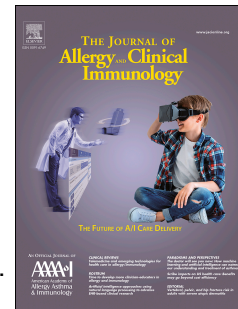


Journal Pre-proof

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PII: S0091-6749(20)30809-5

DOI: <https://doi.org/10.1016/j.jaci.2020.05.039>

Reference: YMAI 14610

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 25 December 2019

Revised Date: 11 May 2020

Accepted Date: 13 May 2020

Please cite this article as: Steinke JW, Lawrence MG, Teague WG, Braciale TJ, Patrie JT, Borish L, BAL Cytokine Patterns in Children with Severe Neutrophilic and Pauci-granulocytic Asthma, *Journal of Allergy and Clinical Immunology* (2020), doi: <https://doi.org/10.1016/j.jaci.2020.05.039>.

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BAL Cytokine Patterns in Children with Severe Neutrophilic and Pauci-granulocytic Asthma

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This work was supported by The Ivy Foundation of Charlottesville, NIH/NHLBI Severe Asthma Research Program U10HL109250-07 (WGT), and NIAAID U01A123337 (LB). American Lung Association/AAAAI Allergic Respiratory Diseases Award (ML)

24 Conflicts of interest: John Steinke-has changed jobs and now works for Genentech,
25 however all work was completed prior to starting the job. No other conflicts related to
26 the paper. W Gerald Teague-no conflicts of interest. Larry Borish- no conflicts of
27 interest. Monica Lawrence- no conflicts of interest. James Patrie- no conflicts of interest.
28 Thomas Braciale- no conflicts of interest.

29

Abstract

Background: Asthma is a complex heterogeneous disease occurring in adults and children that is characterized by distinct inflammatory patterns. While numerous studies have been performed in adults, little is known regarding the heterogeneity of severe asthma in children, particularly inflammatory signatures involving the air spaces.

Objective: To determine the relationship of bronchoalveolar lavage (BAL) cytokine/chemokine expression patterns in children with severe-therapy resistant asthma stratified according to neutrophilic versus non-neutrophilic BAL inflammatory cell patterns.

Methods: Children with severe asthma with inadequate symptom control despite therapy, underwent diagnostic bronchoscopy and BAL. Inflammatory cytokine/chemokine concentrations were determined using a multiplex protein bead assay.

Results: Analysis of BAL constituents with an unbiased clustering approach revealed distinct cytokine/chemokine patterns and these aligned with pathways associated with type 2 innate lymphoid cells, monocytes, neutrophil trafficking, and T effector cells. All cytokines examined (27) with one exception (VEGF) were over-expressed with BAL neutrophilia compared to non-neutrophilic asthma and this was confirmed in a cross-validation analysis. Cytokines specifically responsible for Th17 (IL-17, IL-6, G-CSF) and Th1 differentiation and expression (IL-12, TNF- α , IFN- γ) were enhanced in the neutrophilic cohorts. Neutrophilic groups were also characterized by higher prevalence

of bacterial and viral pathogens, however, cytokine expression patterns manifested independently of pathogen expression.

Conclusion: The results demonstrate that children with refractory asthma and neutrophilic inflammation had a BAL cytokine pattern consistent with a mixed Th17/Th1/Th2 response. In contrast, non-neutrophilic asthma presented independently of cytokine overexpression.

Word counts

Abstract = 234

Body = 2567

Keywords: severe asthma, BAL granulocytes, neutrophilic asthma, eosinophilic asthma, asthma phenotypes, asthma heterogeneity, cytokine, chemokine, rhinovirus

Capsule Summary: Children with refractory asthma and neutrophilic inflammation have a BAL cytokine pattern consistent with a mixed inflammatory response. In contrast, non-neutrophilic asthma presented independently of cytokine overexpression.

Clinical Implications: Children with refractory asthma and neutrophilic inflammation have a Th1/Th17 BAL cytokine pattern while non-neutrophilic asthma presents independently of cytokine overexpression. Both patterns explain non-responsiveness to standard therapeutic approaches.

72

Abbreviations

73 BAL – bronchoalveolar lavage

74 BH – Benjamini and Hochberg

75 FGF – fibroblast growth factor

76 G-CSF – granulocyte colony-stimulating factor

77 GM-CSF – granulocyte-macrophage colony-stimulating factor

78 CCS – corticosteroids

79 IFN – interferon

80 FEV₁ – forced expired volume in one second

81 FVC – forced vital capacity

82 PDGF – platelet-derived growth factor

83 PMN – polymorphonuclear leukocyte

84 RV – rhinovirus

85 SARP – Severe Asthma Research Program

86 SD – standard deviation

87 TNF – tumor necrosis factor

88 VEGF – vascular endothelial growth factor

89 YI – Youden Index

90

Introduction

Asthma presents as a heterogeneous disorder in both adults and children that is characterized phenotypically by various distinct inflammatory patterns and airway hyperreactivity.¹⁻⁵ Attempts have been made to better characterize this heterogeneity in an effort to identify endotypes with the goal of delivering more precise treatment options. Previous studies of adults found sputum granulocyte patterns were associated with specific clinical phenotypes.^{3,6} In one of these studies, the group that had both sputum eosinophils and neutrophils had the lowest lung function, worst asthma control and greater symptoms.⁶ The group with only sputum neutrophils had the highest levels of IL-1 β and CCL20 suggestive of a pro-inflammatory response to infection.⁶ In a similar study, patients with persistent asthma were separated into eosinophilic and non-eosinophilic groups with the non-eosinophilic group characterized by neutrophilic influx with high CXCL8 protein levels.¹

While these and numerous other studies have been performed in adults, the heterogeneity of severe asthma in children has only modestly been explored. We previously published our observations regarding the identification of four phenotypes based on bronchoalveolar lavage (BAL) granulocyte patterns in a sample of children with severe, treatment-refractory asthma.⁷ This was accomplished with the appreciation that all subjects were on high dose inhaled corticosteroids (CCS) and many on oral CCS and this use likely influenced the inflammatory pattern observed. Using criteria established by the severe asthma research program (SARP), these cohorts included subjects with isolated eosinophilia, isolated neutrophilia, mixed granulocytic and paucigranulocytic infiltrates. The groups with neutrophils were younger and had more

114 frequent bacterial and viral pathogens identified in the BAL, while the eosinophilic
115 groups demonstrated increased allergen sensitization, higher IgE levels and higher
116 blood eosinophil levels.⁷ Knowledge of the factors driving the pathogenesis of these
117 clinical phenotypes in children with severe asthma is limited.

118 The aim of the current study was to identify cytokine/chemokine expression patterns in
119 children with severe, therapy- resistant asthma. As all the children in the study had
120 severe asthma, they were all being treated with high dose inhaled steroids and many
121 were also on oral steroids.⁷ Reflecting these aspects of the cohort, few subjects had
122 isolated BAL eosinophilia. Therefore, because the study was exploratory and had no *a*
123 *priori* hypothesis, we stratified the present analysis according to neutrophilic versus non-
124 neutrophilic BAL granulocyte patterns.

125

Methods

Subjects. Study subjects comprised a subset of children reported in our previous publication.⁷ The subjects were selected from the larger study cohort on the basis of sequential enrollment and availability of research samples and relevant clinical information. For the current study, BAL fluid taken at the time of bronchoscopy (n=91) was analyzed for cytokine/chemokine expression and for presence of respiratory pathogens. Briefly, children with severe asthma who failed treatment were referred for evaluation and treatment in our severe asthma clinic. Children who adhered to treatment protocols, but who had inadequate symptom control despite specialty-supervised guidelines-directed therapy, underwent diagnostic bronchoscopy and BAL was collected.⁷ The original study protocol was approved by the Institutional Review Board (UVA IRB-HSR # 17555 and UVA IRB-HSR # 10905). The legal caregivers of the children provided informed consent and children at the appropriate age provided assent to have unused specimens left over from the clinical laboratory banked and analyzed for research purposes.

BALF cytokine analysis. Protein concentrations were detected using a multiplex protein bead assay (Bio-Rad, Hercules, CA). A panel of 27 cytokines and chemokines was utilized (IL-1 β , IL-1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8 (IL-8), IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, interferon (IFN)- γ , granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF)- α , fibroblast growth factor (FGF)-basic, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), CCL2 (MCP-1), CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL5 (RANTES), CCL11 (eotaxin), CXCL10 (IP-

10)). Concentrations were measured in BAL fluid that was concentrated 5-fold using Amicon filter with an exclusion of 3kD. The lower limits of detection for these assays were 0.32 pg/mL IL-1 β , 5.39 pg/mL IL-1Ra, 0.89 pg/mL IL-2, 0.10 pg/mL IL-4, 0.77 pg/mL IL-5, 1.36 pg/mL IL-6, 0.65 pg/mL IL-7, 1.47 pg/mL CXCL8, 0.73 pg/mL IL-9, 2.65 pg/mL IL-10, 2.27 pg/mL IL-12p70, 0.38 pg/mL IL-13, 1.91 pg/mL IL-15, 2.50 pg/mL IL-17, 1.22 pg/mL IFN- γ , 1.62 pg/mL G-CSF, 0.77 pg/mL GM-CSF, 1.87 pg/mL TNF- α , 8.12 pg/mL FGFbasic, 0.84 pg/mL PDGF, 2.55 pg/mL VEGF, 1.62 pg/mL CCL2, 0.24 pg/mL CCL3, 0.28 pg/mL CCL4, 0.75 pg/mL CCL5, 1.10 pg/mL CCL11, 1.06 pg/mL CXCL10.

Granulocyte phenotyping. Unfiltered BAL fluid was transported fresh to the laboratory for total cell count and differential for assignment to BAL granulocyte categories as previously described.⁷ Granulocyte categories were either neutrophilic ($\geq 6\%$ neutrophils) or non-neutrophilic ($< 6\%$ neutrophils) and confirmed by calculating an absolute neutrophil count based on the total cell count.

BAL respiratory pathogen expression. All subjects were assiduously screened for presence of symptoms of a respiratory infection within 1 month of the bronchoscopy and none of them had physical findings suggesting infection. BAL was submitted fresh to the hospital cytopathology laboratory for cytospin preparations and staining. Smears were examined by a cytopathologist for extracellular or intracellular bacteria and the pattern of inflammation. An aliquot of BAL went to the Clinical Microbiology and Molecular Diagnostics Laboratory for qualitative bacterial gram stain, culture, and sensitivities and PCR for respiratory viral pathogens (Luminex xTAG reagents, LiquiChip) including adenovirus, human metapneumovirus, influenza A (subtype

H1,H3, 2009 H1N1), influenza B, parainfluenza 1,2,3,4, respiratory syncytial virus A-B and Enteroviridae/human rhinovirus (pan RV that captures group A, B and C). Nasal swabs were not taken.

Statistical analysis. Differences in clinical features among the 2 cohorts were determined using the Mann-Whitney test for non-parametric data. A p value of <0.05 was considered significant. See online supplement for additional details on statistical analyses.

Results

Subject characterization. The cohort consisted of highly symptomatic school-age children treated with high-dose inhaled and often, oral, CCS (Table I). Reflecting both the severity of their asthma and the use of CCS, few subjects (only 12%) had isolated BAL eosinophilia and this number was insufficient to perform a separate statistical analysis. We therefore excluded children with isolated BAL eosinophilia from further analysis and stratified the subjects according to neutrophilic versus non-neutrophilic (pauci-granulocytic) BAL granulocyte patterns. Children with neutrophilic asthma were younger ($p < 0.002$) and were treated with relatively lower doses of inhaled CCS ($p=NS$). The overall prevalence of allergen sensitization also trended lower in neutrophilic children ($p=NS$). The extent of airflow limitation was similar in both cohorts.

Respiratory pathogen detection. No subjects had symptoms or findings suggestive of current infection at the time of the bronchoscopy. Despite this, we were able to detect viral and bacterial pathogens in both the neutrophilic and non-neutrophilic groups (Table I). The presence of any virus or any bacteria was statistically higher in the neutrophilic group compared to the non-neutrophilic group; 44% vs 16% ($p<0.002$) and 38% vs 16% ($p<0.03$), respectively. The most prevalent virus in both groups was RV (not separated by type) and the most prevalent bacteria, was *Haemophilus influenzae*.

Correlates of BAL cytokine expression with neutrophilic granulocyte pattern.

BAL was analyzed using a multiplex protein bead-based assay for expression of 27 cytokines/chemokines. Concentrations of IL-2, IL-15, PDGF, GM-CSF and FGF-basic were consistently below the level of detection and therefore are not included in the analysis. Protein concentrations were compared between children with a neutrophilic or

non-neutrophilic granulocyte pattern. Representative graphs are shown in Figure 1, with full summary statistics in supplemental Table I. Univariate logistic regression analysis was used to test for the association of each cytokine/chemokine with the neutrophilic phenotype (Table II). The results were highly consistent: concentrations of every cytokine/chemokine, except for VEGF, were significantly greater in children with neutrophilic BAL. And, conversely, the non-neutrophilic pattern was characterized by the striking relative absence of cytokine/chemokine expression.

Predictors of neutrophilic phenotype. Cross-validation derived estimates for the accuracy for classifying neutrophilic/pauci-granulocytic phenotype based on cytokine/chemokine concentrations are shown in Figure 2. Proteins are ranked in descending order from most accurate to least accurate; with VEGF based neutrophilic phenotype classifications performing worst. Cytokine/chemokine neutrophilic phenotype classification performance ranking based on the Youden Index (YI) is shown Figure 3. A YI gives equal weight to false positive and false negative classification errors with a numeric range between 0 and 1; higher values indicate greater classification accuracy (1 indicates that there are no false positives or negatives (i.e., the test is perfect)). The strongest predictors of BAL neutrophilia based on the YI ranking were CXCL8, CXCL10, G-CSF, and IL-17, although IL-4 was also predictive, whereas VEGF and IL-7 performed the worst (Figure 3).

Association between cytokines and infection. As previously noted, BAL neutrophilia was often associated with asymptomatic expression of respiratory viruses and/or pathogenic bacteria. We therefore performed further analyses to determine whether the cytokine patterns observed developed independently of pathogen expression.

Specifically, we tested CXCL8 as it was the highest factor based on the YI that accurately classified neutrophilic/pauci-granulocytic disease. No differences in CXCL8 levels were detected in those individuals with or without bacterial infection (Figure 4). The same was true when viral infection taken into account (data not shown). When the association between cytokine level and neutrophil status was adjusted for any pathogen, again all cytokines/chemokines with the exception of VEGF predicted neutrophil/pauci-granulocytic status (supplemental Table II).

BAL cytokine co-expression. Finally, using unbiased hierarchical dendrogram plots, we analyzed the patterns of cytokines observed in these subjects. Five clusters of cytokines/chemokines in BAL were identified and are highlighted by different colors (Figure 5).

Discussion

In children with severe, treatment-refractory asthma, we previously identified four phenotypes based on BAL granulocyte patterns⁷ including isolated eosinophilia, isolated neutrophilia, mixed granulocytic, and pauci-granulocytic patterns. The aim of the current study was to identify cytokine/chemokine expression patterns underlying the pathogenesis of neutrophilic versus non-neutrophilic BAL phenotypes. BAL fluid was analyzed and statistical analyses were performed on 22 detectable cytokines/chemokines. Unexpectedly, no cytokine or chemokine was over-expressed or otherwise positively associated with the non-neutrophilic phenotype (Table II). All cytokines (with the exception of VEGF) were over-expressed in children with BAL neutrophilia. The best predictors of a neutrophilic phenotype were cytokines/chemokines known to potentiate (or stimulate) neutrophil hematopoiesis, recruitment, and activation (Figure 3). Specifically, strong associations were identified with cytokines/chemokines responsible for neutrophil chemotaxis (CXCL8, GCSF, CXCL10, and TNF- α) and Th17 differentiation (IL-6) and expression (IL-17). Linkages also likely to be important were observed with cytokines responsible for Th1 differentiation (IL-12) and expression (IFN- γ) – cytokines previously associated with neutrophilic asthma⁸ and children.^{9,10} These results are consistent with findings reported in adults with refractory neutrophilic asthma.^{1,4}

As noted, because there were so few subjects with isolated eosinophilic inflammation, the current study could only focus on associations with neutrophilia. It is important to appreciate that all of these children were on high dose inhaled CCS and many were also taking oral CCS (Table I) that was certain to impact the expression of eosinophilic

inflammation in the airway. Thus, the absence of observable – surviving – eosinophils does not preclude the presence of a type 2^{high} cytokine milieu. Our expectation was that the non-neutrophilic cohort would reflect a type 2^{high} (especially IL-5^{high}) milieu – with the expected associated eosinophilic infiltrate mitigated by the impact of CCS. However, all cytokines analyzed including those specific for type 2 inflammation were over-expressed in and predictive of a neutrophilic process.

Even though clinical evidence of an active or recent symptomatic infection precluded performing elective bronchoscopy, many of these children nonetheless demonstrated respiratory pathogens in their airways. Importantly, the presence of bacterial and viral pathogens in BAL samples was associated with presence of BAL neutrophils (Table I). It is also possible that latent isolated *upper* airway infections may have contributed to expression of *lower* airway PMNs in some of these subjects. The presence of pathogenic microorganisms in the airway is associated with more severe asthma characterized by sputum neutrophils and CXCL8 expression.¹¹ For example, RV infection in asthmatics drives increased G-CSF and CXCL8 levels that correlate with an increase in bronchial PMNs.¹² Similarly, *Haemophilus* expression is associated with neutrophilic asthma driven in part by induction of Th17 cellular responses.¹³ Surprisingly, CXCL8 and all other cytokines were independent predictors of neutrophil status (Figure 4 and supplemental Table II).

In contrast to the high expression of cytokines/chemokines associated with PMN recruitment and Th1/Th17 inflammation was the association of neutrophilic disease with IL-4, the canonical Th2 cytokine. It is worth noting that IL-4 also modulates recruitment and activation of PMNs.¹⁴⁻¹⁶ The best explanation for the linkage of IL-4 to neutrophilic

disease is that, as with adults, children with severe asthma comprise a phenotype associated with mixed Th2/Th17 and/or mixed Th2/Th1 inflammation.

We next performed unbiased clustering analysis of cytokine/chemokine patterns that revealed five main cytokine/chemokine patterns (Figure 5). The groupings were consistent with prior associations and these clusters could logically be assigned to functional groups. IL-5 and IL-13 were almost universally co-synthesized (blue). These type 2 cytokines can be produced by numerous cell types, however, their close association – in the absence of linkage to their Th2 partner IL-4 – is perhaps more indicative of an ILC2 cytokine signature. IL-4 and all the remaining T effector cell cytokines clustered together (purple) with little or no separation seen between typical type 1 (IFN- γ), type 2 (IL-4, IL-9) or type 3 (G-CSF, IL-17) cytokines. This result is not surprising as numerous studies have argued that severe asthma is characterized by heterogeneous patterns of inflammation including mixed Th1/Th2/Th17 T effector cells.⁸⁻¹⁰ A pattern for mononuclear phagocytes (orange) emerged as indicated by the clustering of IL-10, IL-12p40 and VEGF. The mediators in the remaining two groups (red and green) are typical of innate inflammatory responses involved in cell trafficking.

Perhaps the most unexpected but most consistent result of this study was that the expression of any cytokine (except VEGF) was predictive of neutrophilic disease *and no cytokine predicted a finding of non-neutrophilic inflammation*. These results suggest that children with pauci-granulocytic severe asthma have novel – cytokine-independent – mechanisms driving the pathogenesis of their severe refractory airway disease. We cannot exclude a role for cytokines not evaluated in our analysis nor can we exclude expression of either these cytokines (or of granulocytes themselves) in the airway tissue

itself. Such studies would require obtaining transbronchial biopsy samples, a procedure not without risk in infants and children. Our data are most consistent with a highly prevalent pauci-granulocytic endotype in severe asthmatic children, that is likely characterized by rigid airways, smooth muscle hypertrophy, fibrosis, goblet cell hyperplasia, and other facets of airway remodeling and possibly via neurogenic mechanisms, *but not by prominent inflammation*.

While preliminary, these and previous observations from our group^{7,10} provide a foundation for clinically-indicated BAL as a tool to inform treatment in children with severe, poorly-controlled asthma who have failed treatment with high-dose corticosteroids. As we have reported previously⁷, a substantial number of these children have pauci-granulocytic BAL (52%), an endotype with lower pathogen recovery and lower markers of type 2 inflammation. Treatment with higher doses of corticosteroids or biological therapies targeting eosinophilic type 2 inflammation henceforth are not likely to be effective in these children. Instead other approaches including exercise, weight loss, and bronchial thermoplasty should be studied. In contrast, identification of BAL fluid neutrophilia, found in isolation (16%) or admixed with BAL eosinophils (22%), is highly associated with pathogen recovery, notably respiratory viruses, and thus is an opportunity for treatment with azithromycin for episodes of poor symptom control.¹⁷

Furthermore, in addition to macrolides, diagnosis of BAL neutrophilia in asthma supports consideration of other anti-neutrophil therapies in the treatment of asthma including anti-oxidants, protease inhibitors, and phosphodiesterase inhibitors.¹⁸ As important, we have shown in this report that the BAL neutrophilia inflammatory signature in children already exposed to high dose corticosteroids is diverse,

characteristic of mixed Th17/Th1/Th2 motifs. Revised treatment in such children could safely involve a reduction in corticosteroid exposure, an approach that might be validated in future studies.

In summary, these results demonstrate two prominent BAL inflammatory and cytokine/chemokine signatures in children with severe treatment refractory asthma who have failed conventional treatment. One endotype consists of prominent BAL neutrophilia that is associated with a mixed Th17/Th1/Th2 response. In contrast, a pauci-granulocytic endotype is associated with reduced expression of cytokines/chemokines. Isolated airway eosinophilic inflammation was an unusual finding. All children were being treated with high dose inhaled CCS and in many cases also oral CCS. The salient characteristics of all of these endotypes point to the basis of their refractory disease. Arguably, the persistence of eosinophilia in those children with that rare endotype reflected steroid resistance and predicts a lack of efficacy of CCS administration. In those subjects with neutrophilic inflammation, current therapeutics, including especially CCS are largely ineffective and thus lack of treatment response is not surprising. Similarly, the pauci-granulocytic, non-inflammatory endotype is likely driven by tissue remodeling. Each of these endotypes comprise a critical unmet medical need requiring the development of unique and novel therapeutic approaches.

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Acknowledgements

351 The authors would like to acknowledge Kristin Wavell, Andrea Garrod, Deborah Froh,
352 Steve Early, and Kimberly DeRonde who were involved in the characterization of the
353 children and sample preparation and storage.

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Tables

Table I			
Features of 102 Adolescents and Children with Poorly-Controlled Asthma Compared by BAL Neutrophil Pattern			
	Non-neutrophilic	Neutrophilic	Eosinophilic
Sample, n (%)	57 (63)	34 (37)	11 (12%)
Age (years), median 25 th -75 th	10 (5-14)*	6 (3-9)	11 (8-14)
Male sex, n (%)	34 (60)	23 (68)	6 (55)
Race Caucasian, n (%)	36 (63)	24 (71)	3 (27)
Atopic (n (%); any positive ImmunoCap)	33 (58)	17 (50)	9 (82)
Geometric mean total blood IgE (IU/ml), median 25 th -75 th	100 (23-480)	53 (16-293)	275 (92-1096)
Number of controller medications, median 25 th -75 th	2 (1-3)	2 (1-2)	3 (2-4)
Daily inhaled CCS dose (fluticasone equivalents), median 25 th -75 th percentile	500 (160-800)	320 (160-680)	800 (320-800)
Maintenance prednisone, n (%)	6 (11)	9 (26)	6 (55)
Any virus, n (%)	9 (16)*	15 (44)	1 (9)
Rhinovirus, n (%)	8 (14)**	12 (35)	1 (9)
Any bacteria, n (%)	9 (16)**	13 (38)	2 (18)
<i>Haemophilus influenzae</i> , n (%)	3 (5)**	7 (21)	0 (0)
FEV ₁ (% predicted), median 25 th -75 th	99 (79-108)	95 (77-107)	ND
FEV ₁ /FVC ratio	0.78±0.10	0.82±0.12	ND

*p<0.002; **p<0.03 non-neutrophilic vs neutrophilic; comparisons not made to eosinophilic due to low numbers; ND= not determined

Table II: Univariate logistic regression summaries for testing the association between the values of each cytokine and neutrophilic phenotype.

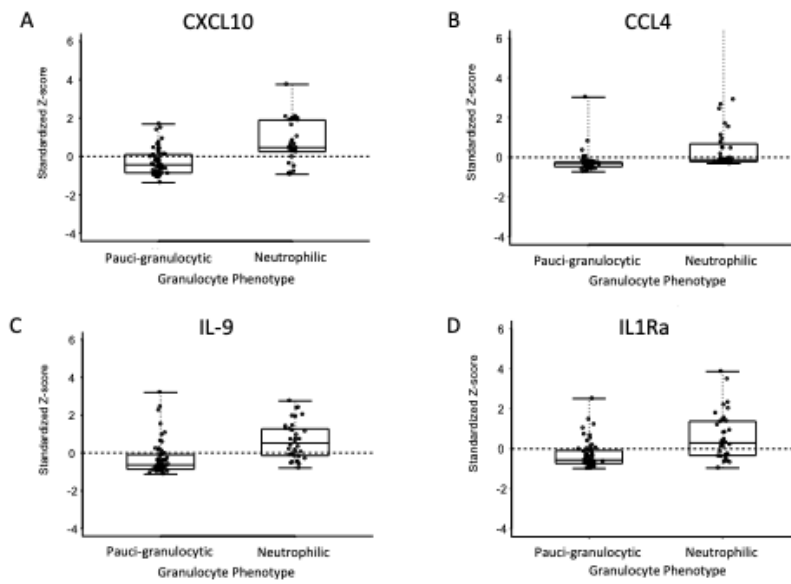
Cytokine	Odds Ratio (OR)	Lower	Upper	Observed P-value	BH False Discovery Threshold P-value	Reject Ho	ROC AUC
TNF α	3.49	1.52	7.99	0.0031	0.0273	Yes	0.84
IL4	2.84	1.61	5.04	0.0003	0.0136	Yes	0.81
IL17	2.61	1.52	4.50	0.0005	0.0227	Yes	0.86
IL13	2.52	1.23	5.19	0.0120	0.0409	Yes	0.80
CXCL8	2.37	1.47	3.85	0.0004	0.0182	Yes	0.89
CCL3	2.27	1.28	4.02	0.0049	0.0318	Yes	0.88
CCL4	2.13	1.46	3.11	0.0001	0.0046	Yes	0.84
IL5	2.09	1.22	3.56	0.0069	0.0341	Yes	0.73
IL6	2.05	1.20	3.51	0.0085	0.0364	Yes	0.88
IL10	2.05	1.08	3.89	0.0279	0.0455	Yes	0.69
IL 1 β	2.01	1.10	3.67	0.0232	0.0432	Yes	0.85
CXCL10	1.89	1.40	2.56	0.0001	0.0028	Yes	0.79
GCSF	1.89	1.35	2.64	0.0002	0.0114	Yes	0.82
IFN γ	1.86	1.31	2.63	0.0005	0.0205	Yes	0.80
IL1Ra	1.79	1.32	2.43	0.0002	0.0091	Yes	0.78
CCL2	1.70	1.23	2.35	0.0013	0.0250	Yes	0.72
IL9	1.64	1.27	2.13	0.0002	0.0068	Yes	0.81
IL12p70	1.60	1.23	2.08	0.0004	0.0159	Yes	0.70
IL7	1.49	1.14	1.96	0.0039	0.0296	Yes	0.69
CCL11	1.44	1.09	1.91	0.0110	0.0386	Yes	0.70
CCL5	1.35	1.03	1.77	0.0319	0.0477	Yes	0.73
VEGF	1.20	0.96	1.49	0.1012	0.0500	No	0.62

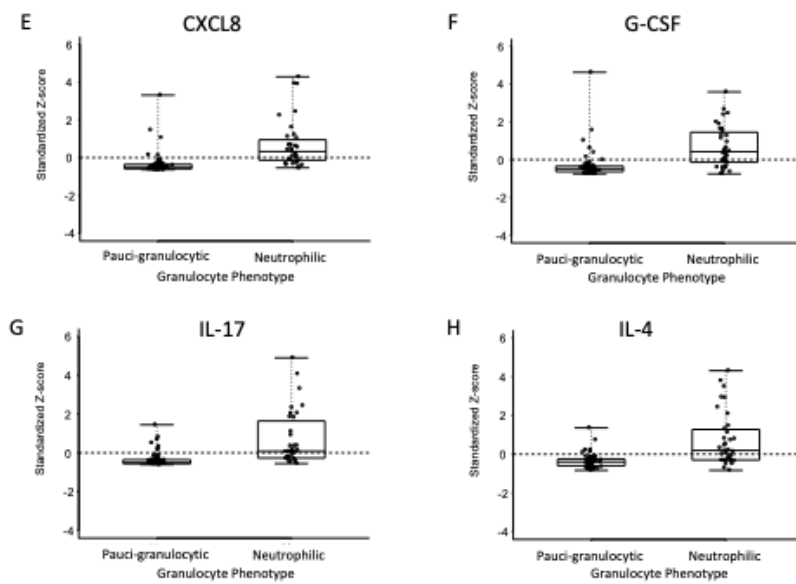
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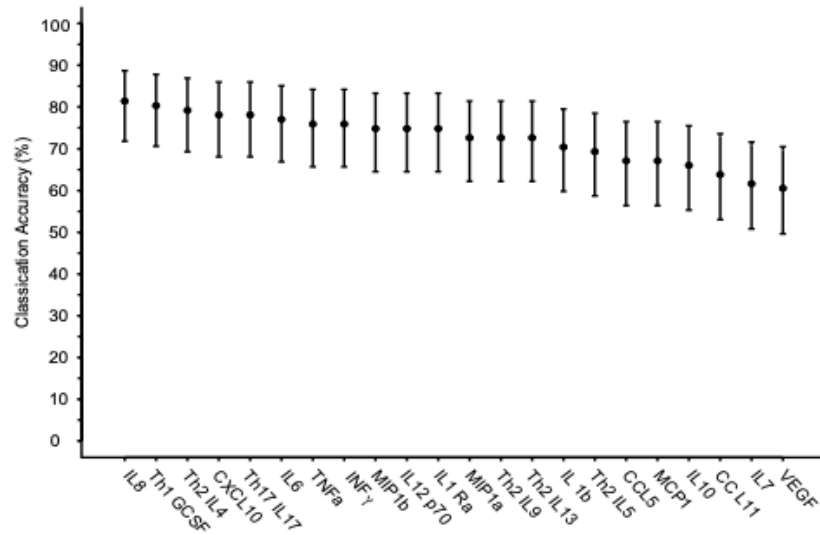
- Figure 1.** Empirical cytokine/chemokine distributions stratified by granulocytic (neutrophil/pauci-granulocytic) phenotype (p values for all cytokines are ≤ 0.0005 (Table I).
- Figure 2.** Cross-validation estimates for accuracy (A) in classifying patients as neutrophilic or pauci-granulocytic. Proteins are ranked in descending ordered from the classifier with highest classification A to the classifier with the lowest A. Vertical lines identify the 95% confidence interval.
- Figure 3.** Ranking of cytokines/chemokines as classifier of granulocytic (neutrophilic/pauci-granulocytic) status. Proteins are ranked in descending ordered from the classifier with highest Youden Index to the classifier with the lowest Youden Index. Note that the Youden Index is a dimensionless number ranging from 0 to 1, where the Youden Index = classification sensitivity + classification specificity – 1. The greater the Youden Index the smaller the overall classification error rate.
- Figure 4.** Empirical CXCL8 distributions based on presence or absence of bacterial infection detected in BAL (differences are not significant).
- Figure 5.** Hierarchical clustering of the cytokine/chemokine variables based on Spearman rank correlation.

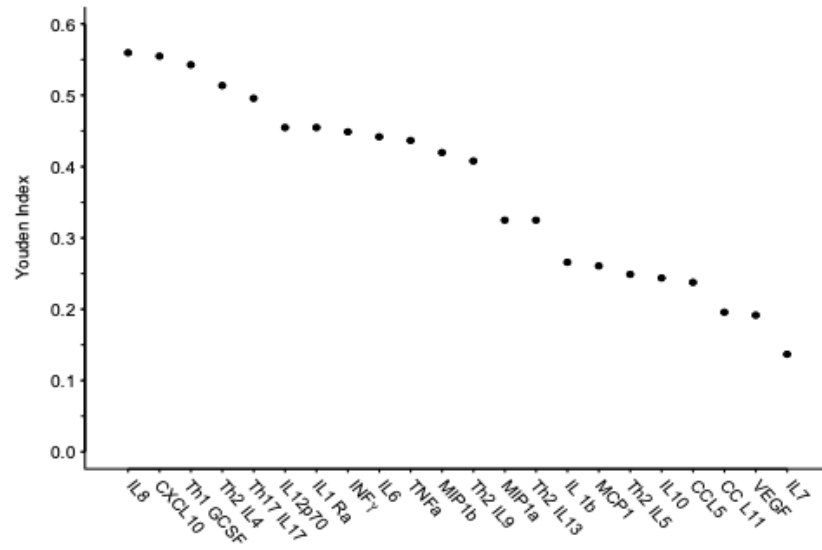
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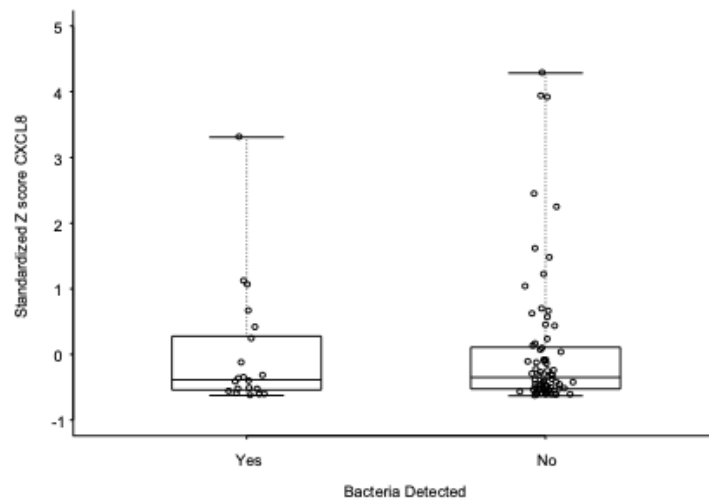
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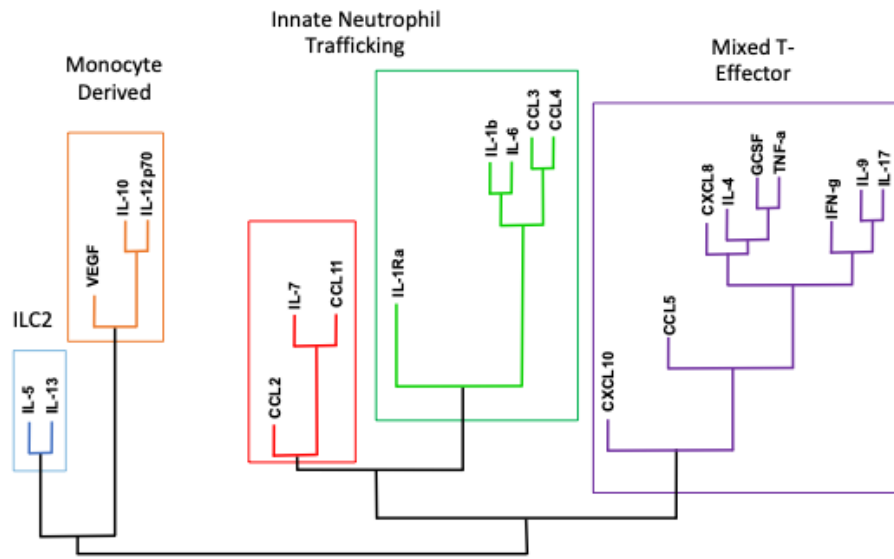












Supplemental Table I. Cytokine empirical distribution summary statistics.

Cytokine	Neutrophilic Phenotype	n	Mean	SD	SE	Median	P.25th	P.75th	Min	Max
IL4	No	57	-0.356	0.371	0.049	-0.398	-0.600	-0.254	-0.835	1.356
IL4	Yes	34	0.740	1.391	0.239	0.192	-0.300	1.274	-0.835	4.304
IL5	No	57	-0.227	0.382	0.051	-0.227	-0.373	-0.216	-0.878	1.346
IL5	Yes	34	0.537	1.517	0.260	-0.196	-0.227	0.604	-0.232	6.677
IL13	No	57	-0.273	0.382	0.051	-0.260	-0.540	-0.220	-0.556	1.737
IL13	Yes	34	0.498	1.504	0.258	-0.158	-0.214	0.382	-0.540	5.738
L9	No	57	-0.291	0.895	0.119	-0.635	-0.865	-0.085	-1.122	3.198
IL9	Yes	34	0.641	0.986	0.169	0.517	-0.143	1.277	-0.801	2.756
IL 1 β	No	57	-0.222	0.428	0.057	-0.311	-0.333	-0.285	-0.349	2.837
IL 1 β	Yes	34	0.475	1.539	0.264	-0.228	-0.269	0.475	-0.340	6.905
IL17	No	57	-0.344	0.399	0.053	-0.519	-0.549	-0.362	-0.606	1.449
IL17	Yes	34	0.713	1.402	0.240	0.082	-0.270	1.642	-0.549	4.900
IFN γ	No	57	-0.323	0.647	0.086	-0.490	-0.661	-0.284	-0.801	3.010
IFN γ	Yes	34	0.634	1.267	0.217	0.392	-0.349	1.076	-0.813	4.664
GCSF	No	57	-0.311	0.788	0.104	-0.481	-0.632	-0.342	-0.750	4.620
GCSF	Yes	34	0.682	1.107	0.190	0.419	-0.132	1.452	-0.752	3.589
IL1 Ra	No	57	-0.308	0.670	0.089	-0.562	-0.741	-0.091	-0.991	2.500
IL1 Ra	Yes	34	0.629	1.185	0.203	0.285	-0.338	1.373	-0.967	3.853
IL6	No	57	-0.266	0.650	0.086	-0.372	-0.491	-0.329	-0.558	4.262
IL6	Yes	34	0.531	1.344	0.230	0.015	-0.282	0.643	-0.383	5.293
IL7	No	57	-0.237	0.638	0.084	-0.421	-0.651	0.003	-1.314	2.083
IL7	Yes	34	0.502	1.357	0.233	0.003	-0.397	0.644	-0.881	3.947
IL8	No	57	-0.330	0.612	0.081	-0.492	-0.585	-0.356	-0.631	3.308
IL8	Yes	34	0.692	1.281	0.220	0.323	-0.126	0.947	-0.531	4.284
IL10	No	57	-0.193	0.315	0.042	-0.274	-0.387	-0.248	-0.513	0.806
IL10	Yes	34	0.405	1.613	0.277	-0.117	-0.274	0.306	-0.448	6.650
IL12 p70	No	57	-0.319	0.686	0.091	-0.610	-0.674	-0.220	-1.224	1.361
IL12 p70	Yes	34	0.551	1.272	0.218	0.325	-0.630	1.179	-1.359	3.699
CC L11	No	57	-0.191	0.660	0.087	-0.474	-0.565	-0.057	-0.931	2.193
CC L11	Yes	34	0.455	1.376	0.236	-0.048	-0.335	0.901	-0.926	5.621
CXCL10	No	57	-0.286	0.688	0.091	-0.433	-0.854	0.109	-1.361	1.685

CXCL10	Yes	34	0.750	1.122	0.192	0.468	0.245	1.892	-0.925	3.749
CCL2	No	57	-0.296	0.563	0.075	-0.428	-0.623	-0.189	-1.200	2.089
CCL2	Yes	34	0.438	1.155	0.198	-0.113	-0.394	0.808	-1.070	3.266
CCL3	No	57	-0.257	0.505	0.067	-0.278	-0.469	-0.259	-0.727	3.020
CCL3	Yes	34	0.546	1.457	0.250	-0.121	-0.220	0.678	-0.321	7.037
CCL4	No	57	-0.360	0.498	0.066	-0.566	-0.638	-0.295	-0.772	1.624
CCL4	Yes	34	0.744	1.311	0.225	0.254	-0.320	1.596	-0.548	4.037
CCL5	No	57	-0.172	0.772	0.102	-0.432	-0.523	-0.229	-0.880	3.992
CCL5	Yes	34	0.387	1.324	0.227	-0.137	-0.307	0.469	-0.560	5.851
VEGF	No	57	-0.113	0.973	0.129	-0.416	-0.794	0.396	-1.325	4.456
VEGF	Yes	34	0.256	1.050	0.180	0.340	-0.517	0.995	-1.369	3.007
TNF α	No	57	-0.317	0.330	0.044	-0.388	-0.474	-0.267	-0.538	1.803
TNF α	Yes	34	0.660	1.473	0.253	-0.061	-0.226	0.830	-0.561	5.408

Supplemental Table II. Multivariate logistic regression summaries for testing the association between the values of each cytokine and granulocyte (neutrophilic/pauci-granulocytic) status adjusted for pathogen status.

Cytokine	Odds Ratio (OR)	Lower	Upper	Observed P-value	BH False Discovery Threshold P-value [‡]	Reject Ho	ROC AUC
IL4	2.83	1.61	4.98	0.0000	0.0023	Yes	0.0023
IL9	1.64	1.27	2.12	0.0000	0.0046	Yes	0.0046
IL17	2.61	1.53	4.47	0.0000	0.0068	Yes	0.0068
GCSF	1.91	1.37	2.69	0.0000	0.0091	Yes	0.0091
IL1Ra	1.79	1.32	2.44	0.0000	0.0114	Yes	0.0114
CXCL8	2.42	1.48	3.95	0.0000	0.0136	Yes	0.0136
IL12 p70	1.60	1.23	2.08	0.0000	0.0159	Yes	0.0159
CXCL10	1.95	1.42	2.68	0.0000	0.0182	Yes	0.0182
CCL4	2.12	1.45	3.09	0.0000	0.0205	Yes	0.0205
IFN γ	1.84	1.31	2.60	0.0010	0.0227	Yes	0.0227
CCL2	1.74	1.26	2.41	0.0010	0.0250	Yes	0.0250
IL7	1.53	1.16	2.01	0.0030	0.0273	Yes	0.0273
TNF α	3.52	1.53	8.11	0.0030	0.0296	Yes	0.0296
CCL3	2.30	1.28	4.12	0.0050	0.0318	Yes	0.0318
IL5	2.23	1.27	3.93	0.0060	0.0341	Yes	0.0341
IL6	2.11	1.21	3.66	0.0080	0.0364	Yes	0.0364
CCL11	1.45	1.09	1.92	0.0100	0.0386	Yes	0.0386
IL13	2.46	1.21	5.03	0.0130	0.0409	Yes	0.0409
IL 1 β	1.99	1.10	3.62	0.0240	0.0432	Yes	0.0432
IL10	2.04	1.07	3.87	0.0290	0.0455	Yes	0.0455
CCL5	1.34	1.02	1.77	0.0360	0.0477	Yes	0.0477
VEGF	1.21	0.98	1.51	0.0810	0.0500	No	0.0500

Supplemental methods

Statistical analysis

Cytokine/chemokine protein data transformation: In order to correct for plate-to-plate variability, the protein values of all cytokines/chemokines were converted to a standardized Z-score. The standardized Z score was computed per cytokine/chemokine by first subtracting the mean of the cytokine/chemokine protein values from each cytokine/chemokine protein value, and then dividing this quantity by the standard deviation of the cytokine/chemokine protein values. It is important to note that the standardized Z-scores were computed per plate without distinguishing between neutrophil category (i.e. neutrophilic, and non-neutrophilic). Accordingly, the standardized Z scores of all cytokines/ chemokines were in standard deviation (SD) units, and the mean of each cytokine/chemokine standardized Z-score distribution was 0 SD and the standard deviation of each cytokine/chemokine standardized Z-score empirical distribution was 1 SD.

Patterns of cytokine/chemokine expression: Patterns of cytokine/chemokine expression were discerned via a hierarchical clustering algorithm, in which the non-parametric Spearman rank correlation coefficient served as the similarity measure to group cytokine and chemokines into clusters based on standardized Z score correlation.^{E1} The “varclus” function of the “Hmisc” library of Spot Fire S+ version 8.2 (TIBCO Inc., Palo Alto, CA) was used to generate a hierarchical clustering cluster tree diagram that graphically reveals the underlying patterns of cytokine/chemokine expression.

Association between neutrophil phenotype and BAL cytokine/chemokine expression: Univariate logistic regression was used to test for neutrophil phenotype versus BAL

cytokine/chemokine expression association for each cytokine and chemokine. The logistic regression outcome variable identified neutrophilic phenotype cases ($Y=1$) and non-neutrophilic phenotype cases ($Y=0$), and the aforementioned set of cytokine/chemokine standardize Z scores served as the set of univariate logistic regression model predictor variables. Due to the large number of cytokine/chemokines ($n=22$), the Benjamini and Hochberg (BH) false discovery error procedure was used in the hypothesis testing decision process to restrict the false positive discovery error rate (i.e. rejecting the null hypothesis when the null hypothesis is true) to be no more than 0.05 (i.e. 5%). The “lrm” function of “Hmisc” library of Spot Fire S+ version 8.2 (TIBCO Inc., Palo Alto, CA) was used to conduct the univariate logistic regression analyses.

Neutrophil phenotype classification base on BAL cytokine/chemokine expression: The leave-one-out cross-validation procedure.^{E2} was used to rank the individual cytokine/chemokine neutrophil phenotype classifiers based on cross-validation classification accuracy. In conducting the leave-one-out cross-validation, the case left out of the univariate logistic regression training set was assigned to the neutrophilic group if the logistic regression model predicted probability for the case belonging to the neutrophilic group exceed the observed prevalence (0.374) of neutrophilic cases in the sample of 91 children with severe asthma who failed treatment. The ordered ranking of the individual cytokines and chemokines as a neutrophil phenotype classifier was established base on the Youden Index.^{E3} The Youden Index (YI) is a unit-less measure of classification accuracy that is computed as the arithmetic sum of classification “sensitivity” and classification “specificity” – 1 (i.e. $YI = \text{sensitivity} + \text{specificity} - 1$). Note that the YI gives equal weight to false positive and false negative classification errors

and has numeric range [0, 1], with higher index values signifying greater classification accuracy. The “cv.glm” function of the “boot” library of the statistical package R.^{E4} was used to conduct the leave-one-out cross-validations.

Supplemental References

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E3. Youden WJ. Index for rating diagnostic tests. *Cancer* 1950;3:32-5.

E4. R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org>.