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Complex association patterns for inflammatory mediators in induced sputum from subjects with asthma

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Summary

Background: The release of various inflammatory mediators into the bronchial lumen is thought to reflect both the type and degree of airway inflammation, eosinophilic Th2, and Th9, or neutrophilic Th1, and Th17, in patients with asthma.

Aims: We investigated whether cytokines and chemokines differed in sputum from subjects with more severe compared with milder asthma and whether unbiased factor analysis of cytokine and chemokine groupings indicates specific inflammatory pathways.

Methods: Cell-free supernatants from induced sputum were obtained from subjects with a broad range of asthma severity (n = 158) and assessed using Milliplex[®] Cytokines/Chemokine kits I, II and III, measuring 75 individual proteins. Each cytokine, chemokine or growth factor concentration was examined for differences between asthma severity groups, for association with leucocyte counts, and by factor analysis.

Results: Severe asthma subjects had 9 increased and 4 decreased proteins compared to mild asthma subjects and fewer differences compared to moderate asthma. Twenty-six mediators were significantly associated with an increasing single leucocyte type: 16 with neutrophils (3 interleukins [IL], 3 CC chemokines, 4 CXC chemokines, 4 growth factors, TNF- α and CX3CL1/Fractalkine); 5 with lymphocytes (IL-7, IL-16, IL-23, IFN- α 2 and CCL4/MIP1 β); IL-15 and CCL15/MIP1 δ with macrophages; IL-5 with eosinophils; and IL-4 and TNFSF10/TRAIL with airway epithelial cells. Factor analysis grouped 43 cytokines, chemokines and growth factors

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CONFLICT OF INTEREST

The authors declare that there was no conflict of interests with the subject matter of the manuscript.

SUPPORTING INFORMATION

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which had no missing data onto the first 10 factors, containing mixes of Th1, Th2, Th9 and Th17 inflammatory and anti-inflammatory proteins.

Conclusions: Sputum cytokines, chemokines and growth factors were increased in severe asthma, primarily with increased neutrophils. Factor analysis identified complex inflammatory protein interactions, suggesting airway inflammation in asthma is characterized by overlapping immune pathways. Thus, focus on a single specific inflammatory mediator or pathway may limit understanding the complexity of inflammation underlying airway changes in asthma and selection of appropriate therapy.

Keywords

chemokines; cytokines; factor analysis; growth factors; severe asthma

1 | INTRODUCTION

All levels of asthma severity show evidence of airway inflammation, with both eosinophils and neutrophils contributing to asthmatic inflammatory patterns.^{1–6} We have reported that asthma subjects, having combined increases in both sputum eosinophils and neutrophils, had the lowest lung function, worse asthma control, increased symptoms and healthcare utilization.⁷ Unsupervised cluster analysis in the Severe Asthma Research Program (SARP) including sputum and blood eosinophil and neutrophil percentages, showed sputum neutrophils associated with more severe asthma.⁶ However, most studies in asthma have focused on eosinophils and Th2 inflammation⁸ with little consideration of the potential role of neutrophils,^{1,9,10} Th1 and Th17 inflammation pathways^{7,11,12} Investigation of inflammatory pathology underlying asthma has shifted to molecular components.^{13–18} However, the list for molecular biomarkers associated with either innate or adaptive immune response is extensive.^{13,19–21} Th2 and sub-set Th9 cells primarily express IL-4, IL-5, IL-9 and IL-13 through STAT6 and GATA3 regulation in response to IL-4, whereas Th1 cells express IFN- γ , IL-12 and IL-10 family members, IL-22 and IL-26, through STAT4 and T-bet regulation in response to IL-12 and IFN- γ ; thus, both Th1 and Th2 have positive autoregulatory reinforcement.²² Th17 inflammation is distinct from either Th1 or Th2 and is suppressed by IFN- γ and IL-4, but responds to TGF- β and IL-6 stimulation.¹³

An approach to investigating airway inflammation in asthma is through use of broad-spectrum multiplex protein assay, which allows assessment of relationships, both protein-cell and protein-protein associations. Moreover, this approach avoids reliance on measures of mRNA which lack linear association with actual protein synthesized.²³ Although several recent reports have examined gene expression patterns in sputum and bronchial epithelial samples from subjects with severe asthma, these have included more limited information on corresponding protein analyses.^{24–27} One report from U-BIOPRED, examining protein differences between 4 clinically identified cluster groups, found 9 differentially abundant sputum proteins in more severe clusters compared to moderate, well-controlled asthma with normal FEV1.²⁸ However, the 1 cluster group with greatest number of differentially abundant proteins was characterized by 16-packyear smoking history which may influence and contribute to these elevated proteins. Biomarkers observed to be associated with clinical phenotypic subgroups in asthma or specific cellular inflammation may be useful to predict

response to steroids or newly developed biologic therapies. Studies in mice suggest that steroid resistant inflammation may be mediated by Th17 inflammation,²⁹ and that neutralization of IL-22, a Th17-associated cytokine, attenuates pro-inflammatory response and lung function decrements elicited by *A fumigatus* conidia in mice sensitized to this allergen.³⁰ From these observations, we hypothesize that different mediator and cell interactions may exist in specific subgroups of patients with asthma, for example, those with mild, moderate or severe asthma, and that release of inflammatory proteins is associated with increased cell influx into the airways, reflecting underlying Th1, Th2, Th9 and Th17 inflammation, or combinations of these.

To address these hypotheses, we measured 75 cytokines, chemokines and growth factors, representing a broad range of airway inflammatory proteins, examined differences in defined subgroups of mild, moderate and severe asthma, and tested for correlations with increased cell constituents in sputum. In addition, we performed factor analyses to identify interactive group associations among increased cytokines, chemokines and growth factors.

2 | METHODS

Patients with mild to severe asthma were comprehensively characterized according to the SARP phenotype protocol at Wake Forest School of Medicine and the University of Wisconsin as previously described.³¹ Briefly, non-smoking subjects (<5 packyears) who met ATS criteria for the diagnosis of asthma provided written informed consent (Wake Forest Institutional Review Board approved protocol: BG01–425; University of Wisconsin Institutional Review Board approved protocol: H-2006–0338). All subjects with asthma were recruited, with special emphasis to enrol those with severe asthma, resulted in a cohort with a broad range of disease severity, but with a larger proportion of severe asthma than observed in the general population. Evaluation, at least 4 weeks after any final corticosteroid treatment for an exacerbation or 2 weeks after any final antibiotic treatment for an infection, included spirometry, bronchodilator reversibility and bronchial responsiveness to methacholine (BHR), allergen skin prick tests to a panel of 12–14 allergens, total serum IgE level, collection of blood, exhaled NO measurement, sputum induction^{6,7} and administration of questionnaires that characterized asthma symptoms, quality of life, medications and healthcare utilization.^{31,32} Subjects enrolled in the observational SARP1 + 2 were classified as severe by the criteria of the ATS workshop (Proceedings of the ATS Workshop on Refractory Asthma 2000³³); those who did not meet the criteria for severe (either high dose inhaled corticosteroids [ICS] or oral corticosteroid as a major criterion, plus 2 or more minor criteria) were considered “not severe” and post hoc grouped into either mild (pre-bronchodilator FEV1% predicted ≥80%) or moderate asthma (FEV1% predicted <80%, on low or moderate dose of ICS).³¹

2.1 | Subjects

SARP subjects had severe (N = 38, as defined by the ATS/ERS Guidelines³³) or not severe asthma (N = 63, mild asthmatics, and N = 57 moderate asthmatics not meeting “severe” criteria).^{31,32} Demographics and clinical characteristics for these subjects corresponded to those previously reported for subjects from all SARP sites³¹ (Table 1).

2.2 | Sputum induction and processing

The sputum induction method was adopted from the NHLBI Asthma Clinical Research Network³⁴ and used in SARP.^{6,7} The induction and processing are described in greater detail in the supporting information. Sputum was induced and processed immediately following spirometry. Cell cytospins were stained for differential counts of at least 500 non-squamous cells per subject slide. Cell-free supernatants were aliquoted and stored at -80° before use in Luminex analyses (see below). Both cytokines and specific cell types were expressed per mL of total sputum sample obtained.

2.3 | Inflammatory cytokine assay

Cell-free sputum supernatants were assayed for 75 different inflammatory protein concentrations using Milliplex Human Cytokine/Chemokine Panels I, II and III (catalogue numbers HCYTOMAG-60K, HCYP2MAG-62K and HCYP3MAG-63K, respectively, Merck Milli-pore) as previously described.³⁵ Standards for determination of linear curve plus 2 control samples representing high and low levels for each cytokine/chemokine were included in each assay.

2.4 | Statistics

Demographic and biomarker data are presented as means $\pm$ standard deviations, or medians (25%–75% quartiles) for continuous variables, and as % positive for categorical variables. Measures not meeting Kolmogorov-Smirnov test for normal distribution were transformed to log or square root values. Continuous variables were tested by parametric or nonparametric tests (SAS 9.2, or SigmaStat 3.1). Analyses with a significant difference were further explored by post hoc pairwise tests (Tukey or Dunn's). Categorical variables were analysed using chi-square tests. Adjustment by Bonferroni for multiple tests was rejected due to possible interrelationship of cytokines which was supported by factor analysis (see below); thus variables found to have a P value $< .05$ were considered significant.

Initial analysis considered the number of subjects with measurable levels for each cytokine, chemokine or growth factor; if more than 100 subjects' samples had undetectable or out-of-range values, further association with specific cell counts in multiple linear regression models was not explored (the 11 cytokines excluded for all subjects are listed in Table S2). The number of subjects in mild, moderate and severe subgroups was examined by chi-square analyses to determine whether disproportionate absence or presence of cytokines in any subgroup occurred. Multiple linear regression models were generated for individual cytokines, chemokines and growth factors to explore association with specific cell types in sputum: macrophage/monocyte, lymphocyte, eosinophil, neutrophil and bronchial epithelial cell counts, without or with inclusion of the categorical variable ICS use. Cell counts and cytokines were both presented per mL of total sputum sample for purpose of regression comparison and to adjust for variable amount of saliva present, although no differences in WBC across groups were observed. Factor analysis investigated associations between proteins for which all of 140 subjects had measurable levels ($N = 43$ cytokines); that is, there were no missing values for any mediators for subjects. The MINEIGEN criterion retained 10 factors after principal component analysis; all mediators having $>40\%$ association with a factor in varimax rotation method were listed with that factor. Restricting the cutpoint to

>50% for association with a factor eliminated only 2 mediators; therefore, >40% was accepted as providing sufficient detection without incurring excess weak associations. Protein groups on each factor were examined in [string_db.org](https://string-db.org) version 10 to independently confirm potential protein associations and networks between cytokine, chemokine and growth factors.³⁶

3 | RESULTS

Subject demographics and clinical characteristics for the whole cohort and the subgroups with mild, moderate or severe asthma are presented in Table 1. There were significant differences in demographics and clinical characteristics between the subgroups comparing those with mild to those with severe asthma. However, the group with moderate compared to severe asthma showed fewer differences. Medications other than ICS, although differing between the mild, moderate and severe groups, were used in much smaller proportions of subjects and were nearly the same for mild and moderate subgroups (Table S1).

3.1 | Cellular characteristics

Table 2 provides sputum inflammatory cell differential percentages and counts/mL. Differences were observed in decreased macrophage % and increased bronchial epithelial cell % and cell count $\times 10^4$ /mL for subjects in the severe asthma subgroup compared to mild asthma. There was a trend towards increased neutrophil % in the severe asthma subgroup ($P = .073$), but no differences were found for lymphocytes or eosinophils. There were no sputum cell differences between moderate and severe subgroups (Table 2).

The difference between mild, moderate and severe groups for ICS use potentially altered sputum cell differentials. Therefore, the subjects in the mild and moderate subgroups were further stratified by no ICS or ICS use and examined for differences in sputum cell counts. (Only 1 severe asthma subject not on high dose ICS but receiving injected corticosteroid precluded similar stratification of the severe subgroup.) The only difference noted for ICS effect on sputum differential counts was for %eosinophils in the moderate subgroup; however, higher eosinophils were observed in those receiving ICS (no ICS: median eosinophils = 0.3% [0.001–2.1%]; +ICS: median eosinophils: 1.5% [0.55–7.55%]; $P = .005$).

3.2 | Molecular characteristics

Inflammatory mediators of sputum were examined through broad-spectrum assay of 75 cytokines, chemokines and growth factors. Eleven of these had measurable levels above the lower limit of detection in less than one-third of the entire subject cohort (in online repository Table S1) and were not analysed further. These 11 mediators did not differ for the presence or absence of signal between mild, moderate or severe subject cohorts. The remaining cytokines, chemokines and growth factors were individually tested for differences between subjects with mild or moderate asthma compared to severe asthma (Table 2, those not significantly differing among severity groups are listed in Table S2). The severe asthma group had 9 inflammatory proteins increased, IFN- γ , PDGF_{BB}, Fit3L, IL2, IL4, IL9, TNF- β , CCL27 and CXCL7, and 4 proteins decreased, IL3, CXCL10/IP-10, CXCL11/I-TAC and

CXCL13, compared to the mild asthma group; in addition, VEGF showed a trend towards decreased levels in the severe group. Although eosinophil % and absolute counts did not differ for severe compared to mild asthma, severe asthma subjects had increased levels of IL-4 and IL-9, but decreased IL-3 and VEGF, mediators associated with Th2 inflammation. Likewise, increased Th1-associated mediators, IFN- γ and TNF- β , were counterbalanced by decreased CXCL10/IP-10 and CXCL11/I-TAC in subjects with severe asthma. Lack of sputum cell count or % differences between moderate and severe asthma subgroups corresponded with only 4 proteins differing between these subgroups: IL-3, CCL3/MIP1 α , CXCL13/BCA-1 and CXCL11/I-TAC, representing both Th2 and Th1 types of inflammation.

3.3 | Cell and molecular associations

Cytokines, chemokines and growth factors were examined by multivariate regression for association with specific cell types found in sputum. Thirty-eight cytokines, chemokines and growth factors had significant associations with specific sputum cells, or more than 1 cell type, within the entire cohort (Table 3). Only CCL3/MIP1 α associated negatively with increased bronchial epithelial cell counts; the other cytokines, chemokines and growth factor associations were positive with increasing cell numbers. There were 26 cytokines, chemokines or growth factors showing no association with any specific cell type in the entire cohort, although a few had significant associations within mild, moderate or severe subgroups (Tables S4–S6). Greater than two-thirds of the significant associations (68%) within the whole cohort were with a single cell type, and the rest occurred with 2 cell types in different combinations. Neutrophils, either alone (42%) or in combination with lymphocytes or bronchial epithelial cells, were the dominant cell type associated with increasing inflammatory mediator levels. Thirteen per cent of the cytokines, chemokines or growth factors were associated with lymphocytes only, 5% with macrophages only, 5% with bronchial epithelial cells only and 3% with eosinophils only. Some of these cell-molecular associations were expected from previous reports,^{13,20,21,37} but others, for example neutrophils with CCL5/RANTES or CCL2/MCP1, were novel associations (see Figures S1a–d, and S2a–c).

Cell-molecular associations in mild and moderate asthma subgroups were more numerous than in severe asthma subgroup (Tables S4, S5 and S6), although these differences may be attributable to greater numbers of subjects in mild and moderate subgroups than in the severe asthma subgroup and to the reduction in subject numbers in each subgroup as compared to the whole cohort. Thirty of the 35 cytokines, chemokines or growth factors having significant cell associations in mild asthma were also observed in the entire cohort, but only 9 had similar associations with the same cell or multiple cell types. For mild asthma, more positive associations were observed with macrophages (48% of mediator associations), and only 1 (3%), CXCL7/NAP2, was associated with lymphocytes (and neutrophils), compared with 11 (28%) lymphocyte or lymphocyte+ another cell-associated mediators observed in the whole cohort. Sixteen of the 21 cytokines, chemokines or growth factors having significant cell associations in moderate asthma were also observed in the entire cohort, but only 4 of these showed associations with the same cell type or types. As observed in the mild asthma subgroup, the moderate subgroup had fewer mediator

associations with lymphocytes, 4 (19%), compared to the whole cohort. Fourteen of the 16 cytokines, chemokines or growth factors having significant cell associations in the severe subgroup were observed in the entire cohort; 6 associated with the same cell type. Thirteen of the 16 mediators in the severe subgroup were also observed in the mild subgroup, but only 3 mediators in the severe subgroup were also observed in the moderate subgroup. As in the whole cohort, neutrophils were the dominant cell type in cell-molecular associations (85%, 62% and 94% of significant associations in mild, moderate and severe asthma, respectively); however, different cell-molecular associations were observed in mild, moderate and severe asthma subgroups. For example, CCL24/eotaxin 2 was associated with macrophages in the mild subgroup, with epithelial cell count in the severe subgroup, but not any cell type in the moderate subgroup. IL-1 β and IL-23 were associated with macrophages and neutrophils in the severe subgroup, but did not associate with any cell type in mild or moderate subgroups.

One clinical variable potentially altering cell-molecular associations is corticosteroid use, which differed between the asthma severity subgroups (see Table 1), although there was little indication that ICS use altered sputum cells. Therefore, multivariate regressions were repeated adding the independent categorical variable of ICS. Only 4 cytokines showed altered cell associations, TNFSF10/TRAIL, CCL11/eotaxin, CXCL5/ENA78 and CXCL8/IL8, and for only 2 of these, CXCL8/IL8 and TNFSF10/TRAIL, were ICS, a significant independent variable ($P = .023$, and $.025$, respectively), but these mediators did not differ between the mild, moderate and severe subgroups (see Table 2).

Another approach was to examine whether associations occurred among the cytokines, chemokines and growth factors analysed.

3.4 | Molecular associations by factor analysis

Factor analysis was performed to determine which cytokines, chemokines and growth factors were strongly correlated, and therefore represented potential underlying molecular relationships or biologic pathways. Factor analysis included 43 cytokines, chemokines and growth factors for which all of 140 subjects had measurable levels (Table 4). These 43 cytokines, chemokines and growth factors grouped onto Factors 1–10; 16 of the mediators showed association with 2 separate factors (the higher eigenvalue indicated by bold font in Table 4). Each factor contained varying combinations of pro-inflammatory proteins associated with Th1, Th2, Th9 or Th17 inflammation, as well as anti-inflammatory proteins or those associated with repair processes. Factor 1 was predominantly characterized by Th1 inflammatory proteins including IFN- γ , whereas IL-5 and IL-13, representing Th2 inflammation, were associated with Factor 6. Although growth factors variously associated with Factors 1–8, Factor 2 had 4: VEGF, EGF, PDGF_{AA} and TGF- α . Pro-inflammatory IL-17a is identified as a distinct T cell group, Th17,¹³ including IL-25 and CXCL13/BCA1. However, IL-17a was primarily associated with Factor 2, containing multiple growth factors, and among other mediators, IL-6 and CXCL8/IL-8, which are induced by IL-17a.¹³ IL-17a was secondarily associated with Factor 5 which contained CXCL13/BCA1, a chemokine preferentially expressed by Th17 lymphocytes.¹⁴ IL-9, considered a Th2 inflammatory subtype,¹³ was associated with Factor 9, and not Factor 6 which included other Th2

cytokines, IL-5 and IL-13. Other Th2-associated chemokines, for example CCL5/RANTES, CCL11/eotaxin, CCL24/eotaxin2 and CCL26/eotaxin3, were not included in factor analysis because measurable levels were not observed in all 140 subjects.

Cytokines associated with Factors 1–7 were examined in [string_db.org](https://string-db.org)³⁶ to investigate whether the proteins on each factor corresponded to recognized linkage groups as independent confirmation of mediator groups identified in factor analysis (see Figure 1A–G). String did not recognize or indicate linkages for certain proteins in factor group 2 (TNFSF10), group 3 (TGF- α and IL-16) and group 4 (EGF and CCL15). However, TNFSF10, TGF- α , EGF and IL-16 were interconnected with other proteins on factor groups 7, 2, 2 and 6, respectively. Only CCL15 (Factor group 4) failed to interact with constituents on either its primary factor or any other factor. All other proteins on Factors 1–7 appeared to link with at least one other component, forming networks for each factor group.

4 | DISCUSSION

Broad assessment of 75 inflammatory mediators in induced sputum from 158 subjects with a spectrum of asthma severity revealed the following: (i) significant differences in cytokines, chemokines and growth factors between mild and severe asthma subgroups but few differences between moderate and severe asthma subgroups; (ii) inflammatory proteins predominantly associated with increasing numbers of neutrophils, but differences in specific cell-molecular associations were observed between mild, moderate and severe asthma; and (iii) complex patterns of associations between Th1, Th2, Th9 and Th17 inflammatory proteins were identified by factor analysis and confirmed in String v10.³⁶ Despite differences between asthma subgroups, analysis of the whole cohort by factor analysis identified mediators associated with innate inflammation and repair processes on the first 2 primary factors.

Severe asthma may be characterized by different inflammatory characteristics compared to less severe mild and moderate asthma.^{6,7} Our results found the severe subgroup compared to the mild subgroup had fewer sputum macrophages and increased bronchial epithelial cells than observed in mild asthma, but no sputum cell differences compared to moderate asthma. Lymphocytes, eosinophils and neutrophils did not significantly differ across severity groups. However, 13 inflammatory cytokines, chemokines and growth factors differed in concentration between mild and severe subgroups; only 4 of these also differed between moderate and severe. These results may reflect that those with “moderate” asthma may overlap with “severe” classification and, or that the smaller N for the severe asthma subgroup produced less power to reveal differences as observed in other, larger cohorts.^{6,28} U-BIOPRED’s cluster groups with severe asthma had 9 sputum proteins increased compared to moderate asthma, but 5 of these were elevated in subjects with significant smoking packyear history which may contribute to the differences.²⁸ In our severe subgroup, both Th1 mediators, IFN- γ and TNF- α , and Th2 cytokines, IL-4 and IL-9, were elevated. It should be noted that these samples and analyses characterize subjects during baseline periods of stable asthma and 62% of subjects received inhaled corticosteroid therapy which, in responsive subjects, suppresses Th2 adaptive inflammation.³⁸ Thus, Th1 immune

mediators might predominate over Th2, but both were observed in subjects with severe asthma.

We have previously observed decreased lung function associated with increased bronchial epithelial cells in sputum from a larger cohort,⁷ suggesting greater airway injury, lack of repair or remodelling as asthma severity increases. In addition to increased bronchial epithelial cells, the sputum for the current severe subgroup had more than twofold increased PDGF_{BB}, which contributes to airway smooth muscle proliferation³⁹ and fibroblast collagen I production.⁴⁰ However, VEGF, another growth factor considered important in airway repair and remodelling,²⁰ showed a trend towards reduction in our severe group compared to mild.

Unsupervised cluster analysis including blood and sputum eosinophil and neutrophil cell counts with clinical variables for a larger group of SARP subjects observed that sputum neutrophil % was a significant variable for determining cluster assignment associated with more severe asthma.⁶ A number of the cytokines, chemokines and growth factors demonstrating significant associations with increasing sputum cells are considered Th1 or Th17 inflammatory mediators and elicit marked neutrophil infiltration.^{37,41} Thus, molecular associations predominantly with neutrophils were expected, but some novel associations were observed. IL-4 was associated with increased bronchial epithelial cells instead of the expected association with eosinophils and/or lymphocytes.¹³ CCL2/MCP1 was associated with macrophage/monocytes,⁴² but only in the mild subgroup, in contrast to strong associations with neutrophils in the entire cohort, and in moderate and severe subgroups. Association of IL-4 with bronchial epithelial cells may reflect greater airway damage and release of airway epithelial cells into sputum as this Th2 cytokine increases. However, other Th2 cytokines, IL-5 and IL-13, were associated positively with increased eosinophils, conforming to expectation. This data for IL-5 and IL-13 correspond to similar IL-5 and IL-13 increases for “eosinophilic” subjects’ sputum in the report of Manise et al,¹⁷ but their “neutrophilic” group did not show significant elevations in Th1 and Th17 cytokines and mediators, such as IL-10, IL-12, IL-17, TNF- α , and IFN- γ , as we observed here. Moreover, we found the Th2 chemokines, CCL5/RANTES and CCL11/eotaxin, known to attract eosinophils, were instead significantly associated with neutrophil and neutrophil+epithelial counts, respectively. Thus, our results provide examples of Th1, Th2 and Th17 mediators interacting with both neutrophils and eosinophils, and demonstrate overlap between different T cell types for airway inflammation.

There are other immune cell types, such as mast cells, dendritic cells and Treg lymphoid cells which play important roles in asthma,¹³ but are not identified in differential sputum counts due to required specific immunostaining, and therefore could not be examined for mediator associations and possible subgroup alignment with asthma severity in this study. There is also the limitation of undetected differential activation for lymphocytes or macrophages which are present. In addition, cells which may be present at very low frequency, such as basophils, may be missed in differential counts of at least 500 non-squamous cells. Moreover, eosinophils may have degranulated, thereby reducing identification and accurate enumeration in differential counts. The differences observed within the severity subgroups for cell/mediator associations may reflect these limitations.

Nevertheless, multivariate regression identified mediator associations with both eosinophils and lymphocytes not only in the whole cohort, but also in severity subgroups. Large cohorts with specific immunostaining will be necessary to explore immune cell subclass associations with inflammatory mediators further. Factor analysis identified cytokines, chemokines and growth factors which group together. Ten factors were found with 2 or more proteins grouped on each factor. Although factors are considered independent, 16 cytokines, chemokines and growth factors were associated with each of 2 separate factors and 1 chemokine (CXCL9/Mig) with 3 separate factors in our analysis. Restriction of the accepted varimax rotation value from >40% to >50% for mediator association with factors, eliminated only 2 mediators, M-CSF and IL-13; the remaining mediators had stronger associations with a separate factor. Characteristic pathways or activities that each factor may represent are suggested by the reported functions for cytokines, chemokines or growth factors identified with a factor. For example, interferons α and γ , and interleukins 10 and 12p40 on Factor 1 suggest Th1 and anti-inflammatory pathways, whereas 4 growth factors on Factor 2 suggest airway remodelling and repair processes. Furthermore, the association of several neutrophil chemoattractants (IL-17a, CXCL5/ENA-78, CXCL8/IL-8) with 4 growth factors found on Factor 2 corresponds to a recent report that in vitro EGF-stimulated bronchial epithelial cells induce neutrophil chemotaxis and survival.⁴³ Th1 inflammatory cytokines and chemokines were distributed over Factors 1 through 8, indicating overlap with both Th2 (Factor 6) and Th17 inflammation (Factors 2 and 5). Our factor analysis results also supported the concept that Th9 inflammation, as represented by IL-9 found on Factor 9, may be distinct from Th2 inflammation,¹³ as represented by IL-5 and IL-13 on Factor 6. [string_db.org](https://string-db.org) confirmed linkage groups for the majority of mediators identified with each factor, thereby providing independent validation of the factor analysis results. Thus, factor analysis of these cytokines, chemokines and growth factors observed in subjects with a spectrum of asthma severity confirms current understanding of inflammatory mediator relationships, but in addition, indicates novel interactions and underscores complex patterns of Th1, Th2, Th9 and Th17 inflammation combined.

As indicated in the introduction, other recent studies in severe asthma have reported mainly transcriptomic gene expression data from various cell sources, including sputum, epithelial brush and biopsy,^{24–28} in addition to more limited information for protein analysis. Proteins identified by Somascan in sputum supernatants of UBIOPRED subjects presented only mild/moderate asthma, or severe asthma data (either smoking or not) compared to healthy controls subjects, without comparison between mild/moderate and severe asthma groups.²⁵ They noted upregulation of IL-1 receptor family members by gene expression in those with severe asthma²⁵ not only compared to healthy controls but also to the mild/moderate asthma group. Although we do not have gene expression data from our subjects, IL-1 β protein was significantly associated with sputum neutrophils in our severe subgroup, but not in mild or moderate subgroups.

The clinical importance of this study is not only the differences observed for subjects with severe asthma, but the identification of multiple inflammatory pathways existing simultaneously in airways for patients with a broad range of asthma severity. The implication from these observations is that assessment of a single biomarker may mislead in determination of airway inflammatory status and could fail to recognize patient response to

therapeutic interventions. A multi-assessment approach would be better even for trials which target 1 specific component of an immune pathway.

In summary, sputum cytokines, chemokines and growth factors differed between mild and severe asthma subjects, mainly representing Th1 and Th17 inflammation linked to increased neutrophils with few Th2 inflammatory mediators associated with eosinophils. Factor analysis found cytokines, chemokines and growth factors separating into 10 groups comprised of complex associations of Th1, Th2, Th9 and Th17 inflammatory and anti-inflammatory mediators. These results emphasize the multifactorial character of overlapping inflammatory pathways in asthma and highlight differences for more severe asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Cytokines, chemokines and growth factors identified with each factor in Table 4 were examined in string-db.org for interactions. Only CCL15 (on Factor 4) did not interact with any other constituents on different factors. TNFSF10, TGF- α and IL-16, and EGF, lacked interactions with proteins on Factors 2, 3 and 4, but were interactive on Factors 7, 2, 6 and 2, respectively

TABLE 1

Demographics of subjects, entire cohort and stratified by mild, moderate or severe asthma

Variable	Entire Cohort	Mild Asthma	Moderate Asthma	Severe Asthma	P value mild vs severe	P value moderate vs severe
N	158	63	57	38		
Age (y)	35 (26–45)	31 (23–37)	37 (27–46)	43 (34–52)	.001	.023
Asthma duration (y)	22 + 12	19 + 10.5	23 ± 12	26 + 13	.004	.270
Gender (% Female)	62	73	47	66	.586	.120
Racial background (%Cauc/% AA/%other)	61/33/6	66/29/5	56/35/9	61/37/3	.631	.425
BMI	29 (25–35)	28 (24–35)	27 (24–34)	32 (28–37)	.022	.026
FEV1% predicted	80 (65–92)	92 (85–99)	66 (61–74)	75 (62–92)	<.001	.006
FVC% predicted	91 (81–101)	100 (92–107)	81 (75–87)	87 (74–99)	<.001	.096
FEV1/FVC	75 (67–82)	78 (75–82)	68 (60–74)	73 (62–79)	<.001	.192
Max FEV1 reversal (%)	11.5 (7.6–23)	9.3 (6.7–12.3)	16 (9–30)	18.3 (7.9–31.9)	<.001	.973
PC20	1.18 (0.4–3.5)	1.628 (0.66–5.77)	0.62 (0.22–2.00)	1.36 (0.73–2.84)	.335	.123
IgE	172 (69–370)	139 (51–266)	204 (89–470)	150 (69–336)	.47	.334
Blood eosinophil count (K/iL)	0.25 (0.10–0.31)	0.20 (0.10–0.30)	0.30 (0.14–0.35)	0.20 (0.10–0.34)	.597	.180
Number of + allergens	5	4	5	5	.99	.64
Total ICS (% positive)	62	44	58	97*	<.001	<.001

* One severe subject on injected corticosteroids rather than inhaled. Values are presented as either means±SD or medians (25%–75% interquartile range). The significant values are indicated in bold font.

TABLE 2

Sputum cells and soluble proteins in supernatant for the entire cohort and for subjects stratified by mild, moderate and severe asthma

Variable Cells	Entire Cohort	Mild Asthma	Moderate Asthma	Severe Asthma	P value mild vs severe	P value moderate vs severe
WBC%	61 (43–77)	55 (42–75)	66 (35–81)	62.9 (49–78)	.204	.408
Macrophage (%)	47.2 (29–69)	53.6 (37–81)	44 (27–65)	34.2 (22–57)	.002	.220
Lymphocyte (%)	1.2 (0.6–2.4)	1.5 (0.7–2.7)	1.1 (0.6–2.2)	1 (0.6–2.5)	.457	.643
Neutrophil (%)	39.7 (25–63)	36 (16–57)	39.5 (31–67)	46.3 (25–70)	.073	.735
Eosinophil (%)	0.7 (0.2–3.4)	0.4 (0.1–2)	1 (0.2–6)	0.75 (0.2–5.2)	.598	.344
Bronchial Epith (%)	3 (1–6)	2 (1–5)	3 (1–8)	3 (1–6.4)	.014	.427
Total cell count (x10 ⁶)	1.6 (0.9–2.8)	1.7 (1–2.6)	1.7 (1–2.9)	1.25 (0.8–3.0)	.466	.152
WBC (x10 ⁶)	0.9 (0.55–2.0)	0.9 (0.50–1.9)	0.97 (0.6–2.1)	0.865 (0.52–2)	.790	.732
Bronchial epith (x10 ⁴)	4.1 (1.8–8.6)	3.0 (1.3–5.2)	5 (1.5–14.4)	5.1 (2.5–8.6)	.031	.876
Macrophage (x10 ⁴)	38 (19–69)	38 (29–80)	39 (18–94)	31.3 (14–60)	.103	.376
Lymphocyte (x10 ⁴)	1.2 (0.3–3.5)	1.2 (0.4–3.4)	1.5 (0.3–3.5)	1.04 (0.3–5.3)	.888	.843
Neutrophil (x10 ⁴)	38 (14–85)	33 (8.7–78)	40.1 (20–85)	38.1 (19–113)	.221	.885
Eosinophil (x10 ⁴)	0.7 (0.1–4.1)	0.6 (0.06–1.7)	1.1 (0.15–6.0)	0.97 (0.1–6.2)	.453	.579
Proteins						
IFN- γ	2.5 (1.5–4.4)	1.97 (1.2–3.7)	3.8 (1.8–5.0)	2.5 (1.7–4.2)	.027	.442
PDGF-BB	11 (4.9–23)	8.8 (4.7–17.6)	10.4 (5–19)	25 (6.2–43)	.034	.066
Fit3L	5.1 (3.7–7.1)	4.6 (2.3–6.1)	5.2 (3.7–7.6)	5.2 (4.3–6.9)	.038	.946
IL-9	0.7 (0.4–0.9)	0.6 (0.3–0.8)	0.7 (0.4–1.0)	0.9 (0.7–1)	.001	.172
IL-2	1.0 (0.5–1.5)	0.7 (0.4–1.3)	1.22 (0.45–2.1)	1.1 (1–1.6)	.001	.747
IL-3	1.0 (0.7–1.3)	1.1 (0.7–1.3)	1.1 (0.78–1.3)	0.7 (0.6–1.0)	.006	.011
IL-4	2.0 (0.7–5.0)	1.3 (0.5–2.2)	2.54 (1.5–5.1)	4.2 (0.7–7.8)	.039	.605
TNF- β	1.1 (0.6–1.9)	0.55 (0.2–1.5)	1.1 (0.6–2.4)	1.2 (0.7–2.0)	.029	.893
VEGF	191 (75–371)	215 (96–399)	197 (104–371)	124 (40–319)	.058	.112
CCL3/MIP1a	9.9 (2.8–21.8)	11 (3–23)	8 (2–14)	22 (8–69)	.479	.038
CCL27/CTACK	2.5 (1.7–3.4)	2 (1.4–3.2)	2.6 (1.8–3.4)	2.7 (2.4–8.5)	.046	.192
CXCL7(NAP2)	33 (21–69)	27 (17–58)	36 (26–62)	51 (26–89)	.050	.322
CXCL10/IP-10	1439 (735–2647)	1455 (877–2704)	1566 (788–3301)	1181 (275–2076)	.047	.068
CXCL11(I-TAC)	14 (5.4–35)	13.6 (6.4–32)	20 (8–52)	9.7 (3.3–19)	.018	.006

Variable Cells	Entire Cohort	Mild Asthma	Moderate Asthma	Severe Asthma	P value mild vs severe	P value moderate vs severe
CXCL13/BCA-1	14 (3.3–42)	14.3 (3.8–59)	14.9 (4.9–41.6)	5.1 (1.7–25)	.005	.011

Values are presented as medians (25%–75% interquartile ranges).

The *P* values in bold font indicate significant difference between severe and mild or moderate groups.

TABLE 3
Multiple linear regression for mediator concentration in entire cohort with specific sputum cell counts

Cytokine/chemokine	R, P value	Epithelial count	Macrophage count	Lymphocyte count	Neutrophil count	Eosinophil count
IL-15	.45, <.001		0.041			
CCL15/MIP1d	.45, <.001		0.048			
CCL17/TARC	.51, <.001	0.050		0.039		
IL-7	.50, .001			0.039		
IL-16	.47, .001			0.050		
IL-23	.51, .001			0.013		
IFN- α	.46, .001			0.037		
CCL4/MIP1b	.46, .002			0.007		
CXCL7(NAP2)	.58, .001			0.032	<0.001	
IL-6	.52, <.001			0.029	0.003	
IL-12p40	.55, .001			0.008	0.009	
CCL7/MCP3	.55, .001			0.019	0.006	
CCL22/MDC	.48, .001			0.032	0.037	
CXCL5/ENA-78	.40, .002				0.009	
CXCL6/GCP2	.46, .001				0.05	
CXCL9/MIG	.45, .001				0.039	
CXCL11/I-TAC	.43, .001				0.016	
G-CSF	.51, .001				0.001	
GMCSF	.46, .001				0.022	
CX3CL1/Fractalkine	.37, .005				0.014	
IL-1b	.33, .026				0.017	
CXCL8/IL-8	.53, .001				<0.001	
IL17a	.49, .001				<0.001	
CCL20/MIP3a	.54, .001				<0.001	
CCL19/MIP3b	.50, .001				<0.001	
PDGF-AA	.42, .001				0.005	
CCL5/RANTES	.52, .001				<0.001	
TGF- α	.52, .001				0.004	
TNF- α	.52, .001				<0.001	

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Cytokine/chemokine	R, P value	Epithelial count	Macrophage count	Lymphocyte count	Neutrophil count	Eosinophil count
CCL11/eotaxin	.49, .001	0.016			0.036	
FGF2	.54, .001	0.009			0.036	
CCL3/MIP1a	.57, .013	0.011 neg			0.054	
CCL2/MCP1	.48, .001	0.052			<0.001	
VEGF	.45, .001	0.054			0.052	
IL-4	.43, .01	0.016				
TNFSF10/TRAIL	.41, .002	0.010				
IL-13	.42, .001	0.024				0.031
IL-5	.50, .001					0.001

R and P values for overall regression model; P values for individual cell count appear under cell type(s) which were significantly associated. All were positive associations with increasing cell count except for CCL3/MIP1a which was negatively associated with epithelial cell count ("neg" suffix).

TABLE 4

Cytokines, chemokines and growth factors identified with each factor were characterized according to inflammation type

Factor 1	Th1 —IL-15, IL-10, CXCL11/I-TAC , IFN- α 2, IFN- γ , IL-12p40, (IL-15 & CXCL11 induced by IFN- γ) Th2 —GMCSF, CCL7/MCP3, CCL8/MCP2 Anti-inflammatory —IL-10, sCD40L (increased by IFN- γ +TNF- α), IFN- γ , IFN- α , IL-12p40 Growth Factors —GMCSF
Factor 2	Th1 —IL-6, CXCL8/IL-8 , CXCL5/ENA78 Th2 —CCL2/MCP1, CCL7/MCP3 , CCL22/MDC, IL-7 Th17 — IL-17a Anti-inflammatory —TGF- α , TNFSF10/TRAIL Growth Factors —VEGF, EGF, PDGF _{AA} , TGF- α
Factor 3	Th1 —CXCL10/IP10, IL-1b, IL-16 , TNF- α Th2 —CCL2/MCP1, IL-3 Anti-inflammatory —TGF- α Growth Factors —TGF- α
Factor 4	Th1 —CXCL1/GRO α , CXCL8/IL-8, CXCL9/Mig Th2 —CCL15/MIP1d, CX3CL1/Fractalkine Growth Factors —EGF
Factor 5	Th1 —CXCL6/GCP2, CXCL9/Mig, Th2 —G-CSF Th17 —IL-17a, CXCL13/BCA-1, CCL20/MIP3a Growth Factors —G-CSF
Factor 6	Th1 —IL-12p40, IL-16, CXCL5/ENA78 , CXCL6/GCP2 , Th2 —IL-5, IL-13 Anti-inflammatory —IL-12p40
Factor 7	Th1 — IL-6 , CXCL9/Mig, CXCL10/IP10 , CXCL11/I-TAC Th2 —M-CSF Anti-inflammatory —TNFSF10/TRAIL Growth Factors —M-CSF
Factor 8	Th1 —IL-1a, IL1Ra Th2 —M-CSF Growth Factors —M-CSF
Factor 9	Th9 —IL-9 Anti-inflammatory —CCL21/6Ckine
Factor 10	Th2 —CCL8/MCP2, CCL13/MCP4

Those that may be induced by other proteins identified on the same factor are noted in parentheses. Cytokines, chemokines or growth factors observed on 2 different factors are presented in bold font for the stronger association (higher Eigenvalue).