.21 $\pm$ 0.21 (0-0.75)	0.34 $\pm$ 0.29 (0-1.00)	0.22 $\pm$ 0.25 (0-1.32)	0.929	.119
TNF- α	0.19 $\pm$ 0.17 (0.04-0.74)	0.51 $\pm$ 0.91 (0.04-4.44)	1.47 $\pm$ 5.04 (0.06-25.71)	0.953	.259

Data represent the mean $\pm$ SD with minimum and maximum concentrations shown in parentheses.

*Smaller values indicate better discrimination between groups.

†To adjust for multiple comparisons, significance was defined as $\alpha \leq 0.002$.

‡Indicates significant differences between groups after Bonferroni correction.

§P < .01 vs adult control.

identified as the single differentiator of all the groups using stepwise methods ($F = 6.952$; $P = .004$) and explained 100% of the variance through 1 discriminant function (Wilks $\lambda = 0.623$; $\chi^2 = 10.875$; $P = .004$).

DISCUSSION

To our knowledge, this is the first study to use high-dimensional data reduction techniques on BAL and AM inflammatory markers in children with severe asthma. By using the supervised method of linear discriminant analysis, we provide the first preliminary evidence of the molecular phenotype of severe asthma in children. Whereas cytokine and chemokine concentrations in the BAL and AM cell lysate did not differ between children with moderate and severe asthma, the linear combination of these biomarkers resulted in clear separation of the 2 asthmatic groups. Thus we conclude that severe asthma in children is associated with unique patterns of airway inflammation that persist despite corticosteroid treatment.

Airway macrophages are abundant in the airways and are important regulators of the innate immune response. These vital AM functions are compromised in patients with severe asthma. We and others have previously demonstrated a 2-fold reduction in AM phagocytosis^{14,16} accompanied by increased AM apoptosis.¹⁴ Although it might appear that AM function is suppressed in patients with severe asthma, these same patients are characterized by paradoxical AM activation. Compared with controls, AMs from subjects with asthma have increased basal spontaneous generation of superoxide anion,^{26,27} an increased ratio of proinflammatory to anti-inflammatory cytokines,^{15,28,29} and increased

inflammatory gene expression evidenced by increased histone acetyl-transferases and decreased histone deacetylases.³⁰ Whereas corticosteroids suppress LPS-induced AM inflammation in subjects with mild to moderate asthma,²⁸ this same effect is not observed in patients with severe asthma.^{15,29} This unopposed secretion of inflammatory mediators, combined with impaired AM phagocytosis of apoptotic toxic granulocytes, may ultimately contribute to the airway inflammation we observed in the BAL fluid of children with severe asthma. Alternatively, the inflammatory products released by other cell types such as airway epithelial cells may induce AM inflammation and dysfunction in patients with severe asthma.

Although the models generated by this study result in relatively discrete clustering of children with severe asthma, they do not address the issue of multiple phenotypes that may exist within this group of children. Whereas children with severe asthma share similar clinical features (eg, airflow obstruction and increased bronchial hyperreactivity despite corticosteroid treatment), their airway cellular profiles may differ considerably.⁷⁻⁹ Indeed, both a neutrophilic phenotype and an eosinophilic phenotype have been reported in similar samples of children with severe asthma.^{2,7-9} The results from this study do not provide clear evidence to support of one pattern of airway inflammation over the other. Although the neutrophil chemoattractants GRO (CXCL1) and IL-8 (CXCL8) were highly expressed in children with severe asthma, we also observed high concentrations of the T_H1 cytokine IFN- γ as well as the T_H2 cytokine IL-13, which is associated with eosinophilia and airway remodeling.³¹ Other chemoattractants and leukocyte activation products such as IL-6, IP-10 (CXCL10), MIP-1 β (CCL4), RANTES (CCL5), MCP-3 (CCL7), MDC

TABLE III. Chemokine concentrations (pg/mL) in the BAL fluid

Chemokine	Adult control (n = 30)	Moderate asthma (n = 22)	Severe asthma (n = 31)	Wilks λ^*	P value [†]
GRO	455 $\pm$ 391	3,054 $\pm$ 1,971§	2,657 $\pm$ 1,615§	0.600	<.001‡
(CXCL1)	(126-1,596)	(166-10,000)	(111-6,363)		
IL-8	2.8 $\pm$ 2.9	28.3 $\pm$ 26.6§	45.2 $\pm$ 49.1§	0.780	<.001‡
(CXCL8)	(0.7-11.7)	(4.0-104)	(6.1-245)		
IP-10	248 $\pm$ 284	762 $\pm$ 637§	1,302 $\pm$ 2,145§	0.902	<.001‡
(CXCL10)	(16-1294)	(114-2,674)	(46-10,000)		
MCP-1	27.6 $\pm$ 19.5	43.5 $\pm$ 34.2	177 $\pm$ 603	0.961	.090
(CCL2)	(8.3-82.5)	(5.9-1,494)	(3.52-2,934)		
MIP-1 α	1.2 $\pm$ 1.2	5.3 $\pm$ 8.8	57.9 $\pm$ 268	0.970	.076
(CCL-3)	(0-2.9)	(0-37.8)	(0-1,288)		
MIP-1 β	3.0 $\pm$ 3.2	20.8 $\pm$ 23.6§	19.1 $\pm$ 32.3§	0.881	<.001‡
(CCL-4)	(0-15.0)	(1.6-106)	(0-161)		
RANTES	25.6 $\pm$ 49.1	108 $\pm$ 78.2§	201 $\pm$ 235§	0.776	<.001‡
(CCL5)	(0.04-199)	(15.1-266)	(7.6-1,145)		
MCP-3	0	8.7 $\pm$ 7.8§	12.2 $\pm$ 15.3§	0.771	<.001‡
(CCL7)	(0)	(0-27.9)	(0-73.5)		
Eotaxin	0.1 $\pm$ 0.4	0.8 $\pm$ 2.9	6.8 $\pm$ 29.9	0.967	.175
(CCL11)	(0-1.5)	(0-13.4)	(0-144)		
MDC	34.3 $\pm$ 24.2	57.4 $\pm$ 26.5§	90.8 $\pm$ 98.2§	0.885	.002‡
(CCL22)	(0-85.9)	(16.6-106)	(6.1-357)		
Fractalkine	60.7 $\pm$ 26.8	142 $\pm$ 88.1§	141 $\pm$ 79.4§	0.744	<.001‡
(CX3CL1)	(20.7-132)	(0-358)	(0-312)		

Data represent the mean $\pm$ SD, with minimum and maximum concentrations shown in parentheses.

*Smaller values indicate better discrimination between groups.

[†]To adjust for multiple comparisons, significance was defined as $\alpha \leq .002$.

‡Indicates significant differences between groups after Bonferroni correction.

§P <.01 vs adult control.

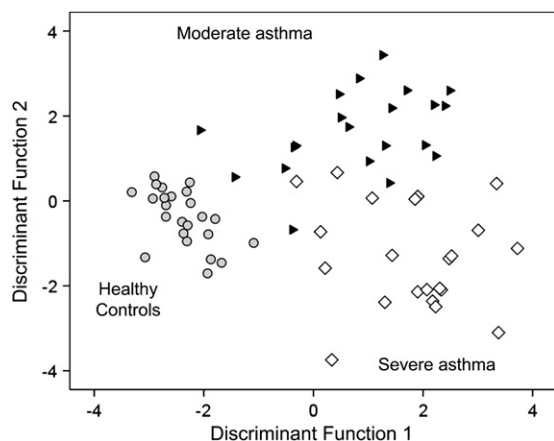


FIG 1. Scatterplot of the discriminant functions generated from 23 BAL fluid cytokines and chemokines. Each data point represents a single subject. The plot depicts clustering and clear separation of children with severe asthma (white diamonds) from children with moderate asthma (black triangles) and healthy controls (gray circles).

(CCL22), and fractalkine (CX3CL1) were also elevated. Whereas IL-13 and IL-6 differentiated subjects with asthma from controls by using stepwise methods, GRO (CXCL1), RANTES (CCL5), IL-12, IFN- γ , and IL-10 differentiated severe from moderate asthma in children. These data confirm that severe asthma in children is not simply a T_H2-mediated disease with relative loss of T_H1-mediated cytokine production. Furthermore, given that both neutrophils and eosinophils were elevated in this sample of children with severe asthma, the patterns of cellular inflammation are also complex and likely do not involve one cell type independent of others.

This study had a number of limitations. First, although the AM was the primary cell of interest, our findings may also reflect activation of other airway cells, including epithelial cells. Indeed, other cell types aside from AMs are equally relevant in the pathogenesis of severe asthma and warrant further study. Second, because bronchoscopy cannot be ethically performed in healthy asymptomatic children, our sample was restricted to children with moderate and severe asthma undergoing bronchoscopy with BAL for clinical indications. This likely biased the sample toward children with more severe disease. Third, the lack of a healthy pediatric control group does raise the question whether age is a confounding factor in our results. Fourth, because the American Thoracic Society criteria for severe asthma are based primarily on high-dose corticosteroid treatment,¹⁹ our moderate asthma group may have included children who were undertreated. However, the fact that pulmonary function in this group of patients was similar to that of healthy controls discredits this notion. Furthermore, the relatively discrete grouping of subjects with moderate and severe asthma by our linear discriminant analyses suggests that moderate and severe asthma are indeed phenotypically distinct.

The pathophysiology of severe asthma is complex and not completely understood. The defining feature of severe asthma is the need for high doses of corticosteroids to achieve an acceptable level of asthma control.¹⁹ Because corticosteroids are the cornerstone for the diagnosis of severe asthma, we cannot exclude the possibility corticosteroids influenced our results. Although we did not strictly measure corticosteroid adherence with digital dosing meters or systemic cortisol concentrations, we did attempt to monitor asthma prescription refills. Therefore, to our knowledge, all of the children included in this study were compliant with their prescribed asthma medications. Given the immunosuppressive properties of corticosteroids, it is possible that our AM findings

TABLE IV. Cytokine and chemokine concentrations (pg/mg protein) in the BAL macrophages

Cytokine/chemokine	Adult control (n = 14)	Moderate asthma (n = 6)	Severe asthma (n = 6)	Wilks λ^*	P value [†]
IL-6	0.4 $\pm$ 0.6 (0-1.8)	1.1 $\pm$ 0.5§ (0.5-1.7)	1.7 $\pm$ 1.1§ (0.2-2.9)	0.623	.002‡
TNF- α	0.9 $\pm$ 0.6 (0.2-2.7)	0.5 $\pm$ 0.4 (0.2-1.1)	1.1 $\pm$ 1.5 (0.1-4.1)	0.931	.332
GRO (CXCL1)	824 $\pm$ 923 (61.2-3777)	1218.8 $\pm$ 798 (381-2329)	1480 $\pm$ 1095 (354-2894)	0.911	.200
IL-8 (CXCL8)	29.8 $\pm$ 50.4 (2.7-200)	58.1 $\pm$ 53.4§ (25.9-164)	199 $\pm$ 190§ (21.8-536)	0.655	<.001‡
IP-10 (CXCL10)	120 $\pm$ 117 (0-401)	232 $\pm$ 314 (19.2-858)	1202 $\pm$ 1985 (7.6-5192)	0.799	.236
MCP-1 (CCL2)	60.4 $\pm$ 46.2 (7.4-176)	33.4 $\pm$ 21.3 (8.8-72.9)	76.2 $\pm$ 58.9 (4.4-150)	0.893	.578
MIP-1 α (CCL3)	37.2 $\pm$ 32.3 (6.5-125)	31.4 $\pm$ 13.1 (7.6-43.9)	104 $\pm$ 116 (0-320)	0.789	.809
MIP-1 β (CCL4)	34.1 $\pm$ 30.5 (8.1-30.5)	47.7 $\pm$ 10.5 (38.0-63.0)	118 $\pm$ 123 (0-303)	0.747	.471
RANTES (CCL5)	180 $\pm$ 123 (54.6-417)	352 $\pm$ 327 (23.1-901.4)	218 $\pm$ 309 (5.3-793)	0.907	.253
MDC (CCL22)	17.2 $\pm$ 11.7 (7.0-45.1)	23.8 $\pm$ 4.7 (15.7-28.9)	45.4 $\pm$ 48.24 (14.4 $\pm$ 142)	0.802	.031
Fractalkine (CX3CL1)	156 $\pm$ 75.9 (73.6-319)	165 $\pm$ 12.2 (153-183)	165 $\pm$ 135 (77.4-438)	0.997	.728

Data represent the mean $\pm$ SD, with minimum and maximum concentrations shown in parentheses.

*Smaller values indicate better discrimination between groups.

†To adjust for multiple comparisons, significance was defined as $\alpha \leq .005$.

‡Indicates significant differences between groups after Bonferroni correction.

§P < .01 vs adult control.

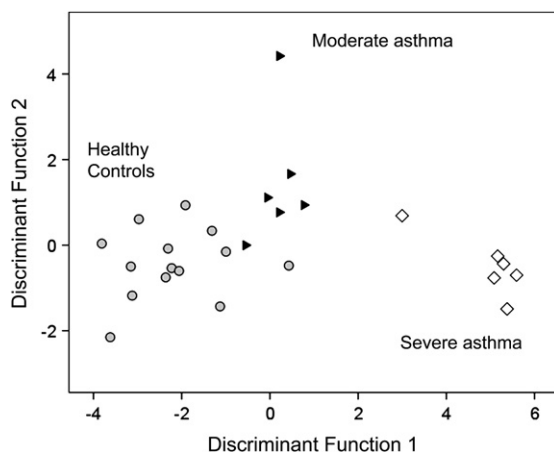


FIG 2. Scatterplot of the discriminant functions generated from 11 AM lysate cytokines and chemokines. Each data point represents a single subject. The plot depicts clustering and clear separation of children with severe asthma (white diamonds) from children with moderate asthma (black triangles) and healthy controls (gray circles).

were influenced by these medications. However, the fact that BAL inflammatory cytokines and chemokines were also elevated is interesting and may reflect altered corticosteroid sensitivity in children with severe asthma.

In summary, we have demonstrated a unique molecular phenotype of severe asthma characterized by increased BAL inflammation and biomarkers of AM activation. Whereas single biomarkers for severe asthma such as airway eosinophils and exhaled nitric oxide have limited discriminatory ability,³² this study provides further support for the use of multiple

discriminators for distinguishing severe from moderate asthma in children. The supervised method of linear discriminant analysis used here provides a possible means for classifying severe asthma in children based on the patterns of BAL and AM cytokine and chemokine expression. However, further testing of our models in an independent study population is necessary to determine the validity of our findings. We hope these findings and future ones will permit better understanding of the severe asthma phenotype in children. Although these data likely will not aid clinical diagnosis, they might convey a risk for exacerbations or response to clinical treatment. As we and others pursue the relevance of these results, other high-dimensional methods of data analysis in children with severe asthma are warranted. Ultimately, high-dimensional methods may be needed to develop targeted interventions for children with severe asthma who are otherwise very difficult to treat.

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Clinical implications: Children with severe asthma are characterized by unique patterns of airway inflammation, which may account for ongoing symptoms despite corticosteroid treatment.

REFERENCES

1. Fitzpatrick AM, Gaston BM, Erzurum SC, Teague WG. Features of severe asthma in children: atopy and increased exhaled nitric oxide. *J Allergy Clin Immunol* 2006;118:1218-25.
2. Chipps BE, Szefer SJ, Simons FE, Haselkorn T, Mink DR, Deniz Y, et al. TENOR Study Group. Demographic and clinical characteristics of children and adolescents with severe or difficult-to-treat asthma. *J Allergy Clin Immunol* 2007;119:1156-63.
3. Payne DNR, 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.
4. Fitzpatrick AM, Teague WG, Holguin F, Yeh M, Brown LAS. Airway glutathione homeostasis is altered in children with severe asthma: evidence for oxidant stress. *J Allergy Clin Immunol* 2009;123:146-52.
5. Moore WC, Bleecker ER, Curran-Everett D, Erzurum SC, Ameredes BT, Bacharier L, et al. Characterization of the severe asthma phenotype by the National Heart, Lung and Blood Institute's Severe Asthma Research Program. *J Allergy Clin Immunol* 2007;119:405-13.
6. Miranda C, Busacker A, Balzar S, Trudeau J, Wenzel SE. Distinguishing severe asthma phenotypes: role of age at onset and eosinophilic inflammation. *J Allergy Clin Immunol* 2004;113:101-8.
7. de Blic J, Tillie-Leblond I, Tonnel AB, Jaubert F, Scheinmann P, Gosset P. Difficult asthma in children: an analysis of airway inflammation. *J Allergy Clin Immunol* 2004;113:94-100.
8. Hauk P, Krawiec M, Murphy Boguniewicz J, Schiltz A, Goleva E, et al. Neutrophilic airway inflammation and association with bacterial lipopolysaccharide in children with asthma and wheezing. *Pediatr Pulmonol* 2008;43:916-23.
9. Lex C, Payne DNR, 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.
10. Gibson PG, Norzila MZ, Fakes K. Pattern of airway inflammation and its determinants in children with acute severe asthma. *Pediatr Pulmonol* 1999;28:261-70.
11. Norzila MZ, Fakes K, Henry RL, Simpson J, Gibson PG. Interleukin-8 secretion and neutrophil recruitment accompanies induced sputum eosinophil activation in children with acute asthma. *Am J Respir Crit Care Med* 2000;161:769-74.
12. Peters-Golden M. The alveolar macrophage: the forgotten cell in asthma. *Am J Respir Cell Mol Biol* 2004;31:3-7.
13. Goleva E, Hauk PJ, Hall CF, Liu AH, Riches DWH, Martin RJ, et al. Corticosteroid-resistant asthma is associated with classical microbial activation of airway macrophages. *J Allergy Clin Immunol* 2008;122:550-9.
14. Huynh MN, Malcolm KC, Kotaru C, Tilstra JA, Westcott JY, Fadok VA, et al. Defective apoptotic cell phagocytosis attenuates prostaglandin E₂ and 15-hydroxyicosatetraenoic acid in severe asthma alveolar macrophages. *Am J Respir Crit Care Med* 2005;172:972-9.
15. Bhavsar P, Hew M, Khorasani N, Torrego A, Barnes PJ, Adcock I, et al. Relative corticosteroid insensitivity in severe asthma compared with non-severe asthma. *Thorax* 2008;63:784-90.
16. Fitzpatrick AM, Holguin F, Teague WG, Brown LAS. Alveolar macrophage phagocytosis is impaired in children with poorly controlled asthma. *J Allergy Clin Immunol* 2008;121:1372-8.
17. National Heart, Lung and Blood Institute, National Asthma Education and Prevention Program. The NAEPP Expert Panel report: guidelines for the diagnosis and management of asthma—update on selected topics 2002. Bethesda (MD): National Institutes of Health; 2002. Publication no. 02-5075.
18. American Thoracic Society. Lung function testing: selection of reference values and interpretative strategies. *Am Rev Respir Dis* 1991;144:1202-18.
19. American Thoracic Society. Proceedings of the American Thoracic Society Workshop on Refractory Asthma: current understanding, recommendations, and unanswered questions. *Am J Respir Crit Care Med* 2000;162:2341-51.
20. 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.
21. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. ATS/ERS Task Force. Standardisation of spirometry. *Eur Respir J* 2005;26:319-38.
22. Hankinson JL, Odencrantz JR, Fedan KB. Spirometric reference values from a sample of the general U.S. population. *Am J Respir Crit Care Med* 1999;159:179-87.
23. American Thoracic Society and the European Respiratory Society. 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.
24. Fisher RA. The use of multiple measurements in taxonomic problems. *Ann Eugen* 1936;7:179-88.
25. Hand DJ. Discrimination and classification. New York: Wiley; 1981.
26. Calhoun WJ, Reed HE, Moest DR, Stevens CA. Enhanced superoxide production by alveolar macrophages and air-space cells, airway inflammation, and alveolar macrophage density changes after segmental antigen bronchoprovocation in allergic subjects. *Am Rev Respir Dis* 1992;145:317-25.
27. Jarjour NN, Calhoun WJ. Enhanced production of oxygen radicals in asthma. *J Lab Clin Med* 1994;123:131-6.
28. John H, Lim S, Seybold J, Jose P, Robichaud A, O'Connor B, et al. Inhaled corticosteroids increase interleukin-10 but reduce macrophage inflammatory protein-1 α , granulocyte-macrophage colony-stimulating factor, and interferon- γ release from alveolar macrophages in asthma. *Am J Respir Crit Care Med* 1998;157:256-62.
29. Hew M, Bhavsar P, Torrego A, Meah S, Khorasani N, Barnes PJ, et al. Relative corticosteroid insensitivity of peripheral blood mononuclear cells in severe asthma. *Am J Respir Crit Care Med* 2006;174:134-41.
30. Cosio B, Mann B, Ito K, Jazrawi E, Barnes PJ, Chung KF, et al. Histone acetylase and deacetylase activity in alveolar macrophages and blood monocytes in asthma. *Am J Respir Crit Care Med* 2004;170:141-7.
31. Fulkerson PC, Fischetti CA, Rothenberg ME. Eosinophils and CCR3 regulate interleukin-13 transgene-induced pulmonary remodeling. *Am J Pathol* 2006;169:2117-26.
32. Lex C, Ferreira F, Zacharasiewicz A, Nicholson AG, Haslam PL, Wilson NM, et al. Airway eosinophilia in children with severe asthma: predictive value of non-invasive tests. *Am J Respir Crit Care Med* 2006;174:1286-91.

METHOD OF LINEAR DISCRIMINANT ANALYSIS

Data analysis was performed by using SPSS software (Version 15; SPSS Inc, Chicago, Ill) after graphical screening of the data for normality. Wilks λ tests were used to test the equality of group means for each individual cytokine and chemokine. Because of the large number of comparisons, the threshold of significance was adjusted using a Bonferroni correction (for BAL analytes, $\alpha = 0.002$; for AM analytes, $\alpha = 0.005$). Variables that differed significantly between groups were further analyzed by ANOVA with Tukey post hoc tests using a 2-tailed α of 0.05.

To determine whether the combinations of BAL and AM cytokines and chemokines could discriminate between children with severe versus moderate asthma and healthy controls, a linear discriminant analysis was performed by using the Fisher method, which is robust against departures from normality. Linear discriminant analysis is similar to logistic regression and is useful for building a predictive model of group membership based on observed characteristics. The procedure yields a set of discriminant functions based on the linear combinations of variables that provide the best discrimination between groups. The resulting model takes the form

$$D = a_1x_1 + a_2x_2 + \dots a_ix_i,$$

where D is the discriminant function (a number generated from the equation), x is the predictor variable, and a is the coefficient. With 3 groups, there are 2 resulting discriminant functions produced. The procedure automatically chooses a first function that will separate the groups as much as possible. It then chooses a second function that is both uncorrelated with the first function and provides as much further separation as possible. The discriminant functions can be plotted to visualize the proximity of that subject to others in the same group.

Discriminant analysis was performed by using the raw concentrations of cytokines and chemokines measured in the BAL supernatant and AM lysate. Data were not logarithmically transformed because of acceptable power that was achieved by using the nontransformed data. Power was confirmed by entering the covariance matrix and group means into PASS software (Version 2008; NCSS, Kaysville, Utah) and calculating the power for a multivariate analysis of variance. For the BAL and AM lysate models, powers of 0.97 and 0.70 were achieved, respectively, to test differences between groups with a Wilks λ approximate F test and a 5% significance level. Effect sizes were further generated using the Wilks λ for the entire model such that

$$\eta^2 = 1 - \lambda^{1/3}.$$

The effect sizes (η^2) for the BAL analysis and AM lysate analysis were 0.56 and 0.63, respectively.

For the BAL analysis, the first model was generated from the linear combination of all 23 cytokines and chemokines measured in the raw BAL fluid without correction for the protein content or dilution of the BAL fluid. Because of some missing data points, 24 healthy adult controls, 22 subjects with moderate asthma, and 22 subjects with severe asthma were included in the linear discriminant model of the raw BAL fluid markers.

For the AM analysis, 12 of the 23 cytokines and chemokines measured were undetectable in the AM lysate. Therefore, the second model was generated from the linear combination of only those 11 analytes expressed by the AMs, which included IL-6, TNF- α , GRO (CXCL1), IL-8 (CXCL8), IP-10 (CXCL10), MCP-1 (CCL2), MIP-1 α (CCL3), MIP-1 β (CCL4), RANTES (CCL5), MDC (CCL22), and fractalkine (CX3CL1). These AM concentrations were adjusted for the protein content of the cell lysate and are expressed as picograms per milligram of protein. The second linear discriminant model of the AM cell lysate markers included 14 healthy adult controls, 6 children with moderate asthma, and 6 children with severe asthma.

Variables were entered simultaneously and were not subjected to stepwise methods because of no *a priori* knowledge of logical ordering. Previous probabilities for group assignment were adjusted for the number of cases included in the analysis. Covariance of the predictor variables was assessed using pooled within-groups matrices and Box M tests. The ability of the canonical discriminant functions to distinguish between groups was further evaluated by Wilks λ and χ^2 tests. Cut-points for group membership are depicted in Table E15.

Cross-validation of the models was performed in randomly selected subjects by using Bernoulli variates with a probability parameter of 0.7. For cross-validation, a preset number of cases was extracted once and treated as the test data. For the validation model of the BAL markers, the new dataset (extracted from the existing database) included 14 adult controls, 17 subjects with moderate asthma, and 16 subjects with severe asthma. The validation model of the AM markers included 10 adult controls, 3 children with moderate asthma, and 3 children with severe asthma.

To determine the strongest discriminators of the groups, 2 stepwise linear discriminant analyses were performed. For the first model, all 23 cytokines and chemokines measured in the BAL fluid were entered as predictors. For the second model, the 11 analytes expressed by the AMs, including IL-6, TNF- α , GRO (CXCL1), IL-8 (CXCL8), IP-10 (CXCL10), MCP-1 (CCL2), MIP-1 α (CCL3), MIP-1 β (CCL4), RANTES (CCL5), MDC (CCL22), and fractalkine (CX3CL1), were entered as predictors. Variables were entered simultaneously using the Wilks λ method. Entry and removal probabilities for the F statistic were set at 0.05 and 0.10, respectively.

REFERENCES

- E1. Shore SA, Moore PE. Effects of cytokines on contractile and dilator responses of airway smooth muscle. *Clin Exp Pharmacol Physiol* 2002;29:859-6.
- E2. Wilson MS, Pesce JT, Ramalingam TR, Thompson RW, Cheever A, Wynn TA. Suppression of murine allergic airway disease by IL-2: anti-IL-2 monoclonal antibody-induced regulatory T cells. *J Immunol* 2008;181:6942-54.
- E3. Koya T, Miyahara N, Takeda K, Matsubara H, Swasey C, Balhorn A, Dakhama A, Gelfand EW. CD8+ T cell-mediated airway hyperresponsiveness and inflammation is dependent on CD4+ IL-4+ T cells. *J Immunol* 2007;179:2787-96.
- E4. Zhao LL, Lotvall J, Linden A, Tomaki M, Sjostrand M, Bossios A. Prolonged eosinophil production after allergen exposure in IFN-gammaR KO mice is IL-5 dependent. *Scand J Immunol* 2008;67:480-8.
- E5. Rose-John S, Scheller J, Elson G, Jones SA. Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. *J Leukoc Biol* 2006;80:227-36.
- E6. Kelly EA, Koziol-White CJ, Clay KJ, Liu LY, Bates ME, Bertics PJ, Jarjour NN. Potential contribution of IL-7 to allergen-induced eosinophilic airway inflammation in asthma. *J Immunol* 2009;182:1404-10.
- E7. Commins S, Steinke JW, Borish L. The extended IL-10 superfamily: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, and IL-29. *J Allergy Clin Immunol* 2008;121:1108-11.
- E8. Wills-Karp M. IL-12/IL-13 axis in allergic asthma. *J Allergy Clin Immunol* 2001;107:9-18.
- E9. Kasaian MT, Donaldson DD, Tchistiakova L, Marquette K, Tan XY, Ahmed A, Jacobson BA, Widom A, Cook TA, Xu X, Barry AB, Goldman SJ, Abraham WM. Efficacy of IL-13 neutralization in a sheep model of experimental asthma. *Am J Respir Cell Mol Biol* 2007;36:368-76.
- E10. Isogai S, Athiviraham A, Fraser RS, Taha R, Hamid Q, Martin JG. Interferon-gamma-dependent inhibition of late allergic airway responses and eosinophilia by CD8+ gamma delta T cells. *Immunology* 2007;122:230-8.
- E11. Faith A, McDonald J, Peek E, Richards D, Caulfield J, Chevreton E, Roberts D, Lee T, Corrigan C, Hawrylowicz C. Functional plasticity of human respiratory tract dendritic cells: GM-CSF enhances T(H)2 development. *J Allergy Clin Immunol* 2005;116:1136-43.
- E12. Brightling C, Berry M, Amrani Y. Targeting TNF-alpha: a novel therapeutic approach for asthma. *J Allergy Clin Immunol* 2008;121:5-10.
- E13. Geiser T, Dewald B, Ehrenguber MU, Clark-Lewis I, Baggiolini M. The interleukin-8-related chemotactic cytokines GRO alpha, GRO beta, and GRO gamma activate human neutrophil and basophil leukocytes. *J Biol Chem* 1993;268:15419-24.
- E14. Streiter RM. Interleukin-8: a very important chemokine of the human airway epithelium. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L688-9.
- E15. Lindell DM, Lane TE, Lukacs NW. CXCL10/CXCR3-mediated responses promote immunity to respiratory syncytial virus infection by augmenting dendritic cell and CD8(+) T cell efficacy. *Eur J Immunol* 2008;38:2168-79.
- E16. Schober A. Chemokines in vascular dysfunction and remodeling. *Arterioscler Thromb Vasc Biol* 2008;28:1950-9.
- E17. Maurer M, von Stebut E. Macrophage inflammatory protein-1. *Int J Biochem Cell Biol* 2004;36:1882-6.
- E18. Appay V, Rowland-Jones SL. RANTES: a versatile and controversial chemokine. *Trends Immunol* 2001;22:83-7.

- E19. Shang XZ, Chiu BC, Stolberg V, Lukacs NW, Kunkel SL, Murphy HS, Chensue SW. Eosinophil recruitment in type-2 hypersensitivity pulmonary granulomas: source and contribution of monocyte chemotactic protein-3 (CCL7). *Am J Pathol* 2002;161:257-66.
- E20. Rothenberg ME. Eotaxin. An essential mediator of eosinophil trafficking into mucosal tissues. *Am J Respir Cell Mol Biol* 1999;21:291-5.
- E21. Pinho V, Oliveira SH, Souza DG, Vasconcelos D, Alessandri AL, Lukacs NW, Teixeira MM. *J Leukoc Biol* 2003;73:356-62.
- E22. Guo J, Chen T, Wang B, Zhang M, An H, Guo Z, Yu Y, Qin Z, Cao X. Chemoattraction, adhesion and activation of natural killer cells are involved in the anti-tumor immune response induced by fractalkine/CX3CL1. *Immunol Lett* 2003;89:1-7.

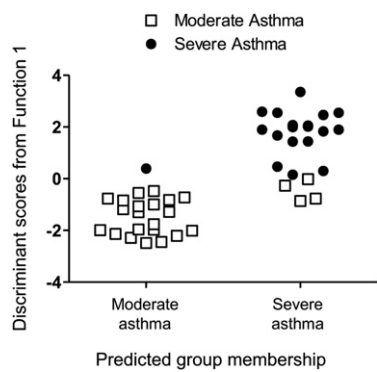


FIG E1. Predicted group membership of children with *a priori*-defined moderate and severe asthma by linear discriminant analysis of 23 cytokines and chemokines in the BAL fluid. To demonstrate the utility of the discriminant analysis in distinguishing asthma severity in children, the analysis was performed excluding healthy controls. The unstandardized cut-point of the canonical discriminant function for the classification of patients was 1.403 (standardized is 0).

TABLE E1. Criteria for severe asthma in children, as defined by the National Institutes of Health/National Heart, Lung, and Blood Institute’s Severe Asthma Research Program

Major criteria for severe asthma (must have at least 1)	
Daily high-dose ICS (at least 6 continuous mo)	
Children <12 y: ≥440 µg fluticasone equivalent/d	
Children ≥12 y: ≥880 µg fluticasone equivalent/d	
Daily oral corticosteroid use (at least 6 continuous mo)	
Minor criteria for severe asthma (must have at least 2)	
Treatment with a daily controller medication in addition to ICS	
Daily short-acting bronchodilator use (at least 5 of 7 d)	
Airway obstruction with FEV ₁ <80% predicted at baseline	
One or more emergency department visits in the previous 12 mo	
Three or more oral corticosteroid bursts in the previous 12 mo	
History of worsening symptoms with a reduction in corticosteroid dose	
History of intubation	

TABLE E2. Rationale for cytokine and chemokine selection

Cytokine/chemokine	Biological actions relevant to asthma	Reference
IL-1 β	Activates T-cells and macrophages, inhibits relaxation of airway smooth muscle	E1
IL-2	Regulates endogenous regulatory T cells, which suppress airway inflammation	E2
IL-4	Activates B cells; induces differentiation into T _H 2 cells, airway eosinophilia, remodeling, and airway hyperresponsiveness	E3
IL-5	Primary cytokine involved in the production, differentiation, maturation and activation of the eosinophils	E4
IL-6	Promotes T-cell and B-cell growth and differentiation, may promote transition from acute to chronic airway inflammation	E5
IL-7	Promotes maturation and survival of T cells, enhances eosinophil activation and survival	E6
IL-10	Potent suppressor of macrophage functions, downregulates T _H 1 cytokines, enhances antibody production	E7
IL-12 (p70)	Activates NK cells, induces CD4 T-cell differentiation into T _H 1-like cells	E8
IL-13	Contributes to airway hyperresponsiveness, mucous secretion, inflammation, and fibrosis	E9
IFN- γ	Macrophage activation, increased expression of MHC molecules, inhibits late airway responses and airway eosinophils	E10
GM-CSF	Stimulates growth and differentiation of myelomonocytic lineage cells, enhances T _H 2 cell development	E11
TNF- α	Induces airway hyperresponsiveness, activates epithelial/endothelial cells, promotes neutrophil and eosinophil recruitment	E12
GRO (CXCL1)	Potent neutrophil chemoattractant, promotes angiogenesis and macrophage adhesion	E13
IL-8 (CXCL8)	Potent neutrophil chemoattractant	E14
IP-10 (CXCL10)	Inhibits angiogenesis, promotes viral clearance and leukocyte recruitment	E15
MCP-1 (CCL2)	Promotes mast cell degranulation, signals leukocyte infiltration, promotes smooth muscle formation	E16
MIP-1 α (CCL-3)	Enhances T-cell chemotaxis and monocyte migration, modulates T _H differentiation	E17
MIP-1 β (CCL-4)	Enhances T-cell chemotaxis and monocyte migration	E17
RANTES (CCL5)	Promotes mast cell, macrophage, and eosinophil infiltration to sites of inflammation	E18
MCP-3 (CCL7)	Signals leukocyte infiltration and eosinophil recruitment, regulates macrophage function	E19
Eotaxin (CCL11)	Potent eosinophil chemoattractant	E20
MDC (CCL22)	Potent T _H 2 cell chemoattractant, activates eosinophils	E21
Fractalkine (CX3CL1)	Promotes leukocyte recruitment and adhesion, activates NK cells	E22

NK, Natural killer.

TABLE E3. Assay sensitivities and precision as reported by the manufacturer

Cytokine/chemokine	Minimal detectable concentration (pg/mL)	Intra-assay precision (% CV)	Interassay precision (% CV)
IL-1 β	0.06	3.11	2.16
IL-2	0.16	4.27	7.48
IL-4	0.13	4.16	9.12
IL-5	0.01	4.50	14.27
IL-6	0.10	3.51	4.48
IL-7	0.12	4.75	6.24
IL-10	0.15	3.31	11.84
IL-12 (p70)	0.11	4.31	5.08
IL-13	0.48	5.86	11.99
IFN- γ	0.10	4.60	5.80
GM-CSF	0.46	4.14	5.03
TNF- α	0.05	3.49	3.78
GRO (CXCL1)	10.10	5.10	11.60
IL-8 (CXCL8)	0.11	3.26	6.48
IP-10 (CXCL10)	1.20	4.70	11.20
MCP-1 (CCL2)	0.90	6.10	12.0
MIP-1 α (CCL-3)	3.50	5.70	14.40
MIP-1 β (CCL-4)	4.50	5.30	10.60
RANTES (CCL5)	1.00	6.20	15.70
MCP-3 (CCL7)	2.00	7.10	13.10
Eotaxin (CCL11)	1.20	7.40	15.0
MDC (CCL22)	3.70	5.10	9.30
Fractalkine (CX3CL1)	6.0	5.30	10.10

CV, Coefficient of variation.

TABLE E4. Features of children with BAL colonization who were excluded from data analysis

Subject	Asthma severity	Sex	Race	Age (y)	ICS dose*	FEV ₁ (%)	BAL neutrophils (%)	Bacteria identified
1	Moderate	Male	White	16	600	106	51.0	<i>S pneumoniae</i>
2	Moderate	Female	White	11	200	82	12.1	<i>H influenzae</i>
3	Moderate	Male	White	10	200	81	88.7	<i>S pneumoniae</i>
4	Severe	Male	White	8	500	104	13.0	<i>H influenzae</i>
5	Severe	Male	White	7	1000	115	5.2	<i>H influenzae</i>
6	Severe	Male	White	9	1000	70	20.8	<i>H influenzae</i>
7	Severe	Female	Black	7	1000	92	6.7	<i>S pneumoniae</i>

*ICS dose is expressed as micrograms of fluticasone equivalents/day.

TABLE E5. Canonical discriminant function coefficients for the linear discriminant model generated from the cytokine and chemokine concentrations measured in the BAL fluid*

Cytokine/chemokine	Standardized	
	Function 1	Function 2
IL-1 β	0.332	0.583
IL-2	-0.046	-0.281
IL-4	-0.195	0.410
IL-5	0.659	-0.167
IL-6	0.590	0.382
IL-7	-0.140	0.071
IL-10	-0.864	0.737
IL-12 (p70)	0.463	-0.280
IL-13	0.848	-0.560
IFN- γ	0.158	0.709
GM-CSF	-0.366	0.323
TNF- α	0.196	-0.673
GRO (CXCL1)	0.470	1.151
IL-8 (CXCL8)	-0.021	-1.007
IP-10 (CXCL10)	0.576	-0.868
MCP-1 (CCL2)	-4.081	1.476
MIP-1 α (CCL-3)	5.470	3.205
MIP-1 β (CCL-4)	-0.426	0.686
RANTES (CCL5)	0.524	-0.638
MCP-3 (CCL7)	0.648	-0.376
Eotaxin (CCL11)	-2.061	-4.208
MDC (CCL22)	-0.131	0.059
Fractalkine (CX3CL1)	-0.177	-0.222

The standardized discriminant function coefficients are similar to the β weights in multiple regression and indicate the partial contribution of each variable to the discriminant functions, controlling for the other independent variables that are entered into the equation. The standardized functions permit comparison of variables that are measured on different scales. Larger absolute values correspond to greater discriminating ability.

*Subjects with missing data because of insufficient BAL volume were excluded.

TABLE E6. Classification function coefficients for the linear discriminant model generated from the cytokine and chemokine concentrations measured in the BAL fluid

Cytokine/chemokine	Adult control* (n = 24)	Moderate asthma (n = 22)	Severe asthma* (n = 22)
IL-1 β	15.549	61.152	32.632
IL-2	4.370	-3.530	5.087
IL-4	1.936	2.205	0.682
IL-5	1.014	3.332	4.924
IL-6	0.299	0.869	0.750
IL-7	0.531	0.326	0.075
IL-10	-0.311	-0.746	-1.913
IL-12 (p70)	1.165	7.594	17.428
IL-13	-2.991	0.256	5.877
IFN- γ	-2.951	2.349	-2.922
GM-CSF	7.695	5.925	0.800
TNF- α	-0.249	-0.489	0.177
GRO (CXCL1)	-0.002	0.001	-0.001
IL-8 (CXCL8)	0.003	-0.065	0.029
IP-10 (CXCL10)	6.71×10^{-5}	4.79×10^{-5}	0.003
MCP-1 (CCL2)	0.069	0.042	0.017
MIP-1 α (CCL-3)	-0.160	-0.005	-0.032
MIP-1 β (CCL-4)	0.075	0.080	-0.029
RANTES (CCL5)	-0.004	-0.002	0.015
MCP-3 (CCL7)	-0.418	-0.290	-0.104
Eotaxin (CCL11)	0.055	-0.831	-0.219
MDC (CCL22)	0.019	0.014	0.009
Fractalkine (CX3CL1)	0.049	0.034	0.041
Constant	-4.950	-11.893	-12.544

The resulting discriminant model takes the form $D = a_1x_1 + a_2x_2 + \dots a_ix_i$, where D is the discriminant function, x is the predictor variable, and a is the coefficient. The coefficients here are the ones used to generate the discriminant functions. For each person in the dataset, the concentration of each analyte was multiplied by the coefficients given here (depending on *a priori* group assignment).

*Subjects with missing data because of insufficient BAL volume were excluded from the model.

TABLE E7. Eigenvalues and Wilks λ values for the linear discriminant model generated from the cytokine and chemokine concentrations measured in the BAL fluid

Discriminant function	Eigenvalue	Percent of variance	Canonical correlation	Wilks λ	χ^2	df	P value
Original model							
1	3.402	67.3	0.879	0.086	132.71	46	<.001
2	1.652	32.7	0.789	0.377	52.67	22	<.001
Validation model							
1	4.725	68.2	0.908	0.054	97.50	44	<.001
2	2.208	31.8	0.830	0.312	39.05	21	.010

Both canonical discriminant functions (1 and 2) were used for the original model and the validation model.

In the original model, the first discriminant function provides separation of controls and subjects with asthma and explains 67% of the total variance in the BAL cytokine and chemokine data. The second discriminant function provides separation of subjects with moderate and severe asthma and explains the remaining 33% of the variance in the BAL analytes. Both discriminant functions are statistically significant. Thus, there are differences between the controls and subjects with asthma, and also between the moderate and severe asthmatic groups.

TABLE E8. Classification results for the original and validation models generated from the cytokine and chemokine concentrations measured in the BAL fluid

Model	Group	Predicted group membership			Total
		Adult control	Moderate asthma	Severe asthma	
Original model*	Adult control	24	0	0	24
	Moderate asthma	3	19	0	22
	Severe asthma	0	2	20	22
Validation model†	Adult control	14	0	0	14
	Moderate asthma	1	15	1	17
	Severe asthma	0	1	15	16

*Of the original group cases, 92.6% were correctly classified.

†Of the validation cases, 83.3% were correctly classified.

TABLE E9. Results of stepwise linear discriminant analysis of cytokines and chemokines in the BAL fluid

Step	Variables included	Tolerance	Significance of F to remove	Wilks λ	Exact F value	P value
1	GRO	1.000	<0.001		21.632	<.001
2	GRO	0.997	<0.001	0.690	15.470	<.001
	IL-13	0.997	<0.001	0.600		
3	GRO	0.997	<0.001	0.532	13.200	<.001
	IL-13	0.988	<0.001	0.494		
	IL-6	0.991	0.003	0.454		
4	GRO	0.892	<0.001	0.431	11.578	<.001
	IL-13	0.981	<0.001	0.432		
	IL-6	0.988	0.003	0.395		
	RANTES	0.885	0.013	0.377		
5	GRO	0.821	0.001	0.368	10.392	<.001
	IL-13	0.970	<0.001	0.388		
	IL-6	0.951	<0.001	0.363		
	RANTES	0.837	0.004	0.350		
	IL-12	0.869	0.029	0.328		
6	GRO	0.786	0.001	0.315	10.028	<.001
	IL-13	0.703	<0.001	0.367		
	IL-6	0.748	<0.001	0.341		
	RANTES	0.810	0.002	0.309		
	IL-12	0.799	0.005	0.297		
	IL-10	0.615	0.009	0.292		
7	GRO	0.775	0.001	0.277	9.673	<.001
	IL-13	0.689	<0.001	0.317		
	IL-6	0.743	<0.001	0.296		
	RANTES	0.790	0.001	0.275		
	IL-12	0.717	0.002	0.268		
	IL-10	0.607	0.007	0.256		
	IFN- γ	0.853	0.016	0.249		

TABLE E10. Canonical discriminant function coefficients for the stepwise linear discriminant model generated from the cytokine and chemokine concentrations measured in the BAL fluid*

Cytokine/chemokine	Standardized	
	Function 1	Function 2
IL-6	0.718	−0.174
IL-10	−0.429	0.632
IL-12	0.396	−0.705
IL-13	0.788	−0.338
IFN- γ	0.095	0.675
GRO (CXCL1)	0.317	0.813
RANTES (CCL5)	0.388	−0.714

The standardized discriminant function coefficients are similar to the β weights in multiple regression and indicate the partial contribution of each variable to the discriminant functions, controlling for the other independent variables that are entered into the equation. The standardized functions permit comparison of variables that are measured on different scales. Larger absolute values correspond to greater discriminating ability.

*Subjects with missing data because of insufficient BAL volume were excluded.

TABLE E11. Canonical discriminant function coefficients for the linear discriminant model generated from the cytokine and chemokine concentrations measured in the AM lysate

Cytokine/chemokine	Standardized	
	Function 1	Function 2
IL-6	−0.725	−0.986
TNF- α	−0.424	0.116
GRO (CXCL1)	−2.482	−0.447
IL-8 (CXCL8)	2.156	0.289
IP-10 (CXCL10)	0.688	0.889
MCP-1 (CCL2)	−0.894	0.842
MIP-1 α (CCL-3)	−1.519	−0.254
MIP-1 β (CCL-4)	4.975	0.565
RANTES (CCL5)	0.930	−0.822
MDC (CCL22)	1.144	−0.162
Fractalkine (CX3CL1)	1.619	−0.006
Constant	—	—

The standardized discriminant function coefficients are similar to the β weights in multiple regression and indicate the partial contribution of each variable to the discriminant functions, controlling for the other independent variables that are entered into the equation. The standardized functions permit comparison of variables that are measured on different scales. Larger absolute values correspond to greater discriminating ability.

TABLE E12. Classification function coefficients for the linear discriminant model generated from the chemokine concentrations measured in the AM cell lysate

Cytokine/chemokine	Adult control (n = 14)	Moderate asthma (n = 6)	Severe asthma (n = 6)
IL-6	-4.064	-3.352	-11.745
TNF- α	0.244	-1.185	-3.981
GRO (CXCL1)	-0.008	-0.013	-0.028
IL-8 (CXCL8)	0.072	0.111	0.236
IP-10 (CXCL10)	0.001	0.000	0.006
MCP-1 (CCL2)	-0.010	-0.089	-0.161
MIP-1 α (CCL-3)	-0.131	-0.175	-0.325
MIP-1 β (CCL-4)	0.258	0.406	0.870
RANTES (CCL5)	0.023	0.038	0.054
MDC (CCL22)	0.152	0.264	0.512
Fractalkine (CX3CL1)	0.079	0.119	0.224
Constant	-8.934	-20.027	-60.069

The resulting discriminant model takes the form $D = a_1x_1 + a_2x_2 + \dots a_ix_i$, where D is the discriminant function, x is the predictor variable, and a is the coefficient. The coefficients here are the ones used to generate the discriminant functions. For each person in the dataset, the concentration of each analyte was multiplied by the coefficients given here (depending on *a priori* group assignment).

TABLE E13. Eigenvalues and Wilks λ values for the linear discriminant model generated from the cytokine and chemokine concentrations measured in the AM cell lysate

Discriminant function	Eigenvalue*	Percent of variance	Canonical correlation	Wilks λ	χ^2	df	P value
Original model							
1	10.645	92.8	0.956	0.05	54.983	22	<.001
2	0.822	7.2	0.672	0.549	10.795	10	.374
Validation model							
1	21.690	78.9	0.978	0.006	40.30	22	.010
2	5.791	21.1	0.923	0.147	15.33	10	.121

In the original model, the first discriminant function provides separation of controls and subjects with asthma and explains 93% of the total variance in the AM cytokine and chemokine data. The second discriminant function provides separation of subjects with moderate and severe asthma and explains the remaining 7% of the variance in the AM analytes.

*Both canonical discriminant functions (1 and 2) were used for the original model and the validation model.

TABLE E14. Classification results for the original and validation models generated from the cytokine and chemokine concentrations measured in the AM cell lysate

Model	Group	Predicted group membership			Total
		Adult control	Moderate asthma	Severe asthma	
Original model*	Adult control	13	1	0	14
	Moderate asthma	1	5	0	6
	Severe asthma	0	0	6	6
Validation model†	Adult control	10	0	0	10
	Moderate asthma	0	3	0	3
	Severe asthma	0	0	3	3

*Of the original group cases, 92.3% were correctly classified.

†Of the validation cases, 100% were correctly classified.

TABLE E15. Cut-points (ie, functions at group centroids) for the classification of patients according to group

Model	Function 1	Function 2
BAL fluid (original model)		
Adult controls	−2.377	−0.390
Subjects with moderate asthma	0.782	1.733
Subjects with severe asthma	1.810	−1.308
BAL fluid (stepwise model)		
Adult controls	−1.643	0.024
Subjects with moderate asthma	0.799	−0.452
Subjects with severe asthma	0.950	0.407
AM lysate (original model)		
Adult controls	−2.240	0.485
Subjects with moderate asthma	−0.165	−1.556
Subjects with severe asthma	5.392	0.423

Values represent the canonical discriminant functions evaluated at group means. The information presented in this table is used to establish the cut-points for classification of patients into their appropriate groups. These values represent the weighted average of the paired values.